The biochemical differences of the superficial digital flexor tendon and the common digital extensor tendon between Warmbloods, Friesians and Thoroughbreds.

Author: E. Hazeleger
Supervisors: W. Back, C.H.A. van de Lest
Utrecht University, Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht, The Netherlands, 2013

Contents

Summary

Reasons for performing this study: Research has shown that the suspensory ligament (tendo interosseus), the superficial and deep digital flexor tendon of Friesians biomechanically differ from the same tendons in ponies. [1] The tendons of Friesians are more elastic compared to those of ponies. [1] It is not clear yet which biochemical components are responsible for this fact. For thoroughbreds, a study showed that the pyrrole and pyridinoline cross-links have an effect on the stiffness and strength of their tendons. [2] There is, as far as known, no research done whether these crosslinks could cause the differences in biomechanical properties of the tendons of Friesians and Warmbloods.

Hypothesis: The crosslinks pyrrole and pyridinoline cause the biomechanically differences in elasticity between the tendons of Friesians and Warmbloods.

Methods: The superficial digital flexor tendon (SDFT) and common digital extensor tendon (CDET) were collected from 12 Warmbloods, 12 Friesians and 8 Thoroughbreds. They were biomechanically tested for water content, crosslink levels (pyrrole and pyridinoline), cellularity, glycosaminoglycans (GAGs) and collagen.

Results: Friesian tendon contain a significant higher pyrrole crosslink level than Thoroughbred tendon. No significant difference is found for the pyridinolines.

Conclusions: The higher content of pyrrole in Friesian tendon could be one of the causes of the biomechanically differences in elasticity, but further research is necessary.

Introduction

Competition horses relatively often suffer from damage to tendons and ligaments. These injuries are the most occurring and the main reason for taking horses (temporarily) out of competition. [2] Tendons which are often affected are the superficial digital flexor tendon and the suspensory ligament (tendo interosseus). [2,3]

Anatomy and physiology

In this paragraph the tendon structures of the distal forelimb will be described.

The common digital extensor tendon passes over the front of the distal limb. [4,5] It originates from the common digital extensor muscle, which in its turn originates from the lateral epicondyle of the humerus. [6] The insertion of the common digital extensor tendon is the extensor process of the distal and the middle phalanx. [4,5,6] It also inserts proximodorsal on the proximal phalanx, together with the lateral digital extensor tendon (Fig. 1). [6] It is a digital and carpal extensor. [6] Contraction of the common and lateral digital extensor muscles brings the bones and joints of the digit into alignment just before the hoof strikes the ground. [4] The common extensor tendon is protected by a synovial bursa as it passes over the dorsal pouch of the fetlock joint. [5]

The lateral extensor tendon lies lateral to the common digital extensor tendon. [5] It originates from the proximal end of the radius and ulna en inserts proximodorsal on the proximal phalanx (Fig. 1). [6] The tendon extends the fetlock joint. [6] The lateral and the common digital extensor tendon are easily palpated in region of the metacarpus. [5]

The superficial digital flexor tendon passes the palmar aspect of the distal limb. [4,5] It originates from the superficial digital flexor muscle, which originates from the medial epicondyle of the humerus. [6] The insertion of the superficial digital flexor tendon is the distal collateral tubercles of the proximal phalanx and the proximal collateral tubercles of the middle phalanx (Fig. 1). [4,5,6] The tendon bifurcates into two branches before it inserts. [4] The superficial digital flexor tendon becomes subcutaneous distal of the carpus. [4,5] It is enclosed, together with the deep digital flexor tendon, by a carpal synovial sheath. [4] The sheath ends at the middle of the metacarpus. The superficial digital tendon flexor is dorsally intimately related to the fascial covering of the deep digital flexor tendon and forms a sleeve around the deep digital flexor tendon at the level of the proximal sesamoid bones. [4,5] The superficial digital flexor tendon flexes the digit and the carpus and extends the elbow joint. [6]

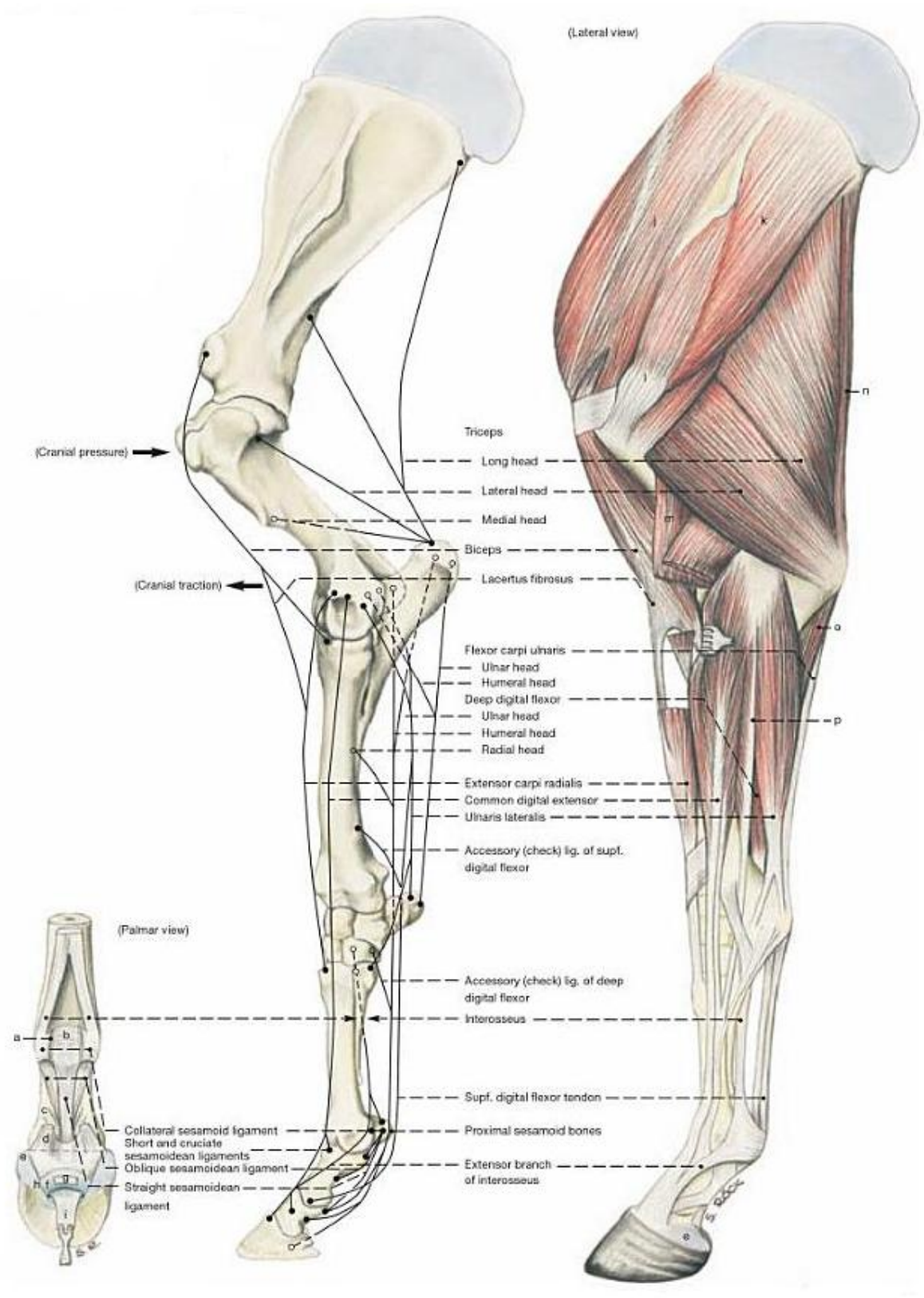

Fig. 1 *The anatomy of the forelimb of the horse* [6]

