EXTRACELLULAR MATRIX SYNTHESIS AND DEGRADATION IN FUNCTIONALLY DISTINCT TENDONS

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A thesis submitted to University College London for the degree of Doctor of Philosophy

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August 2010

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I, Chavaunne Thandiwe Thorpe confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed.....................................

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Abstract

Tendon injury is common in humans and horses, and incidence increases with age. The high-strain energy storing equine superficial digital flexor (SDFT) is injured more frequently than the low-strain positional common digital extensor (CDET). However, previous work indicated that matrix turnover is greater in the CDET than in the SDFT. It was hypothesised that matrix turnover is programmed by the cells' strain environment; therefore high-strain energy storing tendons would have a lower rate of matrix turnover than low-strain positional tendons and the rate of matrix turnover would decrease with increasing age. The rate of matrix turnover was investigated by measuring the potential of the cells to synthesise and degrade matrix proteins, measuring the half-life of the collagenous and non-collagenous matrix proteins and assessing collagen turnover at the protein level. *In vitro* cell phenotype was also assessed in 2D and 3D culture and the effect of load on cells within native tissue was determined. The results show that turnover of collagenous and non-collagenous matrix proteins is differentially regulated in the functionally distinct SDFT and CDET. CDET tenocytes show greater potential for collagen turnover, whereas SDFT tenocytes have a greater potential for proteoglycan turnover; differences that are also present at the protein level. The differences in cell phenotype identified *in vivo* were lost in 2D and 3D culture, but tendon organ culture resulted in the maintenance of tenocyte phenotype. The cells' ability to turnover the matrix does not decrease with increasing age, but collagen within the SDFT appears to become more resistant to degradation with ageing. This results in the accumulation of partially degraded collagen within the SDFT which may have a detrimental effect on tendon mechanical properties. These findings will help to elucidate the mechanisms behind the development of age-related tendinopathy and will be of use when developing treatment regimes.

Table of Contents

5

Acknowledgements

Table of Figures

List of Tables

Publications and Presentations Related to this Thesis

Peer reviewed journal articles:

Thorpe, C.T., Streeter, I., Pinchbeck, G.L., Goodship, A.E., Clegg, P.D., and Birch, H.L. (2010) Aspartic Acid Racemization and Collagen Degradation Markers Reveal an Accumulation of Damage in Tendon Collagen That Is Enhanced with Aging. *J. Biol. Chem.* **285**, 15674-15681.

Thorpe, C.T., Clegg, P.D., and Birch, H.L. (2010) A review of tendon injury: Why is the equine superficial digital flexor tendon most at risk? *Equine Vet. J.* **42**, 174-180.

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Thorpe, C.T., Goodship, A.E., Clegg, P.D., and Birch, H.L. High strain tendons repair less frequently: implications for our Achilles heel. *BSMB satellite meeting: 'Tendon Biology and Pathology.'* September 2010, Norwich, UK.

Thorpe, C.T., Goodship, A.E., Clegg, P.D., and Birch, H.L. Tendinopathies: Is the Ageing Cell Responsible for Reduced Tendon Matrix Turnover in Older Aged Individuals? *ISL&T*. February 2010, Hong Kong.

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Thorpe, C.T., Goodship, A.E., Clegg, P.D., and Birch, H.L. Is the specialised phenotype of tendon cells in functionally distinct tendons an inherent property or programmed by the cell environment? *FECTS*, July 2010, Davos, Switzerland.

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Abbreviations

ACTB   β-Actin

ADAM   A Disintegrin and Metalloproteinase

ADAMTS   A Disintegrin-like and Metalloproteinase with Thrombospondin Motifs

AGE   Advanced Glycation End-product

APMA   Aminophenylmercuric Acetate

Asp   Aspartic Acid

BLAST   Basic Local Alignment Search Tool

C1,2C   Carboxy-terminal ¾ fragment of Type I Collagen

CDET   Common Digital Extensor Tendon

COMP   Collagen Oligomeric Matrix Protein

CSA   Cross Sectional Area

DDFT   Deep Digital Flexor Tendon

deH-HLHNL Dehydro-Hydroxylysinohydroxynorleucine

deH-LHNL   Dehydro-Lysinohydroxynorleucine

DEPC   Diethylpyrocarbonate

DMB   Dimethylmethylene Blue

DMBA   Dimethylaminobenzaldehyde

DMSO   Dimethyl Sulphoxide

DMEM   Dulbecco's Modified Eagle's Medium

DNA   Deoxyribonucleic Acid

ECL   Enhanced Chemi-Luminescent

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ESWT	Extracorporeal Shock Wave Therapy
FCS	Foetal Calf Serum
GAG	Glycosaminoglycan
GapDH	Gylceraldehyde 3-Phosphate Dehydrogenase
GuHCl	Guanidine Hydrochloride
HBSS	Hanks Balanced Salt Solution
HDF	Human Dermal Fibroblast
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HIRP5	HIRA Interacting Protein-5
HLKNL	Hydroxylysino-Keto-Norleucine
HP	Hydroxylysyl-pyridinoline
HPLC	High Performance Liquid Chromatography
HPRT1	Hypoxanthine Phosphoribosyltransferase-1
HYL-ALD	Hydroxylysine-Aldehyde
ICTP	Carboxy-Terminal Telopeptide of Type I Collagen
IL1-β	Interleukin 1-Beta
LKNL	Lysino-Keto-Norleucine
LP	Lysyl-pyridinoline
MAET	Medial Accessory Extensor Tendon

MCP	Metacarpo-phalangeal Joint
MMLV-RT	Moloney Murine Leukaemia Virus Reverse Transcriptase
MMP	Matrix Metalloproteinase
MRPS7	Mitochondrial Ribosomal Protein S7
MSC	Mesenchymal Stromal Cell
NAC	N-acetyl-L-cysteine
OPA	O-phthaldialdehyde
PBS	Phosphate Buffered Saline
PICP	Carboxy-Terminal Peptide of Type I Pro-collagen
PINP	Amino-Terminal Peptide of Type I Pro-collagen
PRP	Platelet Rich Plasma
RFU	Relative Fluorescence Units
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RPM	Revolutions per Minute
RSD	Relative Standard Deviation
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDFT	Superficial Digital Flexor Tendon
SDHA	Succinate Dehydrogenase A
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SL	Suspensory Ligament

SLRP   Small Leucine Rich Proteoglycan

TAPSO   3-[N-Tris (Hydroxymethyl) Methylamino]-2-Hydroxypropanesulfonic acid

TBP   TATA-Box Binding Protein

TFA   Trifluoroacetic Acid

THF   Tetrahydrofuran

TIMP   Tissue Inhibitor of Matrix Metalloproteinase

CHAPTER ONE

1. Introduction

1.1 General Introduction

The primary function of most tendons is to transfer force generated by a muscle to a bone in order to facilitate movement around a joint. For efficient function tendons must be relatively inextensible and able to resist high forces (Ker 2002). This property is conferred by the molecular composition; tendons are comprised mainly of type I collagen molecules organised into a hierarchical structure which results in high tensile strength. Historically, it had been assumed that tendons are simple structures with a simple function. However, recent work has found that this is far from true, in addition to transferring force specific tendons have other functions including energy storage (Alexander 1991). Recent studies have focused on understanding tendon formation and development; however there is much that is not known about mature tendon in terms of tendon turnover and maintenance of properties required for efficient function. This is important as tendon injury is one of the most common musculoskeletal injuries both in athletes and the general population (Jarvinen *et al.,* 2005). The incidence of injury increases with age (Kannus *et al.,* 1989) and recurrence of injury is common as the tendon healing response is slow and inadequate; the defect is replaced by scar tissue which is likely to be weaker than the original tissue (Corr *et al.,* 2009). Tendon injury is frequently preceded by degenerative changes to the extracellular matrix (Birch *et al.,* 1998) rather than occurring as the result of a single overloading event, but it is not clear what causes this initial degeneration. The initial degeneration is not usually accompanied by an inflammatory response, and so degenerative conditions that affect tendons are now referred to as tendinopathies rather than tendinitis (Riley 2008), which would imply an inflammatory component. Specific tendons are more prone to injury than others; for example energy storing tendons such as the Achilles tendon are injured far more frequently than tendons that function purely to position the limb, such as the anterior tibialis tendon (Kausch and Rütt 1998). Tendon injury is also a common cause of wastage in the equine athlete; with a similar epidemiology and aetiology to human tendon injury. In horses, the majority of injuries occur to the energy storing superficial digital flexor tendon (SDFT), while the positional common digital extensor tendon (CDET) is rarely injured (Ely *et al.,* 2009). The horse is a good model for studying the effect of tendon type and age on extracellular matrix turnover which is likely to be important in the aetiology of tendinopathy, the initiation and progression of which appears to be similar between horses and humans.

1.2. Anatomy of the Equine Forelimb

The horse is a cursorial animal, i.e. 'designed' to run at high speed. Evolution has resulted in the lengthening of the limbs, in particular the distal portion, and reduction of muscle mass and digits in this region. In the forelimb all muscles are situated proximal to the carpus, and are connected to the distal part of the limb by long tendons. The four main tendinous structures in the distal part of the equine forelimb are shown in Figure 1-1. The SDFT is situated on the palmar aspect of the forelimb just below the skin. The superficial digital flexor muscle originates on the medial epicondyle of the humerus and inserts via its long tendon onto the proximal and middle phalanges, the tendon also receives an accessory branch from the radius. The SDFT is superficial to the deep digital flexor tendon (DDFT); the deep digital flexor muscle also originates on the medial epicondyle of the humerus and its corresponding tendon inserts onto the distal phalanx, also receiving an accessory branch from the carpus. At the level of the metacarpophalangeal joint the SDFT forms a sleeve around the DDFT. The suspensory ligament (SL) is also known as the interosseus muscle, although it contains little muscular tissue, and is situated deep to the DDFT. It originates on the proximopalmar aspect of the third metacarpal bone. It divides proximal to the metacarpophalangeal joint; the main branches insert onto the proximal sesamoid bones while the extensor branches extend around the limb to insert onto the CDET. The CDET is situated on the dorsal aspect of the third metacarpal; the common digital extensor muscle originates on the lateral epicondyle of the humerus and its corresponding tendon inserts onto the proximal and distal phalanges (Budras *et al.,* 2001; Goody 2001). On the lateral border of the CDET there is a thin accessory branch of the extensor tendon (Denoix 2000), approximately 5 mm in diameter. Although not well documented, this accessory tendon is also often present on the medial border of the CDET (Birch H.L, personal communication). In some horses either the medial or lateral accessory branch, or both branches, are fused to the CDET, whereas in others they are completely separate from the CDET proper. It is likely that as the horse has evolved to have fewer toes these vestigial tendons have persisted to a greater extent in some horses than in others; in animals with a greater number of toes, such as the cow and goat, the extensor tendon divides to insert onto each toe (Dyce *et al.,* 2002).
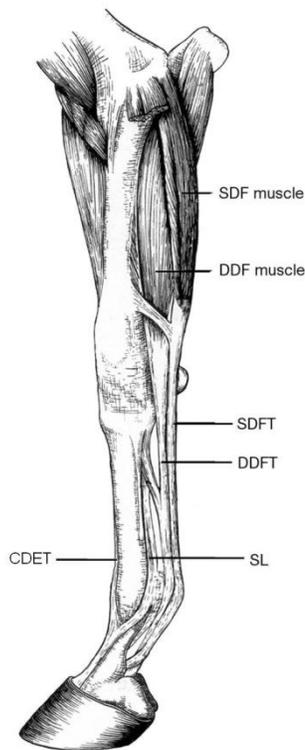
Figure 1-1: Anatomy of the equine forelimb showing the four main tendinous structures. Adapted from Wilson *et al.* (2001).

1.3. Tendon Function

Tendons in the equine forelimb act mainly to position the limb correctly during locomotion and prevent hyperextension of the metacarpophalangeal joint (Butcher *et al.,* 2007). Some tendons have an additional function, acting as springs to store and release energy as they are stretched and recoil during the stance and swing phase of each stride such that the energetic cost of locomotion is reduced (Alexander 1991). In humans, the tendon that acts as the main energy store is the Achilles tendon (Lichtwark and Wilson 2005). The SDFT and SL are the main energy storing structures in the equine forelimb and are subjected to higher strains than the DDFT and CDET, which do not contribute significantly to energy storage (Wilson *et al.,* 2001). Energy storing tendons experience much higher strains than positional tendons; strains of up to 10.3% have been recorded in the Achilles tendon (Lichtwark and Wilson 2005), whereas the anterior tibialis tendon, which functions purely as a positional tendon, experiences maximum strains of 3.1% (Maganaris and Paul 1999). Similar results have been reported in the horse; during gallop, *in vivo* strains of 16% in the SDFT have been recorded (Stephens *et al.,* 1989), which is similar to the failure strains of 15-17% recorded *in vitro* (Dowling *et al.,* 2002; Gerard *et al.,* 2005). In contrast, maximum

25

in vivo strain for the positional CDET has been estimated at 2.5% (Birch *et al.,* 2008b), which is almost four times lower than the failure strain of 9.7% recorded *in vitro* (Batson *et al.,* 2003). Although the DDFT is situated on the palmar aspect of the limb between the SDFT and SL, its corresponding muscle exhibits differences in muscle architecture and fibre type compared to the SDF muscle, and its functions appear to be flexion of the distal phalangeal joint during late swing and stabilisation of the metacarpophalangeal joint rather than storage and return of energy (Butcher *et al.,* 2007; Butcher *et al.,* 2009). Correspondingly, *in vivo* studies have shown than the DDFT experiences lower peak forces and strains during locomotion than the SDFT or SL (Butcher *et al.,* 2007; Platt *et al.,* 1994). Energy storing tendons are therefore subjected to significantly higher stresses than positional tendons; the failure stress of most tendons is approximately 100 MPa and while the majority of tendons in the equine forelimb only experience stresses up to 30 MPa, and those in the human leg experiences stresses of about 20 MPa (Ker *et al.,* 1988), it is possible the equine SDFT and human Achilles tendon may experiences stresses as high as 90 MPa (Brown *et al.,* 2003; Ker *et al.,* 1988).

1.3.1. Tendon Biomechanics

The function of a tendon is reflected in the mechanical properties of the tendon tissue. Tendon is a viscoelastic composite material consisting of collagen fibrils embedded in a softer non-collagenous matrix (Puxkandl *et al.,* 2002). The elastic component is provided mainly by the collagenous matrix whereas viscosity is provided largely by the non-collagenous fraction of the matrix (Puxkandl *et al.,* 2002). When tendon is loaded, the initial lengthening is due to extension of the collagen fibres (Screen *et al.,* 2004; Silver *et al.,* 2003), however above strains of 3% the dominant mechanism of extension is fibre sliding (Screen *et al.,* 2004). Sliding between fibres is thought to be controlled by the non-collagenous proteins (mainly proteoglycans) within the matrix as they provide interactions between adjacent collagen fibres (Scott 2003; Screen *et al.,* 2005a; Screen *et al.,* 2006; Screen 2008). These studies indicate that the viscoelastic response of tendon is dependent on both the amount and rate of applied strain. The response of tendons to loading will also depend on their specific mechanical properties, which have been shown to differ between tendons with different functions; the SDFT is composed of a less stiff material than the CDET, for example (Birch 2007). These differences in mechanical properties arise from

variation in the structure and molecular composition of the tendon matrix; which in healthy tendon is synthesised and maintained by tendon fibroblasts (tenocytes).

1.4. Epidemiology of Tendon Injuries

Tendon injury is one of the most common forms of musculoskeletal injuries that occur to horses competing in all disciplines, although tendon injuries in racehorses are the most investigated. Injuries to the musculoskeletal system have been found to account for 82% of all injuries to racehorses competing in National Hunt and flat races, and of these 46% involved tendons or ligaments (Ely *et al.,* 2004; Williams *et al.,* 2001). Another study reported that tendon or ligament strain accounts for 53% of musculoskeletal injuries that occur during hurdle and steeplechase races (Pinchbeck *et al.,* 2004). A 12 year epidemiological study found that tendon injury was the most common reason for retirement in racing Thoroughbreds in Hong Kong (Lam *et al.,* 2007). Furthermore, it has been found that, over the period of one season, 15% of both National Hunt horses (Ely *et al.,* 2004) and Thoroughbred flat racehorses (Kasashima *et al.,* 2004) in training suffered from a tendon or ligament injury as diagnosed by ultrasound.

The majority of tendon injuries (97-99%) occur to the forelimb tendons (Kasashima *et al.,* 2004; Lam *et al.,* 2007), with the energy storing SDFT being injured in 75-93% of cases, and the remaining injuries occurring to the SL (Ely *et al.,* 2004; Kasashima *et al.,* 2004). Prevalence of SDFT pathology has been found to be 24% in National Hunt horses in training that were assessed ultrasonographically over two seasons (Avella *et al.,* 2009). Injuries to the mid-metacarpal tensional region of the positional DDFT and CDET are rare although injures to the DDFT at both the phalangeal level (Murray *et al.,* 2006a) and within the digital sheath (Smith and Wright 2006) are reported, however the prevalence of such injuries is not yet clear. The risk of tendon injury increases with increasing age (Ely *et al.,* 2004; Ely *et al.,* 2009; Kasashima *et al.,* 2004; Perkins *et al.,* 2005), and is more common in National Hunt horses than in those racing on the flat (Williams *et al.,* 2001). There are several differences between National Hunt horses and flat racehorses which may account for the higher incidence of tendon injury in National Hunt racing. National Hunt horses tend to be older than those that race on the flat, and compete over longer distances and over a greater number of seasons (Williams *et al.,* 2001). Their tendons are also likely to be placed under higher strains when landing over fences. Some studies have reported a higher

risk of tendon injury in entire male horses (Kasashima *et al.,* 2004; Lam *et al.,* 2007; Perkins *et al.,* 2005) but other studies do not support this finding as they are unable to differentiate between entire males and geldings due to the small number of entire males studied (Ely *et al.,* 2004; Ely *et al.,* 2009). Tendon re-injury is a common problem; it has been reported that 23% to 67% of horses with a tendon injury treated using conservative methods will re-injure their tendons within two years of the original injury (Dyson 2004; Marr *et al.,* 1993), and many horses never recover sufficiently from a tendon injury to compete at the same level as they did previously.

There is little information available regarding tendon injury in horses competing in other disciplines. However it has been reported that tendon injury accounts for 43% of injuries that occur to event horses in training, with 33% of injuries to the SDFT, 31% to the SL and 17% to the DDFT (including injuries to the accessory ligament) (Singer *et al.,* 2008). Elite show jumpers have been reported to have a high risk of injury to the forelimb SDFT and DDFT, whereas horses competing in dressage have a high risk of injury to the hind limb SL based on cases referred to one referral centre with orthopaedic injury (Murray *et al.,* 2006b).

Epidemiology of human tendon injury is similar to that reported in horses; overuse injuries occur in athletes, both elite and recreational, and are also a common problem in the workplace (Ferretti 1986; Frost *et al.,* 2002). The majority of overuse injuries occur to the energy storing Achilles tendon; the incidence of Achilles tendinopathy is reported as 6% in the general population, but increases to over 50% in elite endurance athletes, where it is positively correlated with hours of training (Knobloch *et al.,* 2008; Kujala *et al.,* 2005). The incidence of tendinopathy also increases with increasing age and is more common in males (Clayton and Court-Brown 2008; Kannus *et al.,* 1989). Running surface is also a factor in the epidemiology of Achilles tendinopathy, with increased risk of injury when running on sand (Knobloch *et al.,* 2008). Recurrence of tendon injury is also a common problem, and symptoms can persist for several years in some cases (Kettunen *et al.,* 2002). In contrast, positional tendons such as the anterior tibialis tendon are not prone to tendinopathy (Kausch and Rütt 1998).

Identification of factors associated with tendon injury gives important clues to the underlying causes of the injury. Almost all tendon injuries occur to the forelimb tendons

and this is likely to be due to several factors. The forelimbs of the horse carry 60% of the horse's bodyweight when standing and can experience loads in excess of 170% bodyweight when galloping (Swanstrom *et al.,* 2005). There are also differences in function between the hindlimbs and forelimbs which may contribute towards the greater incidence of injury to forelimb tendons; the hindlimbs provide propulsion whereas the forelimbs are involved in braking (Dutto *et al.,* 2004) and therefore are likely to experience higher strains. Forelimb tendons are also likely to experience extremely high strains when landing over fences. It is evident that energy storing tendons such as the equine SDFT and human Achilles are more prone to overload injury than positional tendons such as the equine CDET and human anterior tibialis; this may be due to the fact that energy storing tendons experience much higher strains than those that act purely to position the limb. Ageing has also been identified as an important factor that might predispose an individual to tendon injury; however it is not clear why this is important. There may be an accumulation of micro-damage within the tendon extracellular matrix or alternatively there may be a reduction in the ability of cells in aged tendons to maintain and repair the matrix, possibly due to a decrease in the activity of aged cells (Smith *et al.,* 2002a).