The deep digital flexor tendon also passes the palmar aspect of the distal limb. [4,5] It originates from the deep digital flexor muscle, which originates from the medial epicondyle of the humerus. [6] The insertion of the tendon is on the flexor surface of the distal phalanx (Fig. 1). [5,6] It receives an accessory ligament from the carpus: the carpal check ligament or 'inferior' check ligament.[4,6] The tendon passes through the sleeve of the superficial digital flexor tendon. [4,5,6] A digital synovial sheath encloses the deep and superficial digital flexor tendon, including the branches of the superficial digital flexor tendon and goes further around the deep digital flexor tendon to the middle of the middle phalanx. [4,5, 6] The deep digital flexor tendon flexes the digit and carpus and extends the elbow joint. [6]

The suspensory ligament (tendo interosseus, interosseus muscle) originates from the carpus and the proximal end of the third metacarpal bone. [4,5,6] It inserts on the proximal sesamoid bones (Fig. 1). [4,5,6] Before the insertion the suspensory ligament bifurcates and sends extensor branches around the proximal phalanx to the common digital extensor tendon. [4,5,6] The cruciate, oblique and straight sesamoidean ligaments are a functional continuation of the suspensory ligament beyond the sesamoid bones.[1,5] The suspensory ligament contains little muscular tissue. [4,5,6] It counteracts overextension of the fetlock joint. [6]

There are two different types of tendon: the spring-like tendon and the positional tendon. [2,3,7] Tendons which have the function of storage and release of elastic strain energy thereby increasing the efficiency of locomotion, are spring-like tendons. [2,3,7] The flexor tendons belong to this category. [2,3,7] Spring-link tendons are required to stretch and recoil under physiological loads to ensure efficient return of stored energy. [7] They are subjected to relatively high strains during normal physiological activity. [2,3,7] The main role of a positional tendon is in transmitting muscle generated force to a bone resulting in movement around a joint. [7] They experience much lower strains than spring-like tendons. [2,3,7]

Structure of tendon

Tendons are dense regular connective tissue structures built up in a hierarchical arrangement of increasingly smaller subunits, designed to transmit the force of muscle contraction to bone.[4,8,7,9] They consist of extracellular tendon matrix which is composed of the collagen fibers, elastic fibers, the ground substance and anorganic components.[10]

Collagen fibers

A tendon is a highly hydrated structure, with around 55-70% of its weight consisting of water. [4,8,11] Collagen accounts for 65-80% of the dry mass of the tendon.[7,10,11] This tendon collagen is predominantly type I collagen. [4,10,11,12,13] Collagen molecules are precisely organized into collagen fibrils, fibers, fiber bundles, fascicles and finally the whole tendon.[7,11] One of the smallest units of the tendon is the microfibril. It is formed by five tropocollagen units which join together to form the microfibril. [14] Procollagen undergoes extracellular modifications in maturing to tropocollagen. [15] Microfibrils aggregate into collagen fibrils and a bunch of collagen fibrils forms a collagen fiber. [10] A bunch of collagen fibers forms a primary fiber bundle (subfascicle), and a group of primary fiber bundles forms a secondary fiber bundle (fascicle). A group of secondary fascicles, in turn, forms a tertiary bundle, and the tertiary bundles make up the tendon (Fig. 2). [10]

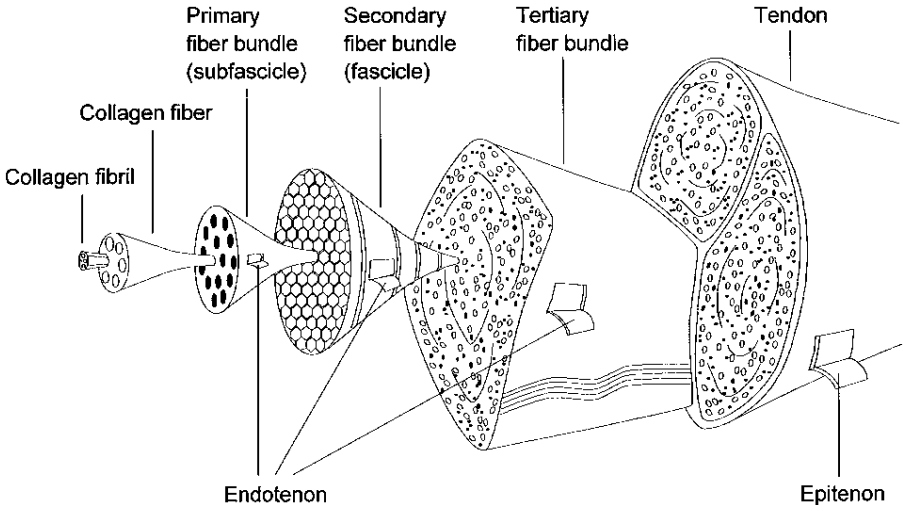

Fig. 2 *The organisation of the tendon structure* [10]

The collagen molecules within a collagen fibril are linked to each other by chemical crosslinks. [4,7] Collagen fibrils are longitudinally, transversely and horizontally oriented. [10] The longitudinal fibrils can form spirals and plaits by crossing each other. [10] Collagen fibers and fibrils have a characteristic wavy pattern in the resting state. That is called crimp. [4,8,10,14] Crimp imparts elasticity to the tendon. [4]

Tendons are often surrounded by loose connective tissue lined with synovial cells. This is called the paratenon. [8,10,11] It functions as an elastic sleeve which reduces frictional forces between the tendon and the surrounding tissues. [8,10]
Under the paratenon, the entire tendon is surrounded by a fine connective tissue sheath called epitenon (Fig 2 and 3). [10] It consists of loose, fatty, areolar tissue and is a relatively dense fibrillar network of collagen. [10,11] The inner surface of the epitenon is adjacent to the endotenon (Fig. 2 and 3). [8,10] It is a fine sheath of connective tissue and it lines each collagen fiber and binds individual fibers together. [10] Sometimes the tendon is surrounded by a tendon sheath. This structure provides a synovial environment for the smooth gliding of tendon over a bony prominence. [8]