1.5. Tendon Composition and Structure

The specialised molecular composition and organisation of tendon results in a high strength structure able to resist uni-directional forces. Tendons consist of a dense fibrous extracellular matrix with a high water content, which is synthesised and maintained by a small population of tenocytes. The matrix is composed mainly of type I collagen, with a small percentage of other collagens and non-collagenous proteins. Collagen fibrils are orientated in the direction of force application and are arranged in a hierarchical structure (Kastelic *et al.,* 1978) which results in high tensile strength (Figure 1-2). Tropocollagen molecules are synthesised by tenocytes and self-assemble to form fibrils, which then form fibres and then fibre bundles (fascicles). Fascicles are surrounded by the endotenon, and are grouped together to form the tendon, which is surrounded by a connective tissue sheath called the epitenon (Kastelic *et al.,* 1978).
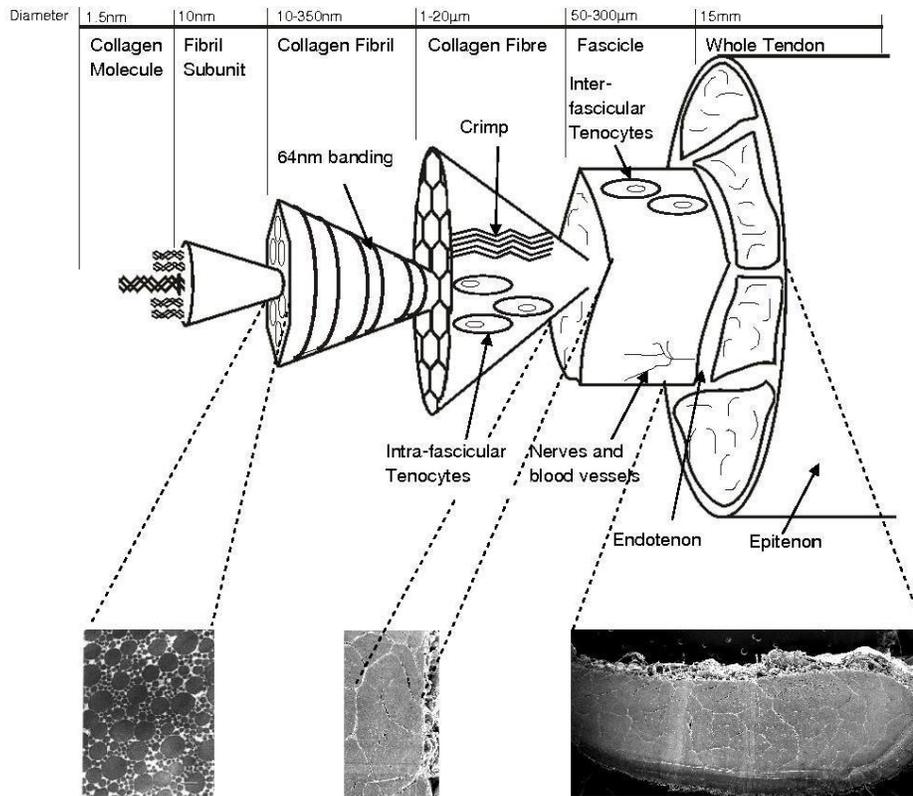
Figure 1-2: Schematic showing the hierarchical structure of tendon.

1.5.1. Molecular Composition

The main component of tendon is water, which makes up 55 - 70% of the wet weight of the tendon. The dry weight of the tendon is comprised mainly of collagen (60% - 85%); currently 28 different types of collagen have been identified but type I collagen is the most abundant in tendon (approximately 95%) with varying amounts of collagen types III, V, XII and XIV depending on tendon type (Kjaer 2004). Approximately 5% of the dry weight is made up of non-collagenous glycoproteins and proteoglycans, the most abundant proteoglycans being the small leucine rich proteoglycans (SLRPs) decorin, biglycan, fibromodulin and lumican with small amounts of aggrecan (Vogel and Meyers 1999). The most abundant glycoproteins found in tendon are collagen oligomeric matrix protein (COMP), tenascin-C and tenomodulin (Docheva *et al.,* 2005; Jarvinen *et al.,* 2000; Smith *et al.,* 1997).

1.5.1.1.   Collagen

The majority of collagen in tendon is the fibril forming type I collagen, which gives the tendon its high tensile strength. The second most abundant collagen found in tendon is another fibrillar collagen, type III, which comprises up to 10% of the total collagen,

depending on several factors including tendon type and age (Birch *et al.,* 1999a; Riley *et al.,* 1994). Collagen type III has been shown to be essential for normal fibrillogenesis and is thought to regulate the size of type I fibrils (Kadler *et al.,* 1990). This is supported by evidence of higher type III content in immature and healing tendons (Banos *et al.,* 2008; Riley *et al.,* 1994). Collagen type V is often located in the core of type I fibrils and is thought to provide a template for fibrillogenesis (Riley 2004). Types XII and XIV collagen are Fibril-Associated Collagens with Interrupted Triple helices and are found associated with type I collagen. It is thought they provide a molecular bridge between fibrillar collagens and other matrix molecules and are involved in stabilising fibrils during development (type XII) and limiting fibril diameter (type XIV) (Banos *et al.,* 2008). The fibrillar collagens are dominated by long triple helical regions and are formed of three polypeptide chains; type I collagen consists of two α1 chains and one α2 chain and therefore there are two genes involved in collagen synthesis, one that codes for the α1 chain (Col1A1) and one for the α2 chain (Col1A2).

Collagen Synthesis
Synthesis of collagen begins with the transcription of the genes that code for the α1 and α2 chains from DNA to messenger RNA (mRNA). The mRNA is transported from the nucleus to a ribosome within the cytoplasm of the cell (Hendershot and Bulleid 2000) (Figure 1-3). The ribosome then attaches to the rough endoplasmic reticulum for translation of the mRNA into pro-polypeptide α chains before assembly of three chains into pro-collagen molecules (Walter and Johnson 1994). The pro-collagen molecule consists of a triple helical region with non-helical amino (N-) and carboxy (C-) terminal telopeptides at each end (Hendershot and Bulleid 2000). Each polypeptide chain that forms the triple helical pro-collagen molecule has a repeating Gly-X-Y triplet, where X and Y are normally proline and hydroxyproline, respectively (Canty and Kadler 2005). Glycine is one of the smallest amino acids and this allows three polypeptide chains to wind together in a triple helix with glycine residues at the core to form 300 nm long pro-collagen monomers.
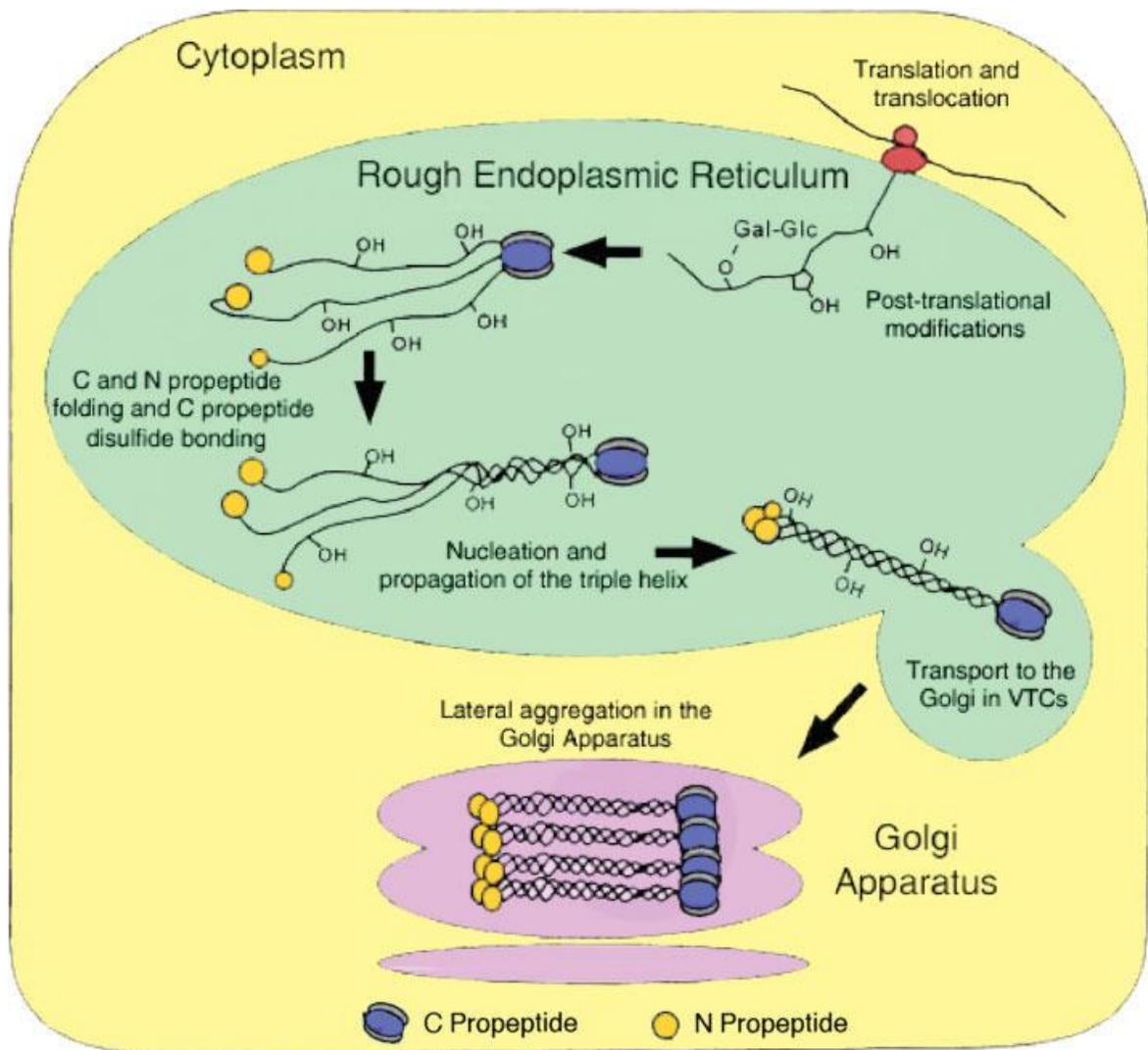
Figure 1-3: Diagram showing synthesis of pro-collagen polypeptide chains, post-translational modifications and formation of the triple helical pro-collagen monomers within the cell. From Banos *et al.* (2008).

Post-translational Modifications

Formation of the triple helical pro-collagen monomers is preceded by several post-translational modifications to the polypeptide chains, including hydroxylation of proline and lysine residues (Myllyla *et al.,* 1981) (Figure 1-3). The three polypeptide chains are then folded into a triple helical structure; the C-terminal pro-peptide plays an important role in this process as it acts as a nucleation site, initiating the formation of disulphide bonds between the polypeptide chains (Bellamy and Bornstein 1971). Once the polypeptide chains have assembled into a triple helix they are transported to the Golgi apparatus in vesicular tubular clusters (Bannykh and Balch 1997), where they aggregate laterally to form pro-collagen bundles (Cho and Garant 1981). The last step in the post-translational modification of pro-collagen is the cleavage of the non-triple helical C- and N-terminal pro-

32

peptides to form tropocollagen. The Kadler model proposes that this occurs intracellularly (Kadler *et al.,* 1996) whereas in the Birk model the cleavage occurs extracellularly (Birk *et al.,* 1995). Cleavage of the pro-peptides and subsequent fibril formation are complex processes; this is an area currently under much investigation (Banos *et al.,* 2008).