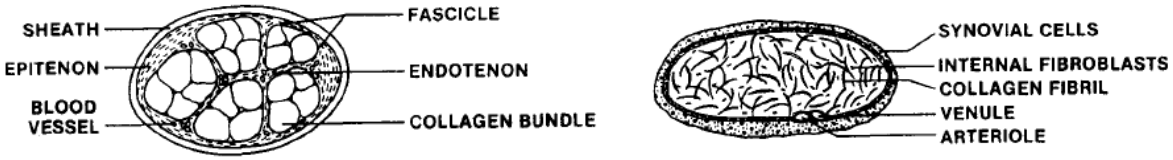

Fig. 3 *Cross-section of a tendon* [9]

Elastic fibers
Elastic fibers account for 1-2% of the dry mass of tendon. [10] The function of the fibers is not clear. They may contribute to the recovery of the crimp after the tendon is stretched. [10]

Ground substance
The ground substance of the tendon surrounds the collagen and it consists of glycosaminoglycans (GAGs), proteoglycans (PGs), structural glycoproteins and other small molecules. [4,10,11,12,16] The ground substance is a hydrophilic gel. [10, 11] PGs are produced by among others tenoblasts.[17] GAGs and PGs have a high water-binding capacity. [10,13] This improves the elasticity of the tendon against compressive forces. [10] It is believed that GAGs and PGs, orienting and ordering the collagen fibrils, act like tissue organizers. [13,16] Proteoglycans are composed of a protein core and one or more glycosaminoglycan sidechains. [7, 10,16] PGs can carry water fifty times their weight. [10] Proteoglycans

such as fibromodulin, decorin and biglycan can interact with collagen fibers and may regulate collagen fibril diameter. [7] The small proteoglycans (fibromodulin, decorin, lumican) are found in the regions of tension. [4] The large proteoglycans (aggrecan and versican) are found in regions of compression where the tendon changes direction over a bony prominence. [4]
Glycoproteins are macromolecules. They have a large protein fraction and a small glycidic component. [10] The tendon tissue also contains many other small non-collagenous proteins. [10] Little is known yet about the functions of these molecules. [10] Examples of molecules which are present in tendon tissue are tenascin-C, fibronectin, thrombospondin, undulin, laminin and adhesive glycoproteins. [10, 11]

Anorganic components
The anorganic components account for less than 0,2% of the dry mass of the tendon. [10] Calcium, magnesium, manganese, silicon, cadmium, phosphor, cobalt, fluoride, copper, lead, zinc, lithium and nickel are detected in tendon tissue. [10]

Tenoblasts and tenocytes are the tendon cells, accounting for 90-95% of the cellular elements of the tendon. [4,10] They are responsible for the formation, organization and maintenance of tendon tissue. [4,10, 13] The tendon cells lie between the collagen fibers, they are elongated fibrocytes and fibroblasts. [10]

Tendons have relatively limited vasculature, it occupies only 1-2% of the extracellular matrix. [11] Blood and nutrients are supplied to the tendons and ligaments through perfusion and diffusion. [4] When blood is delivered via perfusion, it originates from three separate sources: the muscular origin proximally, the osseous insertion distally, and the intra-tendinous and extratendinous vessels. [4,8,11] The extratendinous blood supply comes from vessels within the paratenon (extrasynovially) or mesotenon (intrasynovially) attachments. [4,8] Diffusion primarily occurs where a sheath encloses the structure with synovial fluid. [4]

The collagen molecules within a collagen fibril are linked to each other by chemical crosslinks. [2,7] Crosslinking can arise from two different mechanisms. [18] One is during the development and maturation and consist of a precise enzymatically controlled crosslinking. [18] The other is after the maturation of the tissue and is an accidental non-enzymatic mechanism. [18] The non-enzymatic crosslinking is also known as glycation. [18] With aging, it contributes to additional crosslinking which modifies tissue stiffness. [11] The formation of enzyme-derived crosslinks increases the mechanical strength of the collagen fibrils. [2]
The enzymatic crosslinks are formed at the ends of the collagen molecules in the telopeptide regions following the action of the enzyme lysyl oxidase on specific lysine and hydroxylysine residues. [2,7] Lysyl oxidase converts lysine and hydroxylysine residues into aldehydes. If the hydroxylation is extensive, more hydroxylysine aldehyde will be formed. [2] Lysine aldehydes and hydroxylysine aldehydes undergo a spontaneous reaction with lysine or hydroxylsine residues in the helical region of adjacent molecules to form the immature bivalent crosslinks. [2] Immature crosslinks undergo further spontaneous reactions to form mature trivalent pyridinoline and pyrrole crosslinks. [2] Examples of pyridinoline crosslinks are hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) and examples of pyrrole crosslinks are hydroxylysyl-pyrrole (HL-Pyrrole) and lysyl-pyrrole (L-Pyrrole). [2,7,12] The formation of HP is caused by extensive hydroxylation of lysine residues throughout the collagen molecules. [2] In tissues where less hydroxylation of the helical or telopeptide lysine residues occurs the mature cross-links LP and pyrrole form, respectively. [2]
HP and LP are two major intermolecular crosslinks of collagen that are found in tissues such as cartilage, bone and tendon. HP is the major crosslink found in tendon, while LP levels in tendon are low. [12] Another crosslink is pentosidine. It is a product of the process of non-enzymatic glycation.[12] Pentosidine is found in tissues such as dura mater, skin, cartilage and tendon. [12]

What is known so far and has been researched?
Research has shown that the suspensory ligament (tendo interosseus), the superficial and deep digital flexor tendons of Friesians biomechanically differ from the same tendons in ponies. [1] The tendons of Friesians are more elastic. [1] It is not clear yet which biochemical components are responsible for this fact. However, it is known that the mechanical strength of a tendon is based on the correct orientation of collagen molecules in the fibrils and the stabilization between collagen molecules by the formation of chemical crosslinks. [2] These crosslinks increase the mechanical strength of collagen fibrils. [2] The research focuses on two different crosslinks: pyridinoline and pyrrole. [2] It appears that pyrrole is less present than pyridinoline, but that it has more effect on the tendon strength. [2] The reason could be that pyrrole acts as an interfibrillar crosslink involving three different molecules, while pyridinoline only cross links two collagen molecules within the fibril. [18] Pyrrole shows a positive correlation with the stiffness and the strength of the tendon in Thoroughbred horses. [2] For Warmbloods this has not been researched yet. To my knowledge, no research has been done to establish whether these crosslinks could cause the differences in biomechanical properties of the tendons of Friesians and Warmbloods.

Materials and methods
Tendon collection and storage
The distal parts of the forelimbs of twelve Warmbloods and twelve Friesians were obtained at a slaughterhouse or at a veterinary clinic where the horses were euthanized for reasons other than tendon injury. The Warmbloods were aged 6-12 years (mean ± SD age, 8.8 ± 2.41 years). The Friesians were aged 4-13 years (mean ± SD age, 8.75 ± 2.60 years). The forelimbs were frozen and stored at -20˚C until the collection of the tendon samples. For the collection of the samples, the limbs were thawed in water. The tendons samples were obtained from the mid-metacarpal region of the common digital extensor tendon and the superficial digital flexor tendon (Fig. 4). The samples were stored en frozen again at -20˚C.