Collagen Fibril Formation

Once collagen bundles have been secreted into the extracellular environment they then assemble in a quarter stagger pattern to form fibrils (Figure 1-4). Assembly of collagen molecules into fibrils is precisely organised and results in a characteristic D-band periodicity. This process, known as fibrillogenesis, can also occur spontaneously *in vitro*. It has been proposed that fibronectin and integrins act as organisers during fibrillogenesis, determining the site of fibrillogenesis, while collagen type V and XII are nucleators, initiating the process (Kadler *et al.,* 2008). This would allow the precise control of fibril formation in a variety of tissues. Fibril growth occurs by the lateral and longitudinal fusion of the collagen fibrils results in the formation of larger mature fibrils (Banos *et al.,* 2008). Several molecules have been shown to be involved in the regulation of fibrillogenesis in tendon, these include collagen type III and the SLRPs (Canty and Kadler 2002). Fibril bundles are organised into a variety of complex structures, including orthogonal layers to form structures such as the cornea, basket weaves to form skin and bone, or in parallel to form tendon (Canty and Kadler 2002).
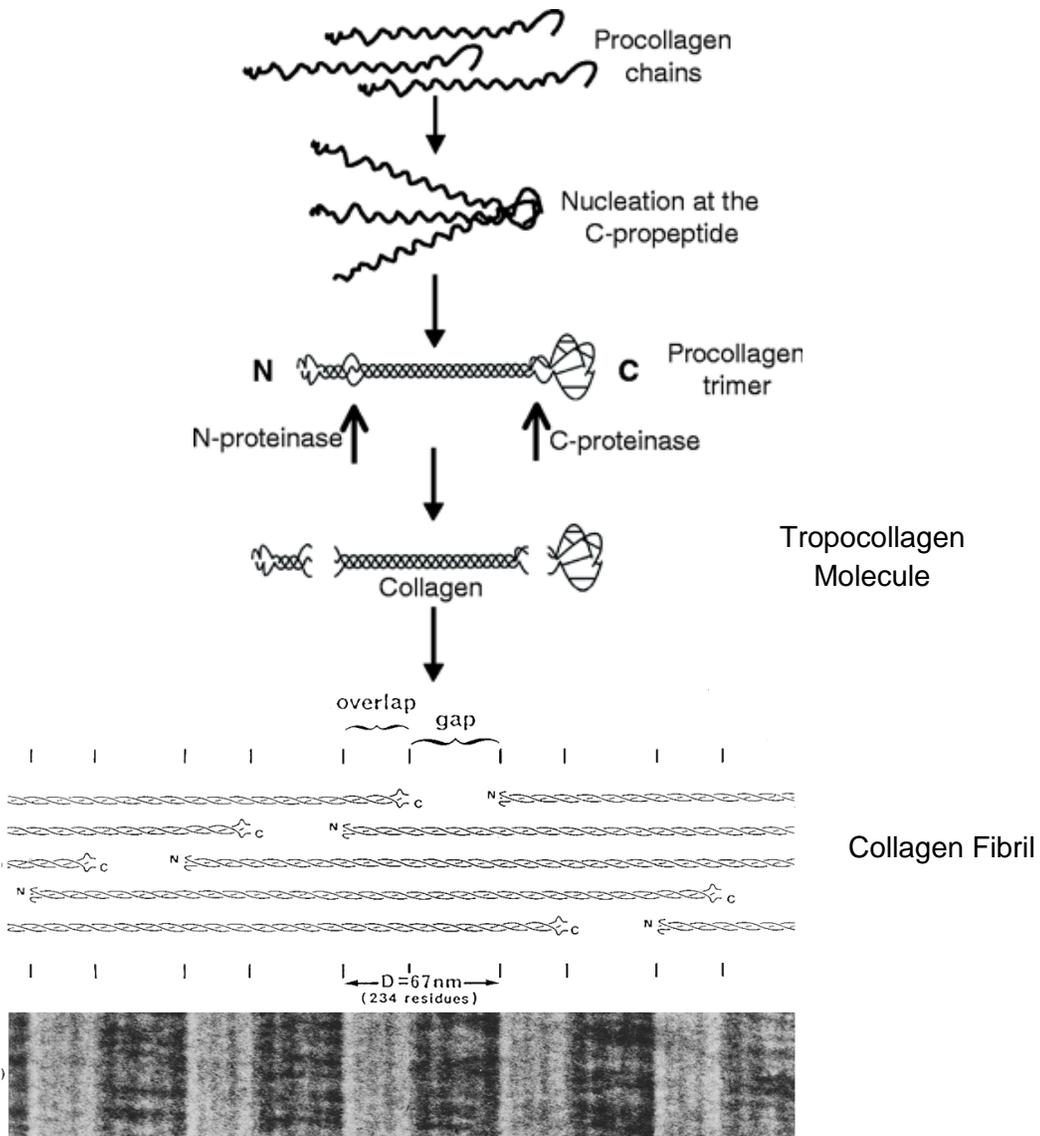
Figure 1-4: Synthesis of collagen fibrils from pro-collagen chains results in the formation of a repeating unit consisting of five collagen molecules arranged in a staggered pattern. When fibrils are stained they exhibit a periodic banding pattern; the repeating light and dark zones are due to preferential stain penetration into areas with lowest collagen packing (gap regions). The D-band is 67 nm long and consists of adjacent overlap and gap regions, where the overlap region is 0.4D and the gap region is 0.6D. Adapted from Kadler *et al.,* (1996) and Canty and Kadler (2005).

Collagen Cross-linking

The fibril structure is stabilised by the formation of crosslinks, which also increase the tensile strength of the tendon. There are two distinct types of crosslinks that form in collagenous tissue, enzymatic and non-enzymatic adventitious crosslinks (also known as glycated crosslinks) (Figure 1-5). Enzymatic crosslinking occurs during growth of immature tendon and is initiated by lysyl oxidase. Initially, the immature crosslinks formed are divalent, but it is thought that they react with other crosslinks to form more stable

34

trivalent crosslinks (Bailey *et al.,* 1998; Bailey 2001). These crosslinks form between lysine residues in the N- and C-terminal telopeptides and bind collagen molecules in the quarter stagger pattern. The crosslinks increase tendon strength by preventing the collagen molecules sliding past each other when the tendon is loaded (Avery and Bailey 2005), instead the triple-helical regions of the collagen molecules stretch contributing to extension of the tendon (Silver *et al.,* 2003). Glycated crosslinks are formed when sugar molecules present in the matrix react with the side-chains of lysine and arginine residues to form advanced glycation end-products (AGEs), which results in crosslinks that can occur along the length of the collagen molecule (Figure 1-5). AGEs form in tissues with a low rate of matrix turnover, such as bone, cartilage and tendon (Avery and Bailey 2005). The concentration of these AGEs increases with age, and cause tendon stiffness to increase so the tendon may be too stiff to function optimally (Avery and Bailey 2006).
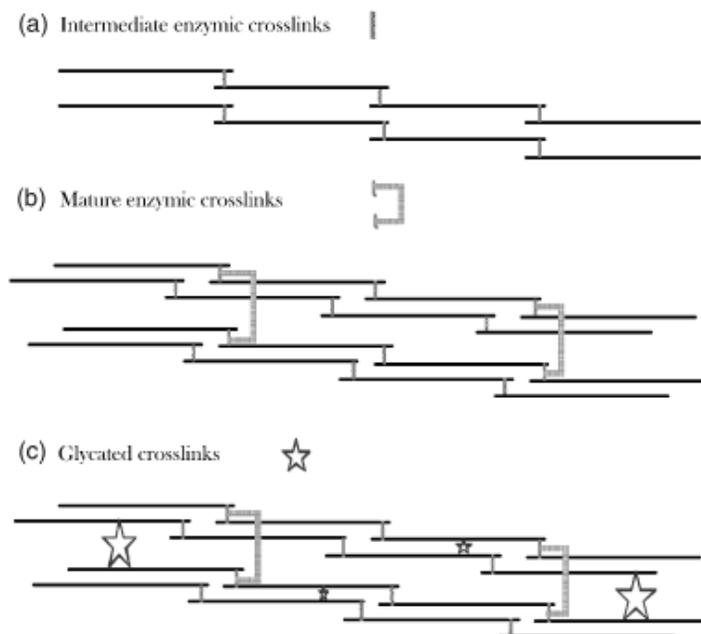
Figure 1-5: Diagram showing the formation of enzymatic and non-enzymatic (glycated) crosslinks between collagen molecules. From Avery and Bailey (2005).

Formation of Enzymatic Crosslinks

Enzymatic crosslinks are formed mainly during growth and maturation and are mediated by the enzyme lysyl oxidase, which deaminates N- and C- terminal lysine residues to form lysyl aldehyde (Bailey *et al.,* 1998). Further maturation of enzymatic crosslinks is spontaneous and the specific crosslinks formed depend on the degree of hydroxylation of the telopeptide lysine residues (Bailey 2001); tendon has a high proportion of lysyl

hydroxylation and so the initial crosslinks are mainly dehydro-hydroxylysinohydroxynorleucine (deH-HLHNL), which forms when the hydroxylysyl-aldehyde reacts with another hydroxylysyl residue. This crosslink is spontaneously converted to the more stable divalent hydroxylysino-keto-norleucine (HLKNL) via an Amadori rearrangement. This divalent cross-link then reacts with another hydroxylysine–aldehyde to form the mature trivalent crosslink hydroxylysyl-pyridinoline (HP) (Figure 1-6). Alternatively, the HLKNL can react with a lysine-aldehyde to form another type of crosslink, hydroxylysyl-pyrrole, the structure of which is undetermined as it is unstable during isolation procedures (Bailey 2001). If the hydroxylysyl-aldehyde forms a divalent crosslink with a lysine residue, dehydro-lysinohydroxynorleucine (deH-LHNL) will be formed and convert to lysino-keto-norleucine (LKNL). LKNL then either reacts with a lysine or hydroxylysine residue to form lysyl-pyridinoline (LP) or lysyl-pyrrole. The structure of HP and LP crosslinks are shown in Figure 1-7.
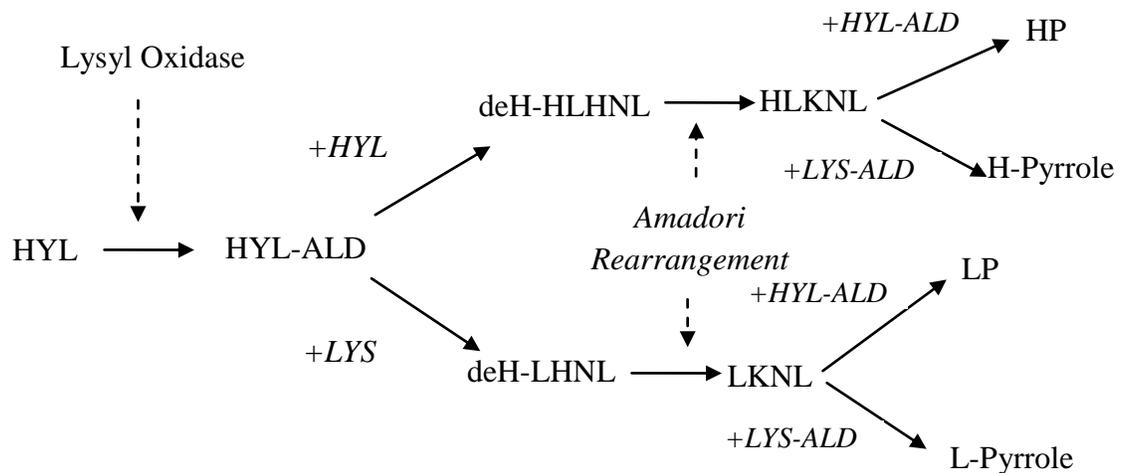
Figure 1-6: Formation of pyridinoline and pyrrolic crosslinks in collagen is mediated by lysyl oxidase and depends on the proportion of lysyl-hydroxylation in the collagen molecule. HYL, hydroxylysine; HYL-ALD, hydroxylysine-aldehyde; LYS, lysine; deH-HLHNL, dehydro-hydroxylysinohydroxynorleucine; deH-LHNL, dehydro-lysinohydroxynorleucine; HLKNL, hydroxylysino-keto-norleucine; LKNL, lysino-keto-norleucine; LYS-ALD, lysine-aldehyde; HP, hydroxylysyl-pyridinoline; LP, lysyl-pyridinoline. Adapted from Bailey *et al.* (1998).
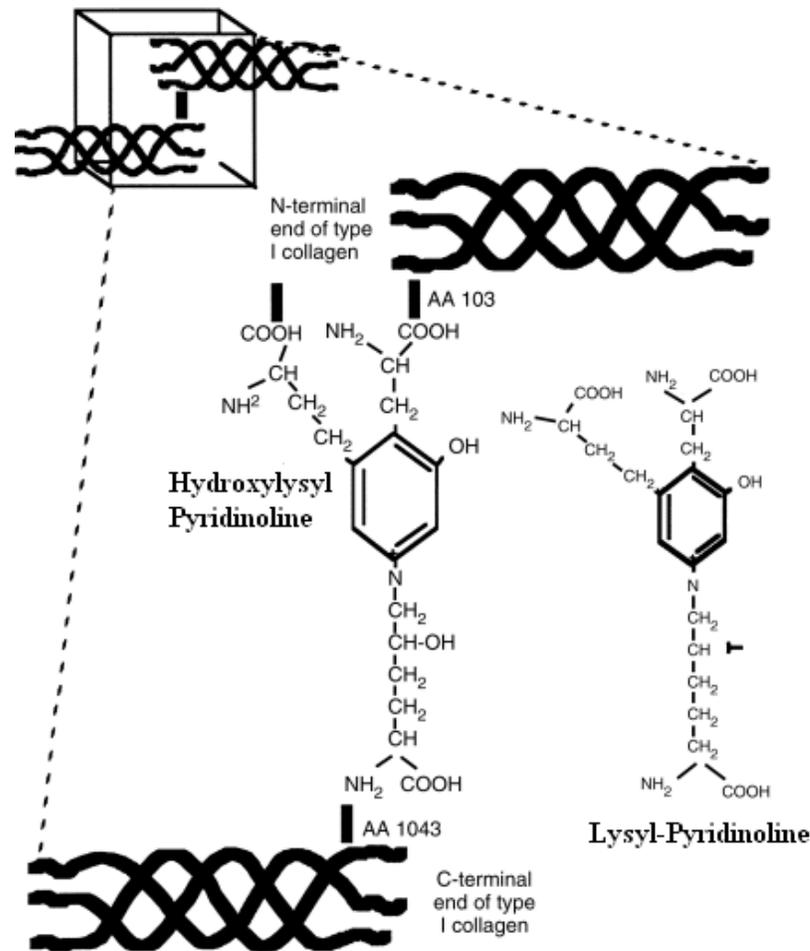
Figure 1-7: Structure of the 2 major enzymatic crosslinks that form in tendon collagen, hydroxylysyl-pyridinoline (HP) and lysyl-pyridinoline (LP). Adapted from Urena and De Vernejoul (1999).

Formation of Non-Enzymatic Crosslinks

Non-enzymatic crosslinks (also known as AGEs) are formed by spontaneous reaction with naturally occurring sugars present in the matrix. As they accumulate with age in tissues with a low rate of matrix turnover they can be used as a marker of matrix age. Pentosidine is the best characterised AGE and is well established as a matrix age marker; it has been shown to accumulate with age in articular cartilage and skin collagen (Verzijl *et al.,* 2000b), connective tissue in the eye (Glenn *et al.,* 2007), intervertebral disc collagen (Sivan *et al.,* 2006a) and tendon (Bank *et al.,* 1999). Pentosidine is formed when a pentose sugar reacts with lysine residues throughout the collagen molecule to form the Schiff base glucosyl–lysine, which undergoes an Amadori rearrangement to form fructose-lysine. Fructose-lysine then undergoes oxidation and reacts with ribose and an arginine residue on an adjacent collagen molecule, forming a pentosidine crosslink (Figure 1-8) (Robins and Bailey 1972; Sell and Monnier 1989). Unlike enzymatic crosslinks, glycation can occur at

37

lysine and hydroxylysine residues throughout the triple helix and telopeptide regions, although glycation occurs preferentially on specific residues (Sweeney *et al.,* 2008). Pentosidine is present at very low concentrations (1 pentosidine molecule per 200-300 collagen molecules) (Bailey *et al.,* 1995; Dyer *et al.,* 1991) and so is unlikely to alter the mechanical properties of the matrix significantly. However, there are other more recently characterised AGEs such as glucosepane (Figure 1-8), which is present in much greater concentrations (Sell *et al.,* 2005), and has been shown to have a detrimental effect on tissue function, resulting in increased stiffness and resistance to degradation (Monnier *et al.,* 2008). AGEs will also accumulate in non-collagenous proteins including proteoglycans if they are present in the matrix for a sufficient amount of time; pentosidine has been shown to accumulate with age in articular cartilage aggrecan (Verzijl *et al.,* 2001) and intervertebral disc aggrecan (Sivan *et al.,* 2006a).
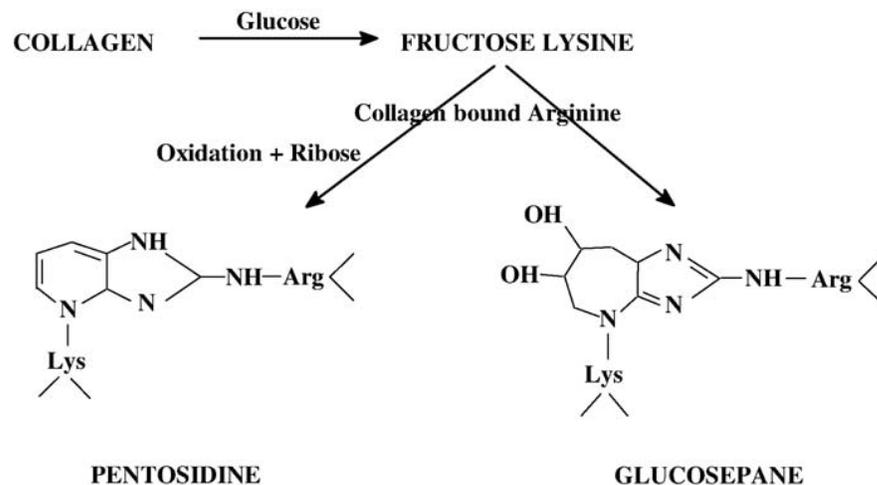
Figure 1-8: Structure of the AGEs pentosidine and glucosepane. From Avery and Bailey (2006).

Additional Age Related Modifications

In addition to the formation of AGEs, modification of the matrix also occurs due to amino acid racemization. All amino acids are incorporated into tissues in the L- form, but over time they slowly convert spontaneously to the D- form. The most rapidly racemizing amino acid is aspartic acid, which has been shown to accumulate with age in human cartilage and tendon (Maroudas *et al.,* 1992; Riley *et al.,* 2002). The ratio of D- to L- aspartate can also be used to calculate half-life of proteins present in the matrix (Sivan *et al.,* 2006b; Sivan *et al.,* 2008).

1.5.1.2. Non-Collagenous Components

The non-collagenous components of the extracellular matrix are mainly glycoproteins and proteoglycans (Yoon and Halper 2005). Glycoproteins are compounds consisting of a protein covalently linked to a carbohydrate, which may range from a monosaccharide to polysaccharides. Proteoglycans are a specific class of glycoprotein, where the core protein is attached to one or several polysaccharide chains which contain amino sugars. These chains are commonly referred to as glycosaminoglycan (GAG) side chains (Sharon 1986).

Proteoglycans within Tendon

Most of the proteoglycans found in the tensile region of tendon are the SLRPs (biglycan, decorin, fibromodulin, lumican) whereas there are higher concentrations of aggrecan in regions of tendon that are exposed to compressive forces (Rees *et al.,* 2000). Aggrecan is the largest proteoglycan found in tendon, consisting of a large core protein (molecular weight of approximately 220 kDa) attached to which are up to 100 chondroitin sulphate side chains and 60 keratan sulphate side chains. The SLRPs have smaller core proteins (molecular weight of about 40 kDa) and fewer side chains. Decorin has the smallest core protein attached to which is one dermatan or chondroitin sulphate GAG side chain, whereas biglycan has two dermatan sulphate side chains (Scott 1992). Fibromodulin and lumican may have up to five covalently bound keratin sulphate side chains, but typically fibromodulin has four side chains and lumican has two to three side chains (Yoon and Halper 2005). The basic structures of the SLRPs are shown in Figure 1-9.
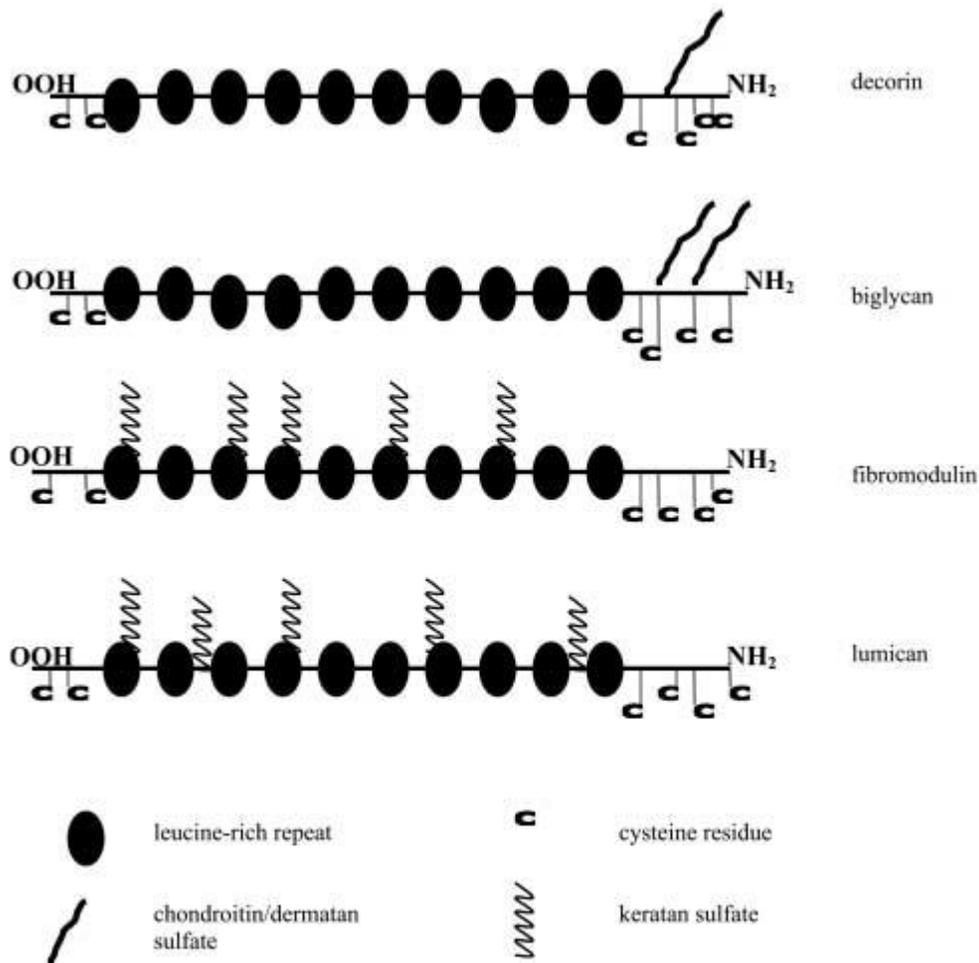
Figure 1-9: Representation of the basic structure of the SLRPs present in tendon. From Yoon and Halper (2005).

The SLRPs are involved in regulating fibrillogenesis and organising the collagenous matrix; the protein core binds to the collagen fibril at specific sites and the glycosaminoglycan side-chains interact with side-chains from other proteoglycans, holding the fibrils at defined distances from each other (Scott 1995).