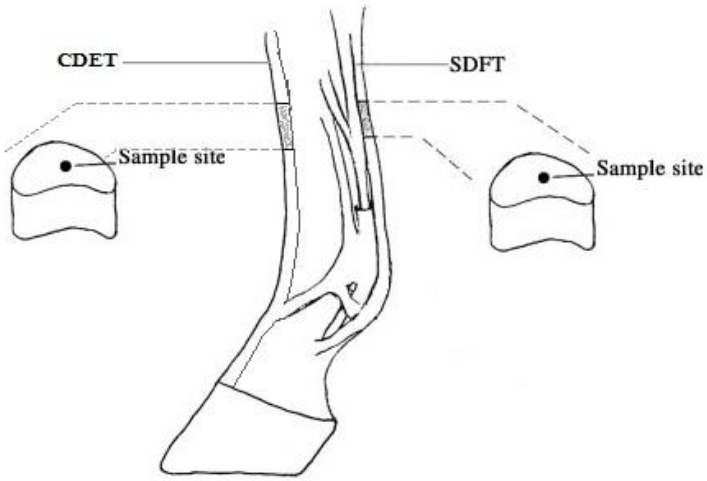

Fig. 4 *Schematic drawing distal part forelimb with the locations where the tendon samples were taken from the CDET and SDFT. And the sample location in a transverse section through the tendon.* [12]

Eight tendon samples from Thoroughbreds were sent from the Royal Veterinary College in London. These samples will also be tested biochemically. Later this year, the tendons of the twelve Warmbloods an Friesians will be send to the Royal Veterinary College where they test them biomechanically and biochemically, together with the tendons of the Thoroughbreds, to compare the different tests (Faculty Veterinary Medicine Utrecht University versus Royal Veterinary College London) for the crosslink pyrrole. The different tests will be discussed in section 'pyrrole crosslink assay'. The Thoroughbreds were aged 3-10 years (mean ± SD age, 7.0 ± 2.73 years).
The tendon samples are analyzed for water content, DNA, GAGs, collagen and crosslinks.

Water content
The water content was determined by weighing the samples before and after it was freeze dried for approximately 20 hours. Before weighing the wet weight, the tendon samples were placed in phosphate buffered saline (PBS) for approximately 30 minutes and were dried (patted) with a filter paper. The following formula is used to calculate the water content of the tendon samples: [(wet weight – dry weight) / wet weight] x 100%.

Papain digestion
For the analysis of DNA and GAGs, the tendon tissue must be digested first. Approximately 25 mg dry weight tendon tissue was digested by papain (Sigma Chemical Co, St Louis, Missouri, USA) for 20 hours at 56 °C. For each tendon sample 400 µl of papain solution is used. The papain solution contains 1 u/ml papain and 0.5 M phosphate buffer (pH 6.5), containing 20 mM $Na_2EDTA\ 2H_2O$ and 20 mM N-acetylcysteine.
After 20 hours, not all the samples were fully digested. 100 µl papain solution, containing 20 µl papain and 1 mg cysteine was added to the samples which were not fully digested and were digested for another 20 hours at 56˚C. To the samples which were digested fully, 100 µl of MilliQ water was added to ensure the same dilution of all samples.

DNA assay
To give an indication of the tissue cellularity, the DNA content was analyzed according to Kim et al. [19] 20 µl papain digested tendon is used, 1 mg/ml Hoechst 33258 color, bisbenzimadazol, (Molecular Probes, Leiden, The Netherlands) is added to the papain digested tendon. The fluorescence was measured immediately after mixing by use of a fluorimeter (Perkin Elmer, Norwalk, Connecticut, USA) with excitation at 352 nm and emission at 455 nm. Calf thymus DNA (Sigma Chemical Co, St Louis, Missouri, USA) is used as a reference. Results are expressed as µg/mg dry weight.

Glycosaminoglycan assay
The papain digested tendon samples were analyzed for S-GAG content using the modified 1,9-dimethylmethylene blue dye, DMMB, (Sigma Chemical Co, St Louis, Missouri, USA) binding assay as described by Farndale et al. [20] To 20 µl of papain digested tendon, 10 µl of 3% bovine serum albumin (BSA) was added. Then, 250 µl of DMMB reagent was added and measured after 20 minutes at room temperature by a Benchmark microtitre plate reader (Bio-Rad Laboratories, Hercules, California, USA) with an absorbency of 525 nm and a reference wavelength of 595 nm. Shark chondroitin sulphate (Sigma Chemical Co, St Louis, Missouri, USA) was used as the standard. Results were expressed as µg GAG/mg dry weight.

Crosslink analysis
600 µl 6 M HCl was added to the freeze dried tendon samples (± 25 mg dry weight). The samples were hydrolyzed at 110 ˚C for approximately 20 hours. Then, 200 µl 2.5 mM homo-arginine (Sigma Chemical Co, St Louis, Missouri, USA) was added. This is an internal standard for mass spectrometric determination, MS (Bio-Rad Laboratories, Hercules, California, USA) of the amino acid hydroxyproline (Hpro) and the collagen crosslinks pentosidine, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). The hydrolyzed samples were vacuum-dried for 24-48 hours. After the samples were dried, 1 ml of MilliQ water was added to the samples. In order to ensure the dried samples dissolved well, a vortex is used. Next, the dissolved samples were centrifuged at 15000*g* for 10 minutes. The supernatants were diluted 100-fold: first, 100 µl of the supernatant was diluted by 900 µl MilliQ water. Second, 100 µl of that solution was diluted by 900 µl solution containing 0.3% heptafluorobutyric acid, HFBA, in 20% methanol. These samples were quantified by high-performance liquid chromatography, HPLC/MS. HPLC/MS analysis was performed using a 4000-TRAP MS at a source temperature of 300°C and a spray voltage of 4.5 kV. Amino acids were separated on a Synergi MAX-RP 80A (250 x 3 mm, 4 µm) column (Phenomenex Inc, Torrance, California, USA) at a flow rate of 400 µl/min, using a gradient from MilliQ water to acetonitrile, both containing 1.2 mmol/l of heptafluorobutyric acid and 2.5 mmol/l ammonium acetate (pH 5.6). Amino acids were identified by MS in multiple reaction mode (MRM) using the following mass transitions

(mz/mz): 132.2/68.2 (hydroxyproline, Hpro), 163.2/128.1 (hydroxylysine, H-lys), 116.1/70.1 (proline), 147.2/130.2 (lysine), 413.3/413.3 (LP), 379.3/379.3 (pentosidine) and 429.3/429.3 (HP). Pentosidine was analyzed simultaneously by fluoroscopy (FP- 1520; Jasco, Dunmow, Essex, UK) with excitation at 297 nm and emission at 400 nm. Data were related to the recovery of internal standard homo-arginine. H-lys, proline, lysine, LP, pentosidine and HP were expressed as nmol/mg dry weight. Hpro was expressed as ng/mg dry weight.