Decorin is the most abundant proteoglycan found in regions of tendon that experience tensile forces and has a role in maintaining and regulating collagen fibril structure, as well as regulating cell proliferation (Yoon and Halper 2005). Decorin is also thought to align and stabilise fibrils during fibrillogenesis (Scott 1996); it inhibits lateral fusion of collagen fibrils and therefore high concentrations of decorin result in the formation of thinner collagen fibrils (Birk *et al.,* 1995). Decorin-null mice exhibit severe tendon defects characterised by the formation of severely disorganised collagen fibrils and bone fragments within the tendon (Kilts *et al.,* 2009). These data suggest that decorin plays a crucial role in

collagen fibrillogenesis and stability. Decorin may also play a role in regulating the formation of enzymatic crosslinks; the decorin binding site is in the C-terminal region of the collagen fibril and is in close proximity to the predominant site for intermolecular crosslink formation (Sweeney *et al.,* 2008).

Biglycan, like decorin, is able to bind to fibrillar collagen and has been implicated in regulation of fibrillogenesis (Yoon and Halper 2005). However, the nature of biglycan collagen interactions have not been fully determined (Zhang *et al.,* 2005). Biglycan knockout mice exhibit tendon defects characterised by thin, disorganised collagen fibrils and ectopic ossification, although this is not as severe as seen in decorin knockout mice (Corsi *et al.,* 2002; Kilts *et al.,* 2009). In addition, biglycan synthesis is upregulated in decorin-null mice, suggesting it may be able to partially compensate for the absence of decorin (Young *et al.,* 2006).

The class II SLRPs, namely lumican and fibromodulin, also interact with fibrillar collagen. These proteoglycans are differentially expressed during tendon development (Ezura *et al.,* 2000) and it has been proposed they are involved in the regulation of fibril fusion; *in vitro* studies have shown they are able to inhibit fibril fusion (Hedbom and Heinegard 1989). Lumican and fibromodulin deficient mice exhibit tendon defects, although these are not as severe as in decorin or biglycan knockout models (Ezura *et al.,* 2000). Aggrecan is found predominantly in areas where the tendon is placed under compressive loads, for example where the tendon wraps round a joint (Rees *et al.,* 2000). Aggrecan is also present in the tensile region of tendon at low concentrations; it is thought to reduce collagen stiffness by increasing the water content within the matrix and may also be able to protect fibrils from collagenases (Jones and Riley 2005; Yoon and Halper 2005).

In addition to regulating collagen fibrillogenesis during tendon development and remodelling it has been suggested that proteoglycans, in particular decorin contribute directly to tendon mechanical properties by aiding in the transfer of strain between discontinuous collagen fibrils. Decorin consists of a horseshoe shaped core protein, attached to which is a single chondroitin or dermatan sulphate side chain; the side chain binds to one edge of the molecule such that the GAG chain can align parallel or perpendicular to the axis of the collagen fibril (Weber *et al.,* 1996). Studies have established that the core protein is able to bind non-covalently to a single collagen triple

helix at a specific amino acid sequence in the gap region (D-band) of the fibril, such that decorin molecules bind approximately every 68 nm along the collagen fibril (Scott 1992; Sweeney *et al.,* 2008; Weber *et al.,* 1996). The side chain interacts with the side chain of another decorin molecule bound to a collagen molecule within an adjacent fibril, forming an interfibrillar bridge (Scott 1992) (Figure 1-10). Individually, these bonds are weak, but combined they are thought to resist sliding between fibres (Gupta *et al.,* 2010).
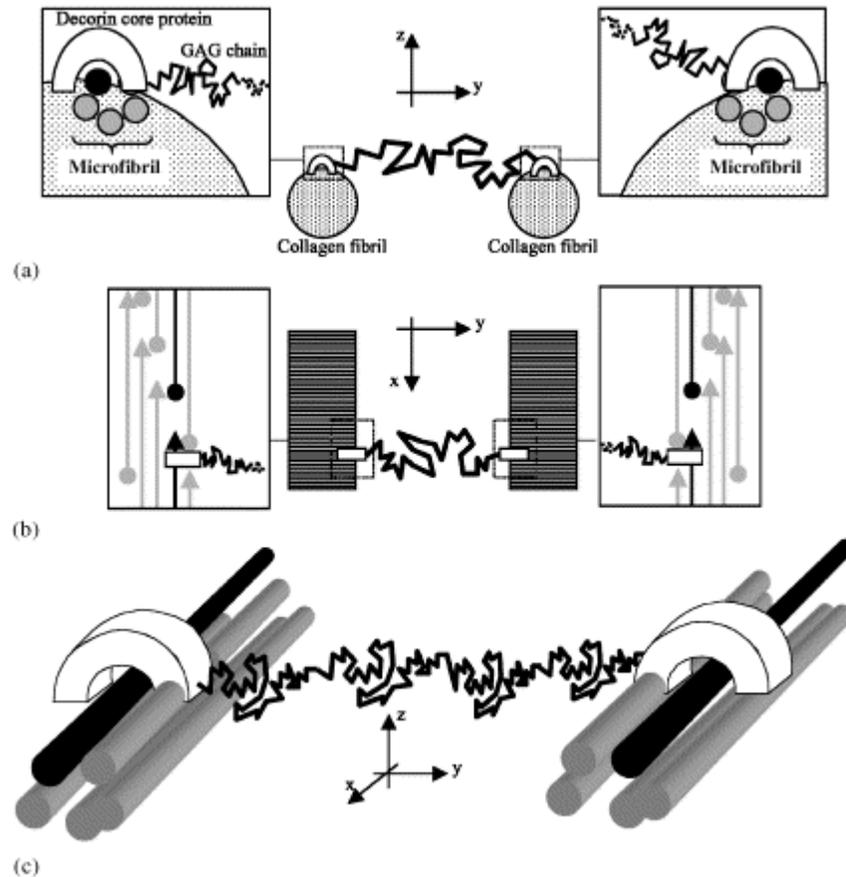
Figure 1-10: Schematic showing interaction between two collagen molecules from adjacent fibrils via decorin core proteins (white horseshoe shaped) and their GAG side chains (black lines). (a) Transverse view of collagen fibrils showing connections formed by decorin. (b) Longitudinal view of collagen fibrils showing decorin binding in the gap region of the collagen fibrils. (c) 3D representation of the decorin protein collagen molecule complex within a microfibril. From Vesentini *et al.* (2005).

It has been shown that modelling collagen fibres as a discontinuous composite material where stress is transferred between collagen molecules by decorin molecules via chondroitin sulphate side chains results in similar values for elastic modulus and ultimate stress as those recorded in mature tendon (Redaelli *et al.,* 2003). However, it has not been established if collagen fibrils within tendon are discontinuous or if they span the length of

the tendon (Provenzano and Vanderby, Jr. 2006). It is clear that fibrils within immature tendon are discontinuous (Birk *et al.,* 1995) and some tendons, including the equine SDFT and human Achilles vary in cross-sectional area along their length (Birch *et al.,* 2002), suggesting that not all fibrils run the entire length of the tendon. However, histological studies have not identified any fibril ends in mature tendon (Provenzano and Vanderby, Jr. 2006). Imaging of tendon is difficult, and as fibrils interweave and are not confined to one plane the likelihood of identifying an end is extremely low (Legerlotz *et al.,* 2010). There is also controversy regarding the strength of the decorin protein and the interactions of this protein with collagen and other decorin molecules, it has been postulated that the bonds formed would be too weak to contribute significantly to strain transfer (Provenzano and Vanderby, Jr. 2006). However, modelling studies have suggested that the structure of GAGs is such that they have a suitable stiffness to transfer stress between fibrils. Collagen fibril mechanical properties have also been linked to fibril length; as fibril length increases there is also an increase in the number of GAGs able to bind to the fibrils and transfer force laterally (Redaelli *et al.,* 2003). A more recent modelling study has reported that the binding strength between the decorin core protein and collagen molecule to be approximately three orders of magnitude greater than the ultimate strength of the GAG chain, indicating that overloading is likely to decrease the mechanical integrity of the collagen fibril by disrupting the GAG side chains rather than causing the decorin core protein to detach from the collagen fibril (Vesentini *et al.,* 2005).

Although modelling studies suggest a mechanical role for proteoglycans, experimental studies have not provided unequivocal data to support this. Several studies have used chondroitinase ABC, which removes the dermatan sulphate and chondroitin sulphate side chains of decorin and biglycan, to disrupt proteoglycans within tendon matrix. Removal of GAGs from rat tail tendon fascicles did not result in changes in fascicle elastic modulus or dynamic viscoelastic properties (Fessel and Snedeker 2009). In contrast, GAG depleted murine Achilles tendons exhibited decreases in elastic modulus, but only in the distal region of the tendon (Rigozzi *et al.,* 2009). Further, analysis of tendon mechanical properties in decorin knock-out mice did not identify differences in the maximum stress or modulus of tail tendon fascicles or flexor digitorum longus tendons when compared to wild type controls (Robinson *et al.,* 2004a). However, the patellar tendons from decorin knock-out mice had higher modulus and stress relaxation when compared to controls. In addition,

tail tendon fascicles from decorin deficient mice have been shown to have reduced sensitivity to the rate of applied strain, whereas fascicles from the tail tendons of immature mice, which have a higher proteoglycan content, are more sensitive to alterations in strain rate (Brent *et al.,* 2003; Robinson *et al.,* 2004b). These conflicting data suggest that the contribution of proteoglycans to tendon mechanical properties may be dependent on tendon type and function, and may also vary throughout the length of the tendon. The alterations in strain rate sensitivity and stress relaxation suggest that proteoglycans may impart viscoelasticity without altering tendon material properties dramatically. However, the mechanisms by which this occurs are yet to be elucidated.

Glycoproteins within Tendon

Collagen oligomeric matrix protein (COMP) is the most abundant glycoprotein present in tendon (Smith *et al.,* 1997). COMP is a large pentameric protein, consisting of 5 sub-units which are arranged as arms around a central cylinder. The precise function of COMP within tendon is unknown, but it has been suggested that it interacts with collagen, cells and other matrix proteins (Smith *et al.,* 2002b) and it is also thought to play a role in fibrillogenesis (Sodersten *et al.,* 2005). The concentration of COMP within tendon is correlated with ultimate tensile stress and elastic modulus in SDFTs from skeletally immature horses, but shows no correlation with mechanical properties of SDFTs from mature horses (Smith *et al.,* 2002b). COMP has also been proposed to contribute to transfer of strain between collagen fibrils. Mutations in the COMP gene are associated with pseudoachondroplasia, a disease characterised by joint laxity and bone abnormalities (Maddox *et al.,* 2000). The mechanisms by which COMP mutations induce this disease are unknown; but it has been shown that COMP mutations affect proteoglycan synthesis in cartilage (Kwak *et al.,* 2009). However, knockout studies have found that COMP-null mice do not exhibit any tendon abnormalities (Svensson *et al.,* 2002) and so this protein may not play a critical role in the function or maintenance of mature tendon matrix.

Tenascin-C is an extracellular matrix glycoprotein that is expressed in low levels in mature musculoskeletal tissues including tendon (Taylor *et al.,* 2009) and shows high expression in immature tissue as well as in disease states and in healing tissue (Jarvinen *et al.,* 2000). Its precise function is as yet unclear but it has been demonstrated that concentration of tenascin-C is greatest in areas that experience high mechanical forces and so it has been

postulated that this protein provides elasticity to the tendon tissue (Jarvinen *et al.,* 2000). Studies have also shown that expression of tenascin-C can be modulated by mechanical loading *in vivo*; immobilisation resulted in a decrease in tenascin-C expression in the rat Achilles tendon, whereas treadmill running caused an increase in tenascin-C compared to controls (Jarvinen *et al.,* 2003). This protein may therefore have a more important role in tendons that experience high strains.

The glycoprotein tenomodulin is a transmembrane protein which is also found in relatively high concentrations in tendon; it has been shown to be essential for normal tenocyte proliferation and formation of regular collagen fibrils (Docheva *et al.,* 2005). Expression of tenomodulin is positively regulated by scleraxis and it is a late marker of tendon formation (Shukunami *et al.,* 2006). In mature tendon, tenomodulin expression is restricted to elongated tenocytes within dense regular collagen fibrils (Shukunami *et al.,* 2006) and tenomodulin null mice exhibit uneven fibril surfaces within their tendons (Docheva *et al.,* 2005), suggesting that tenomodulin is involved in collagen fibril alignment and organisation.

Scleraxis is expressed at high levels in all tendon cells and their progenitors (Brent *et al.,* 2003; Cserjesi *et al.,* 1995; Murchison *et al.,* 2007). The scleraxis gene encodes a basic helix loop helix transcription factor that binds tendon-specific element 2, which is required for Col1A1 expression in tenocytes (Lejard *et al.,* 2007). Scleraxis-null mice exhibit severe tendon defects with a reduced and disorganised matrix, especially in tendons that act to transfer force (Murchison *et al.,* 2007), suggesting it is critical for normal tendon differentiation and development of tendon mechanical properties, possibly via regulation of type I collagen synthesis. However, after maturity the function scleraxis plays in the maintenance of tendon matrix is unknown.

1.5.2. Tenocytes

In healthy tendon the matrix is maintained and repaired by a small resident population of tenocytes which are situated between the collagen fibres within fascicles (intra fascicular) or reside between the collagen fascicles (inter fascicular) (Figure 1-11). Cell density is relatively low in tendon compared to other soft tissues, and numbers decrease during maturation (Stanley *et al.,* 2007). Tenocytes have long cytoplasmic processes which link to other tenocytes via gap junctions, which allows them to communicate with one another and

presumably respond appropriately to mechanical stimuli (Stanley *et al.,* 2007). It is likely that intra-fascicular and inter-fascicular cells within the tendon have different functions although this has not been shown and in general tendon cell phenotype is poorly defined.
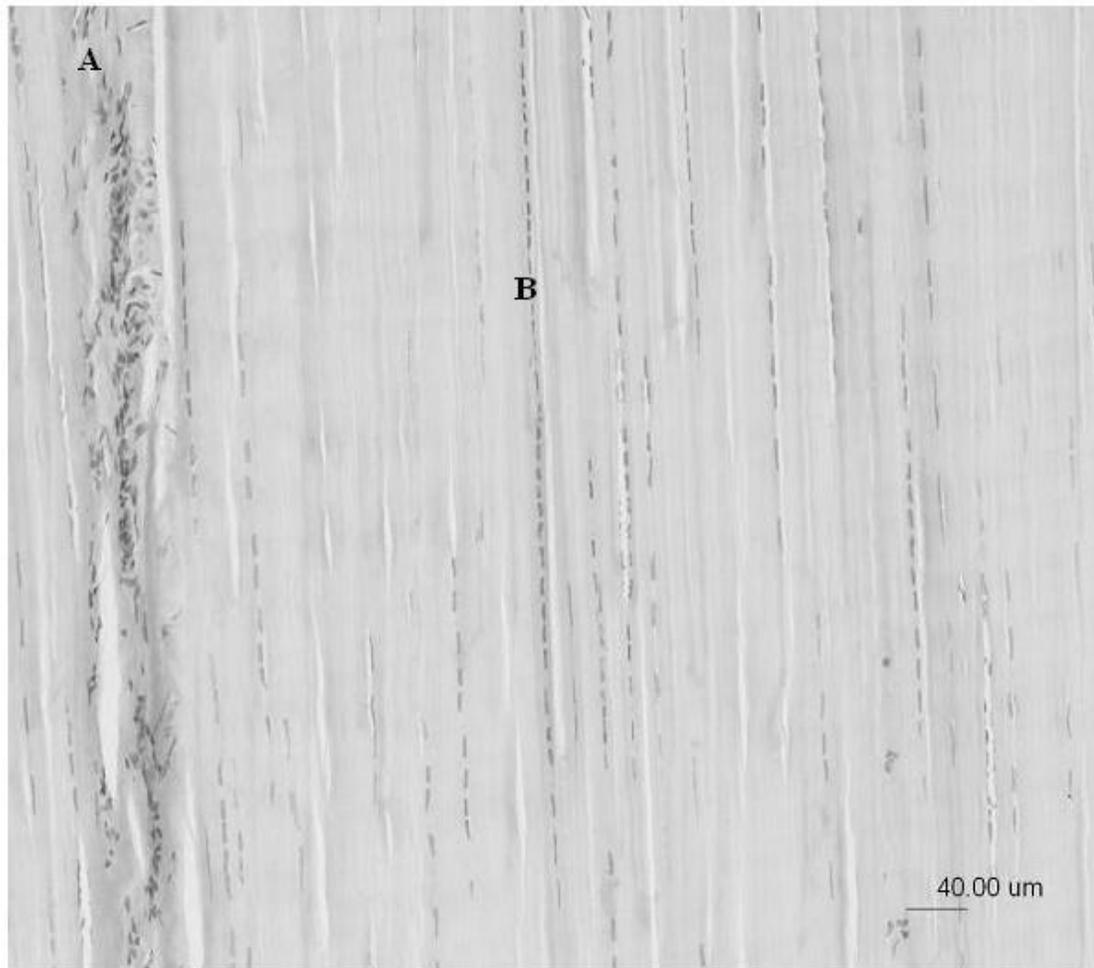
Figure 1-11: Longitudinal haemotoxylin and eosin stained section of a normal mid-metacarpal region of equine SDFT showing areas containing A) Inter-fascicular tenocytes and B) Intra-fascicular tenocytes.

Histological studies have attempted to further characterise cells within tendon by grouping tenocytes into three types according to their morphology (Webbon 1978). Type I cells are elongated with thin nuclei and are the predominant cell type in mature tendon (Stanley *et al.,* 2008). Type 2 cells are often referred to as tenoblasts rather than tenocytes; these cells have ovoid rather than elongated nuclei and are present in greatest concentrations in immature tendons, with a decrease in numbers in mature tendons (Stanley *et al.,* 2007). It has been shown that type 2 cells from human patellar tendons proliferate more rapidly and have higher expression levels of pro-collagen and matrix degrading enzymes than type I

cells from the same tendon (Chuen *et al.,* 2004), suggesting that type 2 cells have greater levels of metabolic activity than type I cells. Type 3 cells are restricted to areas of tendon that experience significant levels of compression and exhibit a morphology that is chondrocyte like; cells in these areas are much more rounded than type 1 or 2 cells (Webbon 1978). In a recent study of the equine SDFT and CDET, it was reported that cells in the tensile mid-metacarpal region of these tendons were either type 1 or 2, with increases in the percentage of type I tenocytes with increasing age (Stanley *et al.,* 2008), suggesting that overall cell activity declines with ageing.

1.5.2.1. Matrix Degradation

Tenocytes are able to remodel tendon matrix by synthesising matrix molecules, including collagen and proteoglycans, and enzymes responsible for degradation, including matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs) and a disintegrin-like and metalloproteinase with thrombospondin motifs (ADAMTS). The activity of matrix degrading enzymes is tightly regulated by a number of mechanisms. These include the presence of tissue inhibitors of metalloproteinases (TIMPs), whether the molecules are in their active or pro-form and their location within the matrix (Nagase *et al.,* 2006).

MMPs are synthesised as inactive pro-enzymes and consist of a pro-peptide region approximately 80 amino acids in length, a catalytic domain about 170 amino acids long, a linker peptide or hinge region and a hemopexin domain about 200 amino acids long (Figure 1-12). Pro-MMPs are inactive as the pro-peptide contains a cysteine switch which binds to the zinc binding motif in the catalytic domain and prevents a water molecule binding to the zinc atom, a step which is essential for activation (Nagase *et al.,* 2006). Once the MMP has been activated by cleavage of the pro-peptide, a water molecule is free to bind to the zinc atom. The substrate binding site is adjacent to the zinc atom and varies in size between MMPs, which determines the substrate specificity. ADAMs consist of a metalloproteinase-like domain, which has proteolytic activity, a disintegrin-like domain which enables binding for integrins and other receptors, a cysteine-rich domain which may enable membrane fusion and a cytoplasmic tail (Wolfsberg *et al.,* 1995). ADAMs are able to cleave a variety of matrix proteins and have also been implicated in control of cell-cell and cell-matrix interactions (White 2003). ADAMTSs are similar in structure to ADAMs, with

up to fourteen additional thrombospondin motifs (Figure 1-12) (Jones and Riley 2005) and also have proteolytic activity against several components of tendon extracellular matrix (Jones and Riley 2005).
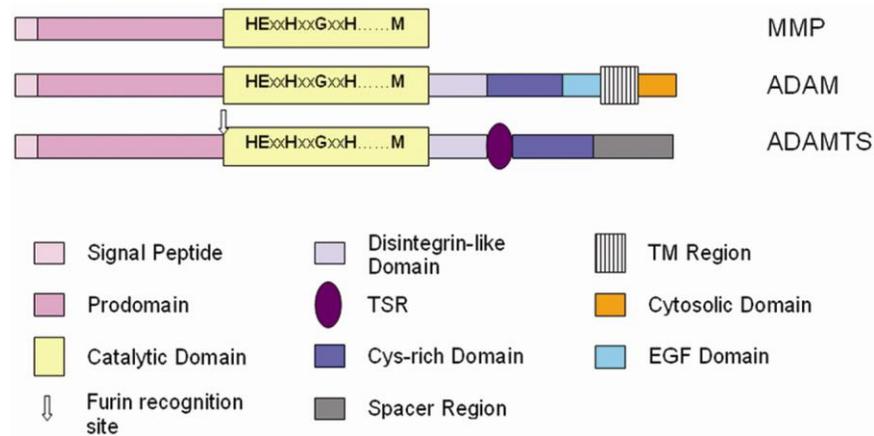
Figure 1-12: Representation of the domain organization of MMPs, ADAMs and ADAMTSs. TSR, thrombospondin type 1-like repeat; EGF, epidermal growth factor; TM, transmembrane. From Jones and Riley (2005).

While some MMPs are activated intracellularly, the majority are secreted as inactive pro-enzymes (Nagase *et al.,* 2006). The pro-peptide can be cleaved by a variety of molecules, including proteinases present within the matrix. As disruption of the cysteine switch is required for activation, addition of agents that affect protein structure, such as aminophenylmercuric acetate (APMA) and sodium dodecyl sulphate (SDS), are also able to activate MMPs without proteolysis of the pro-peptide (Nagase and Woessner, Jr. 1999). Other MMPs are often responsible for activation; for example pro-collagenase is activated by MMP-3 (Suzuki *et al.,* 1990). Once activated, MMPs can still be inhibited by TIMPs, which bind to the active site of the enzyme in a stochiometric ratio (Nagase *et al.,* 2006). TIMPs are also able to inhibit ADAMS and ADAMTSs (Roy *et al.,* 2004).