Collagen content
The collagen concentration is given as µg hydroxyproline per mg dry weight. It is expressed as mg/mg dry weight.

Pyrrole crosslink assay
For the analysis of pyrrole crosslinks, papain digested tendon is used. The samples were centrifuged at 10000 *g* for 15 minutes. The clear supernatants containing digested tendon were used for further analysis. 20 µl supernatant was diluted with 180 µl MilliQ water. 40 µl of Erlich's reagent (500 mg 4-dimethyaminobenzaldehyde in 4.4 ml 60% perchloric acid made up to 10 ml with deionized H_2O) was added to the samples and the reference (1-methyl-Pyrrole Standard). The samples were left for 10 minutes at room temperature before measuring the absorbency at 558 nm and 650 nm as a background reference. Results are expressed as nmol/mg dry weight.

Statistical analysis
The data were analyzed statistically using SPSS 20.0 (SPSS, version 20.0 for Windows, SPSS Inc, Chicago, Illinois), using analysis of variance (ANOVA) for repeated measures, followed by a Tukey Post Hoc Test. The significance level was set at $p < 0.05$. To detect significant outliers, the Grubb's test is used. The significance level was set at $p < 0.05$.

Results

Water content
The SDFT contains a significant higher water content than the CDET. Furthermore, there is a significance difference between each breed for the SDFT and the CDET. Tendon of Warmbloods contain the highest water content, followed by the tendon of the Friesians. Thoroughbreds contain the lowest water content. (See figure 5 and table 1).

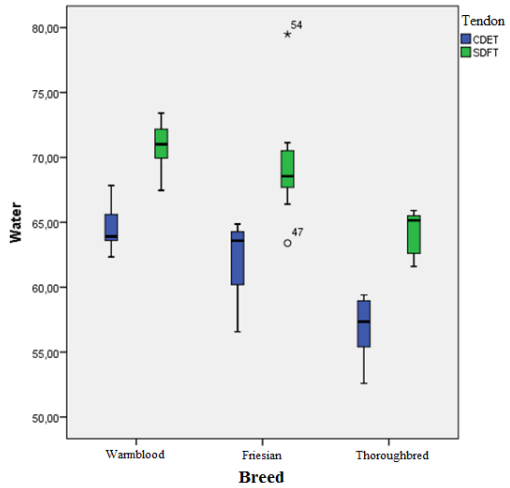

Fig. 5 *Water content (%) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

DNA content

The SDFT contains a significant higher DNA content than the CDET. There is a significant difference between each breed for the SDFT: tendon of Warmbloods contain the highest DNA content, Thoroughbreds have the lowest DNA content. The Friesians are in between. The CDET doesn't show a significance difference between any of the breeds. (See figure 6 and table 1).

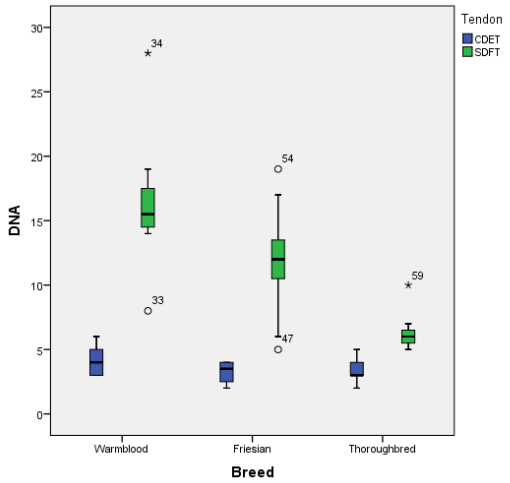

Fig. 6 *DNA content (µg/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

GAG content

The SDFT contains a significant higher GAG content than the CDET for each breed. There is a significant difference between the Warmbloods and the Thoroughbreds in the SDFT in which the Thoroughbreds contain higher GAG content. For the CDET there is no significant difference between the breeds. (See figure 7 and table 1).

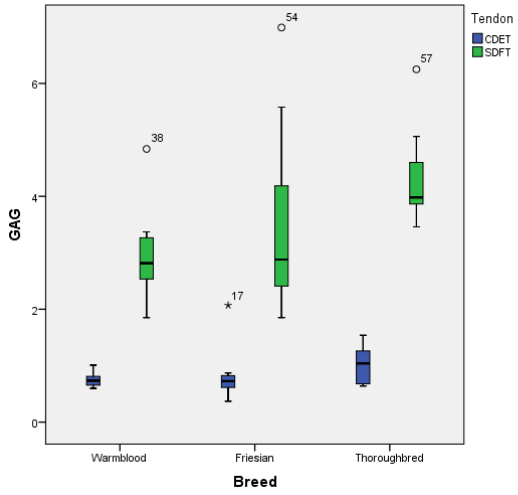

Fig. 7 *GAG content (µg/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds*.

Collagen content

The CDET contains a significant higher collagen content than the SDFT in the tendon of the Warmbloods and the Friesians. There was no significant difference between the CDET and SDFT for the Thoroughbred. In the SDFT, there is a significant difference between Warmbloods and Thoroughbreds. Friesians don't difference significantly. Thoroughbreds being the bred with the highest collagen content. For the CDET, there is no significant difference between any of the breeds. (See figure 8 and table 1).

The Warmblood CDET's and the Friesian CDET's contain significant higher hydroxyproline (Hpro) than the SDFT. For the Thoroughbred this difference isn't significant. For the CDET there isn't a significant difference between any of the breeds. For the SDFT, the Thoroughbred tendons contain significant higher Hpro content than the Warmblood tendons. Friesians don't differ significantly. (See figure 9 and table 1).

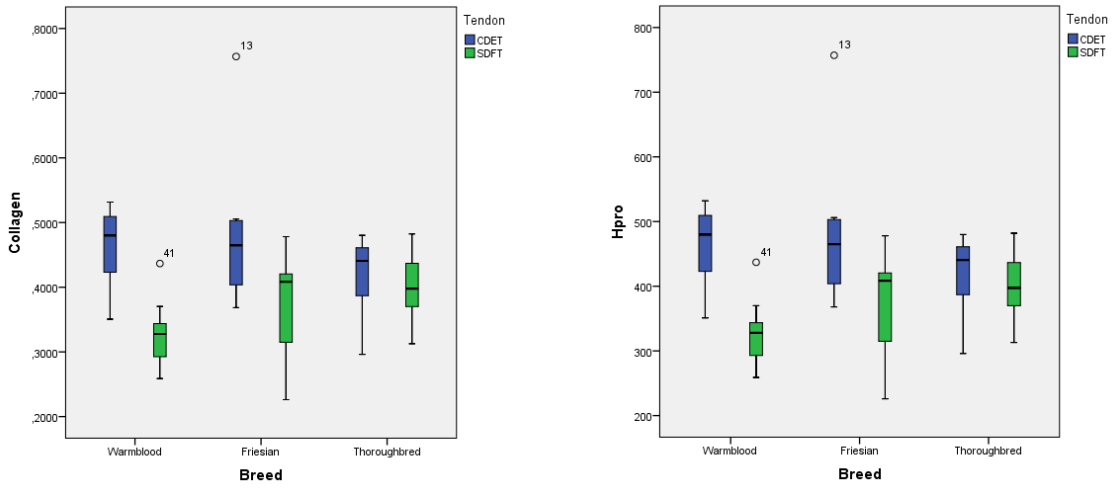

Fig. 8 and 9 *Collagen and Hydroxyproline content (ng/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

Pyrrole crosslink levels

The CDET contains a significant higher collagen content than the SDFT in the tendon of the Friesians and the Thoroughbreds. There was no significant difference between the CDET and SDFT for the Warmbloods. For both CDET and SDFT there is a significant difference between the Friesians and the Thoroughbred. Friesians have significant higher pyrrole crosslinks. There was no significant difference between the Warmbloods and the Friesians or Warmbloods and Thoroughbreds. (See figure 10 and table 1).

There were two outliers (one Warmblood CDET and one Friesian CDET, the statistic test showed a significant difference between the Friesians and the Toroughbreds with and without the two outliers.

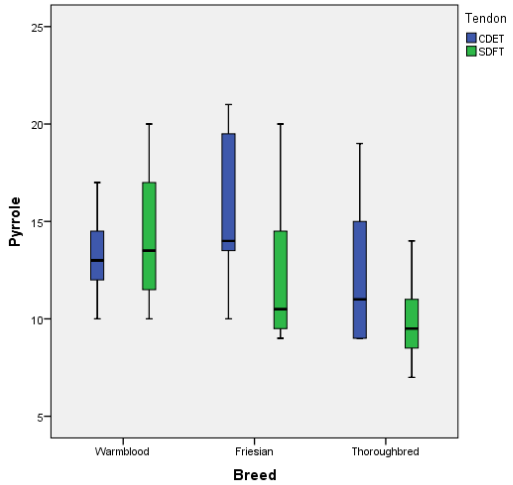

Fig. 10 *Pyrrole content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

Collagen crosslink levels

Hydroxylysine (H-lys) showed a significant difference between the CDET and the SDFT between al breeds. The SDFT contains more H-lys. For both CDET and SDFT there is a significant difference between the Warmbloods and the Thoroughbreds. The tendon of Warmbloods contain more H-lys. (See figure 11 and table 1).

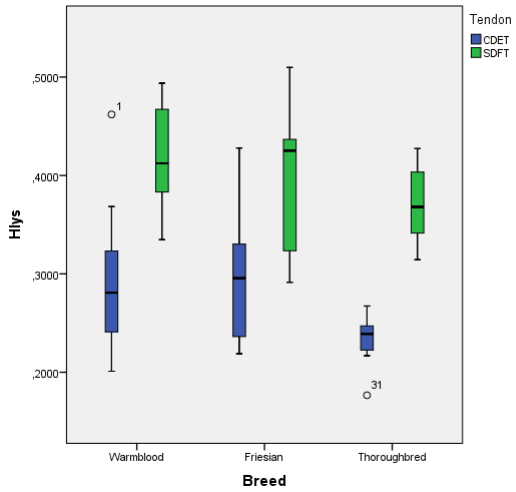

Fig. 11 *Hydroxylysine content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

The CDET contains significant higher proline than the SDFT for all breeds. Warmblood CDET's and Friesian CDET's contain significant higher proline than Thoroughbred CDET's. For the SDFT only the Warmbloods contain significant higher proline than the Thoroughbreds. (See figure 12 and table 1).

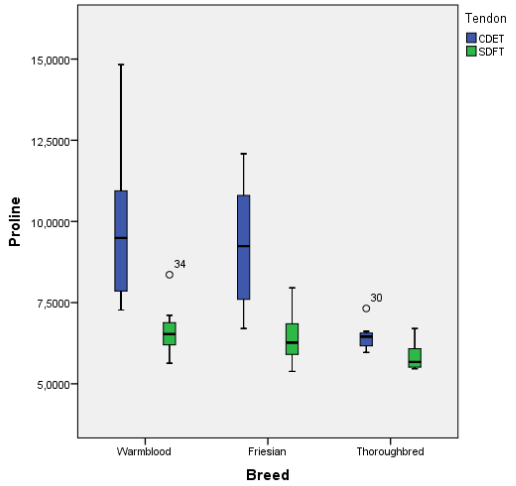

Fig. 12 *Proline content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

Lysine shows a significant difference between the CDET and the SDFT for all breeds. The CDET contains more Lysine. There is no significant difference between the breeds for the SDFT. For the CDET there is a significant difference between Warmbloods and Thoroughbreds (Warmbloods contain more lysine in their tendon) and between the Friesians and the Thoroughbreds (the tendon of Friesians containing more lysine). (See figure 13 and table 1).

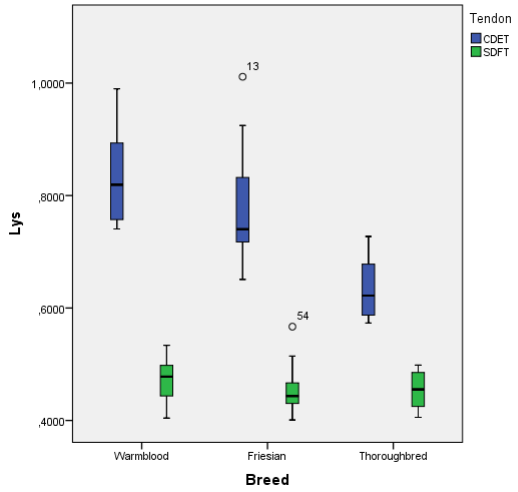

Fig. 13 *Lysine content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

The CDET contains more lysylpyridinoline (LP) for all breeds, but the difference isn't significant. For the CDET, the tendon of Warmbloods and the Friesians contain significant higher LP than the tendon of Thoroughbreds. The SDFT of Warmbloods contain significant higher LP than the tendon of Thoroughbreds. (See figure 14 and table 1).

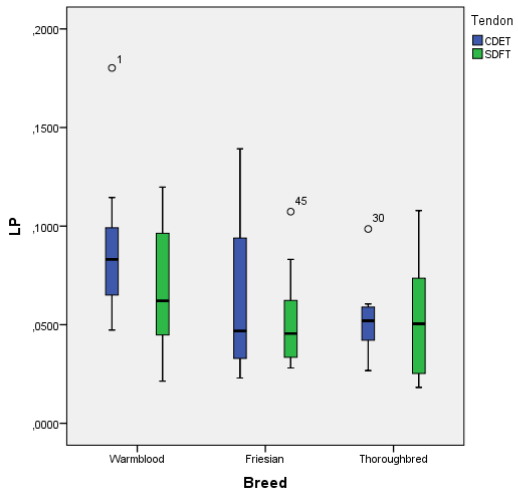

Fig. 14 *LP content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

The CDET contains higher hydroxylysylpyridinoline (HP) for Warmbloods and Friesians. The CDET contains lower HP in Thoroughbred tendons than the Thoroughbred SDFT. These differences between the CDET and the SDFT aren't significant. For the CDET and the SDFT, there are also no significant differences between any of the breeds. (See figure 15 and table 1).

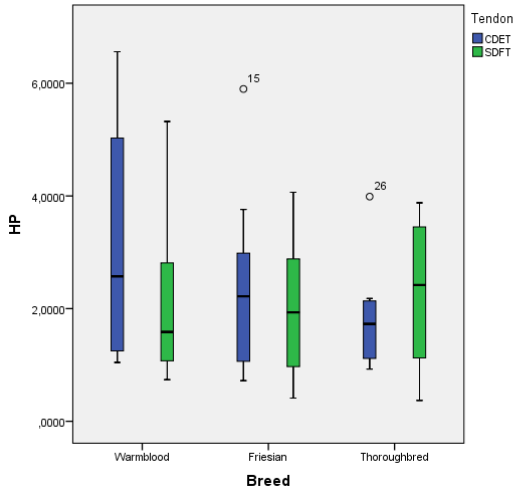

Fig. 15 *HP content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

There is no significant difference between the CDET and the SDFT and there is no significant difference between any of the breeds for pentosidine. (See figure 16 and table 1).

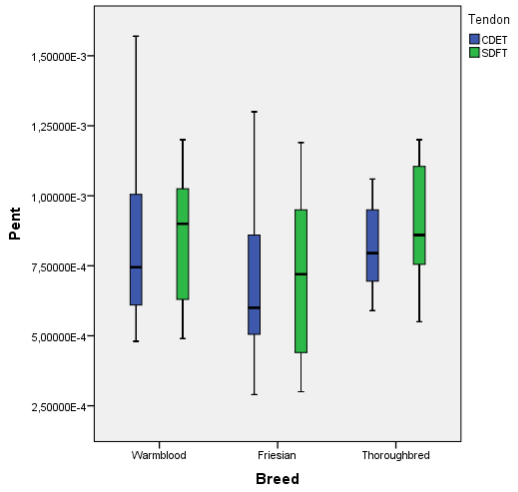

Fig. 16 *Pentosidine content (nmol/mg DW tendon) CDET and SDFT Warmbloods, Friesians and Thoroughbreds.*

Parameter	CDET (mean ± SD)	SDFT (mean ± SD)
Water content (%)		
Warmblood	64.44 ± 1.51	70.82 ± 1.81
Friesian	62.23 ± 2.82	69.24 ± 3.84
Thoroughbred	56.93 ± 2.40	64.25 ± 1.71
DNA, µg/mg		
Warmblood	3.99 ± 1.05	16.23 ± 4.35
Friesian	3.30 ± 0.98	11.78 ± 3.97
Thoroughbred	3.47 ± 0.64	6.51 ± 1.45
GAG, µg/mg		
Warmblood	0.74 ± 0.12	2.96 ± 0.74
Friesian	0.80 ± 0.43	3.44 ± 1.55
Thoroughbred	1.02 ± 0.34	4.33 ± 0.90
Collagen, mg/mg		
Warmblood	0.46 ± 0.06	0.33 ± 0.05
Friesian	0.47 ± 0.10	0.37 ± 0.08
Thoroughbred	0.42 ± 0.06	0.40 ± 0.05
Pyrrole, nmol/mg		
Warmblood	14.43 ± 4.74	13.91 ± 3.60
Friesian	17.56 ± 6.37	12.40 ± 4.05
Thoroughbred	12.50 ± 4.11	10.03 ± 2.10
Hpro, ng/mg		
Warmblood	464.61 ± 57.37	326.97 ± 46.48
Friesian	473.03 ± 100.63	373.43 ± 80.81
Thoroughbred	419.23 ± 61.42	400.58 ± 52.20
H-lys, nmol/mg		
Warmblood	0.29 ± 0.07	0.42 ± 0.05
Friesian	0.29 ± 0.06	0.40 ± 0.07
Thoroughbred	0.23 ± 0.03	0.37 ± 0.04
Proline, nmol/mg		
Warmblood	9.85 ± 2.34	6.60 ± 0.72
Friesian	9.22 ± 1.82	6.42 ± 0.77
Thoroughbred	6.46 ± 0.41	5.84 ± 0.45
Lysine, nmol/mg		
Warmblood	0.84 ± 0.08	0.47 ± 0.04
Friesian	0.78 ± 0.10	0.46 ± 0.05
Thoroughbred	0.63 ±0.06	0.46 ± 0.04
LP, nmol/mg		
Warmblood	0.09 ± 0.03	0.07 ± 0.03
Friesian	0.06 ± 0.04	0.05 ± 0.02
Thoroughbred	0.05 ± 0.02	0.05 ± 0.03
HP, nmol/mg		
Warmblood	3.11 ± 1.95	2.02 ± 1.46
Friesian	2.37 ± 1.48	1.98 ± 1.22
Thoroughbred	1.86 ± 0.98	2.28 ± 1.36
Pentosidine, nmol/mg		
Warmblood	0.00083 ± 0.0003	0.00086 ± 0.0002
Friesian	0.00069 ± 0.0003	0.00071 ± 0.0003
Thoroughbred	0.00082 ± 0.0002	0.00090 ± 0.0002

Table 1. *Biochemical parameters of the CDET and SDFT of Warmbloods, Friesians and Thoroughbreds.*

Discussion and conclusion

This research demonstrates that Friesians have significant higher pyrrole crosslink levels than Thoroughbreds for both CDET and SDFT. There was no significant difference between the tendon of the Warmbloods and the Friesians. This finding doesn't support the hypothesis in the way that the more crosslinks found, the stiffer and stronger the tendon, because Gussekloo et al. found that Friesian tendons are more elastic than tendon of ponies. [1] But this finding still can cause biomechanically differences between the different breeds. Thorpe et al. mentioned that mechanical strength of a tendon is based on the correct orientation of collagen molecules in the fibrils and the stabilization between collagen molecules by the formation of chemical crosslinks. [2] It is possible that the high content of the pyrrole crosslinks in the Friesian tendons aren't orientated correctly causing less mechanical strength than they would if they were orientated correctly. Lindeman et al. showed that human vessel-wall weakening in advanced abdominal aneurysms and aneurysms in Marfan syndrome show dramatically altered collagen architectures with loss of the collagen knitting. [21] They demonstrated that the collagen microarchitecture strongly influence the mechanical properties of tissue and that perturbations in the collagen networks may lead to mechanical failure. [21] Furthermore they showed that there is an increased intramolecular collagen crosslinking in the aneurismal wall in both advanced abdominal aneurysms and Marfan tissue. [21] This could also be the case in the tendons of the Friesians. To determine this, further histological and functional research using an atomic force microscopy (AFM) is necessary. There is also an increase of crosslinks with age. [22, 23] Perhaps the aging process of collagen is increased in Friesian tendons. That could be a possibility of the high pyrrole content in the tendons of Friesians.

Another finding of this research is that the level of hydroxylysylpyridinoline (HP) crosslinks are higher than the lysylpyridinoline (LP) crosslink levels for all breeds. The pyrrole levels are higher than the HP and LP levels in all breeds. This is conflicting with the results of the research of Thorpe et al. [2] The superficial digital flexor tendon of the Friesians shows the lowest HP and LP content of the different breeds. This difference isn't significant. For the common digital extensor tendon the Friesian tendons contain HP and LP levels in between Warmbloods and Thoroughbreds. Thorpe et al. mentioned that LP and HP doesn't show a correlation between these crosslink levels and the mechanical properties. [2] For the other crosslinks the Friesian tendons don't differ significantly from those of the other breeds.

For the water content, DNA content, GAG content and Hydroxylysine content the superficial digital flexor tendon contains a significant higher level than the common digital extensor tendon. The common digital extensor tendon contains significantly more lysine and proline. For pyrrole, the CDET of the Friesians and the Thoroughbreds contain significant higher levels than the SDFT. For Hydroxyproline and collagen, the CDET contains a significant higher level in tendon of Warmbloods and Friesians than the SDFT. For LP, HP and pentosidine there is no significant difference between the CDET and het SDFT. This shows that these two tendons differ from one another biochemically. This can be explained by the different functions of these different type of tendons: the spring-like (or energy storing) tendon and the positional tendon. [2,3,7]

Before concluding, it should be noted that I used only twelve tendons for the Warmbloods and Friesians and eight for the Thoroughbreds. It would be better to increase this number and to have the same number of tendons for each breed. For pyrrole there were two significant outliers which made the significant difference between the Friesians and the Thoroughbreds for the CDET even bigger. The tendon of Friesians contain a significant higher pyrrole crosslink level than the tendon of Thoroughbreds. This could be a cause for the different biomechanically properties of the Friesian tendons. But further research is necessary.

Acknowledgements

I would like to thank W. Back for supervising me during the entire research, C.H.A. van de Lest for helping me with the biochemical tests in the laboratory and the statistics and I would like to thank J. van Dasselaar for helping me collecting the distal parts of the forelimbs of the horses.

References

(1) Gussekloo, S. W.; Lankester, J.; Kersten, W.; Back, W. Effect of differences in tendon properties on functionality of the passive stay apparatus in horses. *Am. J. Vet. Res.* **2011***, 72*, 474-483.

(2) Thorpe, C. T.; Stark, R. J.; Goodship, A. E.; Birch, H. L. Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels. *Equine Vet. J.* **2010***, 42 Suppl 38*, 538-543.

(3) Souza, M. V.; van Weeren, P. R.; van Schie, H. T.; van de Lest, C. H. Regional differences in biochemical, biomechanical and histomorphological characteristics of the equine suspensory ligament. *Equine Vet. J.* **2010***, 42*, 611-620.

(4) Baxter, G. M.; Adams, O. R. In *Adams and Stashak's lameness in horses.* Wiley-Blackwell: Chichester, West Sussex; Ames, Iowa, 2011; .

(5) Dyce, K. M.; Sack, W. O.; Wensing, C. J. G. In *Textbook of veterinary anatomy;* Saunders Elsevier: St. Louis, Mo., 2010; .

(6) Budras, K.; Röck, S. In *Anatomie van het paard : atlas en leerboek;* Bloemendal ; Media Boekservice: Amersfoort; Eerbeek, 2005; .

(7) Birch, H. L. Tendon matrix composition and turnover in relation to functional requirements. *Int. J. Exp. Pathol.* **2007***, 88*, 241-248.

(8) Auer, J. A.; Stick, J. A. In *Equine surgery;* Saunders Elsevier: St. Louis, Mo., 2006; .

(9) Tsuzaki, M.; Yamauchi, M.; Banes, A. J. Tendon collagens: extracellular matrix composition in shear stress and tensile components of flexor tendons. *Connect. Tissue Res.* **1993***, 29*, 141-152.

(10) Kannus, P. Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sports* **2000***, 10*, 312-320.

(11) Kjaer, M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* **2004***, 84*, 649-698.

(12) Lin, Y. L.; Brama, P. A.; Kiers, G. H.; van Weeren, P. R.; DeGroot, J. Extracellular matrix composition of the equine superficial digital flexor tendon: relationship with age and anatomical site. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* **2005***, 52*, 333-338.

(13) Batson, E. L.; Paramour, R. J.; Smith, T. J.; Birch, H. L.; Patterson-Kane, J. C.; Goodship, A. E. Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? *Equine Vet. J.* **2003***, 35*, 314-318.

(14) Kastelic, J.; Galeski, A.; Baer, E. The multicomposite structure of tendon. *Connect. Tissue Res.* **1978***, 6*, 11-23.

(15) Scott, J. E. Proteoglycan:collagen interactions and subfibrillar structure in collagen fibrils. Implications in the development and ageing of connective tissues. *J. Anat.* **1990***, 169*, 23-35.

(16) Scott, J. E. Extracellular matrix, supramolecular organisation and shape. *J. Anat.* **1995***, 187 (Pt 2)*, 259-269.

(17) Morree, J. J. d. In *Dynamiek van het menselijk bindweefsel : functie, beschadiging en herstel;* Bohn Stafleu van Loghum: Houten, 2008; .

(18) Bailey, A. J.; Paul, R. G.; Knott, L. Mechanisms of maturation and ageing of collagen. *Mech. Ageing Dev.* **1998***, 106*, 1-56.

(19) Kim, Y. J.; Sah, R. L.; Doong, J. Y.; Grodzinsky, A. J. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal. Biochem.* **1988***, 174*, 168-176.

(20) Farndale, R. W.; Buttle, D. J.; Barrett, A. J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta* **1986***, 883*, 173-177.

(21) Lindeman, J. H. N.; Ashcroft, B. A.; Beenakker, J. M.; Es, M. v.; Koekkoek, N. B. R.; Prins, F. A.; Tielemans, J. F.; Abdul-Hussien, H.; Bank, R. A.; Oosterkamp, T. H. Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in Marfan syndrome. *PNAS*. **2009***, 107,* 862-865.

(22) Monnier, V. M.; Sell, D. R.; Abdul-Karim, F. W.; Emancipator, S. N. Collagen browning and cross-linking are increased in chronic experimental hyperglycemia. *Diabetes*, **1988***, 37,* 867-872.

(23) Eyre, D. R.; Paz, M. A.; Gallop, P. M. Cross-linking in collagen and elastin. *Annu. Rev. Biochem.* **1984***, 53,* 717-748.