Matrix degrading enzymes are specific for different components of the matrix. The only enzymes capable of degrading intact triple helical collagen are the collagenases (MMP-1, -8 & -13) (Lauer-Fields *et al.,* 2000; Visse and Nagase 2003). These enzymes cleave the collagen into characteristic three-quarter and one-quarter fragments; these fragments can then be further degraded by other enzymes such as the gelatinases (MMP-2 & -9) (Visse and Nagase 2003). However, collagen degradation is further hampered by the hierarchical structure of the tendon; intact fibrillar collagen is highly resistant to degradation and only collagen molecules on the surface of the fibril are accessible for enzymatic degradation

(Perumal *et al.,* 2008). Computational and visualization studies suggest that the C-terminal telopeptide of collagen must be proteolytically cleaved before collagenases can gain access to degrade the triple helical region of the collagen molecule (Perumal *et al.,* 2008). Once the telopeptide has been cleaved the collagenases can then unwind the triple helix to gain access to all three collagen chains (Chung *et al.,* 2004). MMP-3 has been implicated in the cleavage of the C-terminal telopeptide (Sweeney *et al.,* 2008) suggesting that this enzyme may have a role in the control of collagen breakdown. This is likely to be an important regulatory mechanism which would prevent the enzymatic degradation of intact collagen in favour of collagen that is partially damaged such that the triple helical region is more accessible to collagenase. The stromelysin MMP-3 is likely to play an important role in tendon matrix degradation; as well as degrading a variety of matrix proteins, it is also involved in the activation of collagenases and gelatinases (Olson *et al.,* 2000; Suzuki *et al.,* 1990) and is thought to assist in collagen degradation (Sweeney *et al.,* 2008). In contrast, the gelatinase MMP-2 is thought to be a regulator of collagen fibril formation; it has been shown that levels of active MMP-2 increase during fibrillogenesis, and incubating fibrils with the activated form of the enzyme results in lateral growth of the fibrils (Jung *et al.,* 2009). It is possible that MMP-2 is able to influence fibril size and growth by degrading molecules that are regulators of lateral and longitudinal fibril growth, including the SLRPs. As well as degrading collagen fragments generated by collagenases, gelatinases are also able to degrade proteoglycans, as are the stromelysins (MMP-3 & -10), and specific ADAMs and ADAMTSs (Kashiwagi *et al.,* 2004). These studies indicate that degradation of specific components within tendon matrix is tightly controlled; this is important to prevent unregulated degradation which may have a detrimental effect on tendon mechanical integrity. It is therefore likely that concentration and activity of matrix degrading enzymes differ between functionally distinct tendons; previous work has shown greater MMP concentration in the injury prone supraspinatus tendon than the rarely injured biceps brachii tendon (Riley *et al.,* 2002).

1.5.3. Differences in Matrix Composition between Tendons

In order to achieve specificity, the SDFT and CDET have different matrix compositions. The mature SDFT has higher GAG content, water content and cellularity than the CDET (Batson *et al.,* 2003). The SDFT also has a greater proportion of type I and type 2 tenocytes than the CDET, both in immature and mature tendon (Stanley *et al.,* 2008). There are also

differences in matrix composition between the SDFT and DDFT (Birch *et al., 1999a*), although these are not as great as the differences between the SDFT and the CDET. The DDFT has a lower cellularity, percentage of type III collagen and pyridinoline crosslinks, and higher GAG content and percentage of large diameter collagen fibrils than the SDFT. It is thought that small diameter fibrils are more able to prevent interfibrillar slippage due to an increased surface area compared to fibrils with a larger diameter (Birch *et al., 1999a*), and so this, accompanied by higher levels of type III collagen, may enable the SDFT to be more elastic than the DDFT. The differences in proteoglycan content between functionally distinct tendons may also contribute directly to tendon mechanical properties. Although there is a large variation in SDFT strength and stiffness between individual horses (Birch 2007); within an individual the SDFT invariably has a lower elastic modulus (less stiff material) than the CDET. The lower elastic modulus of the SDFT tissue means it can store and return energy more efficiently as it is stretched throughout the stance phase and recoils during the swing phase, returning the stored energy at an appropriate point in the stride cycle (Batson *et al., 2003*). However, at birth and in very young animals, the properties of the digital flexor and extensor tendons are very similar; Shadwick (1990) found no differences between the extensibility, elastic modulus, strength and energy storing ability of pig digital flexor and extensor tendons at birth, but at maturity the tendons were stronger, stiffer and less extensible. The properties of the flexor tendons changed to a much greater degree compared to those of the extensor tendons. The mechanical properties of the flexor and extensor tendons may be modulated by the forces they experience; the flexor tendons are loaded to a greater extent and at a different rate when compared to the extensor tendons so they develop properties that allow them to cope with these loads and also store and return energy efficiently. A more recent study of equine tendon (Batson *et al., 2001*) found that the SDFT and CDET had similar mechanical properties at birth, but after three days the CDET had a higher elastic modulus than the SDFT, suggesting that the tendon matrix undergoes rapid changes in response to the new loads placed upon it.

COMP levels also differ between the functionally distinct tendons in the equine forelimb. COMP is differentially distributed throughout the SDFT and the highest concentration is found in the mid-metacarpal region, which experiences the highest tensile stress, whereas lower levels are found in the CDET (Smith *et al., 1997*). COMP levels are correlated with the number of small diameter collagen fibrils, which may be an adaptation to increased

loading (Sodersten *et al.,* 2005). The ECM composition of the SDFT also differs along its length; collagen content, amount of degraded collagen, cellularity and water content are higher in the mid-metacarpal region than the sesamoid region, whereas GAG levels are lower in the mid-metacarpal region (Birch *et al.,* 2002; Lin *et al.,* 2005b). This may be because the mid-metacarpal region experiences tensile forces, whereas the sesamoid region experiences both tensile and compressive forces (Lin *et al.,* 2005b), so the different regions adapt to fulfil their specific roles.

1.6. Characteristics of Tendon Degeneration

1.6.1. Alterations in Matrix Composition

Degeneration of the SDFT is characterised by discolouration of the central core region of the tendon (Figure 1-13). This is frequently seen at post mortem in horses that exhibit no clinical signs of tendon injury, such as lameness or swelling (Birch *et al.,* 1998; Webbon 1977). Recent attempts to detect these early changes *in vivo* using ultrasonography were not able to predict SDFT injury, although where SDFT pathology was identified an acute injury was more likely to occur (Avella *et al.,* 2009). Abnormal colouration is accompanied by changes in matrix composition; the central core region has increased levels of GAG, type III collagen and cellularity, and decreased collagen-linked fluorescence (a marker of matrix age) compared to the peripheral region of degenerated tendon and the core of normal tendon (Birch *et al.,* 1998). Similar changes occur in degenerated and painful human tendons, with upregulation of collagen types I and III (Ireland *et al.,* 2001), increased proteoglycan content and disorganised collagen fibrils (Riley *et al.,* 2001). Increased production of inflammatory cytokines (Gotoh *et al.,* 2001) and lower concentrations of molecules that are used as markers of matrix age (Bank *et al.,* 1999; Riley *et al.,* 2002) are also common features of tendinopathy.
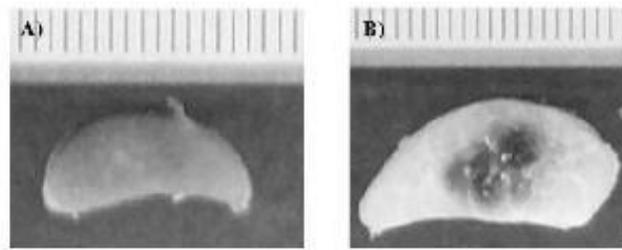
Figure 1-13: Mid-metacarpal region section of A) Normal SDFT and B) Injured SDFT showing central core discolouration.

1.6.2. Alterations in Gene Expression

There are several genes that show changes in expression level with tendon injury; Ireland *et al.* (2001) reported increases in expression of collagen types I and III and the proteoglycan biglycan in samples taken from humans with chronic Achilles tendinopathy. This was accompanied by a decrease in MMP-3 expression. A study that profiled mRNA expression of all the known MMPs, and a selection of ADAMs, ADAMTS genes and TIMPs found differences in gene expression levels in tissue from normal, painful and ruptured human Achilles tendons (Jones *et al.,* 2006). Interestingly, the pattern of gene expression differed between painful and ruptured tendons; the collagenase MMP-1 was found to be upregulated in ruptured tendon, as was the gelatinase MMP-9, along with MMP-19 and -25. Expression of MMP-3 was found to decrease in both ruptured and painful tendon, and mRNA levels of MMP-10 were lower in painful tendons when compared to normal. Painful tendon also showed upregulation of MMP-23 and ADAM-12 when compared to normal tendon (Jones *et al.,* 2006). The increased expression of genes coding for collagen suggests that the cells are attempting to remodel the matrix and repair any micro-damage. These data also suggest that MMPs play an important regulatory role in tendon matrix turnover. The changes in matrix composition that occur with degeneration suggest an increased rate of matrix turnover, which may be an inadequate healing response or an inappropriate cellular response, resulting in the degradation of normal matrix. The stimulus for this change is not known but suggested causes include mechanically induced micro-damage to collagen fibrils, hyperthermia and hypoxia.

1.7. Micro-damage to Collagen Fibrils
Localised damage can occur to the collagen fibrils when tendons are exposed to high strains as in the SDFT during high speed locomotion. *In vitro* experiments have shown that loading tendon fascicles at 80% of failure strain resulted in isolated fibrillar damage and

fibril sliding (Lavagnino *et al.,* 2006). This micro-damage was accompanied by a localised increase in MMP-1 gene expression and production. An *in vivo* model of tendon overload fatigue damage has demonstrated that low levels of damage result in the formation of kinked collagen fibres, which span several fibrils. As damage increases, the matrix becomes disorganised, eventually resulting in the formation of voids between fibres and fibre thinning (Fung *et al.,* 2010a; Fung *et al.,* 2010b), which is accompanied by an increase in MMP-13 expression (Sun *et al.,* 2008). The hierarchical structure of tendon is such that, during a loading cycle, some fibrils may experience higher strains and therefore be at higher risk of damage than others (Kastelic *et al.,* 1978); modelling studies have predicted that there are large strain distributions within the tendon matrix and small overall deformations may be amplified locally (Screen and Evans 2009). Therefore, isolated fibrillar damage may not affect the tendon's gross mechanical properties, but may alter local cell-matrix interactions (Arnoczky *et al.,* 2008a). However if the micro-damage is not repaired by the resident tenocytes it will accumulate and may then result in clinical injury. It may be expected therefore that high strain tendons have a greater capacity for matrix repair than low strain tendons. Although the SDFT has a higher cellularity than the CDET, the cells in the CDET exhibit increased mRNA expression of type I collagen and collagenases, and there are higher levels of the cross-linked carboxy-terminal telopeptide of type I collagen (ICTP), a marker of collagen degradation (Birch *et al.,* 2008b), suggesting the potential for, and the rate of, matrix turnover is higher in the CDET than in the SDFT. It is unclear why tenocyte activity is lower in the SDFT when this tendon is at higher risk of micro-damage, and there are two main hypotheses that need to be considered. Structures that require high mechanical strength to function, such as the SDFT, may be protected from remodelling as this would cause them to weaken transiently as the collagen molecules are degraded and replaced (Laurent 1987). The alternative hypothesis is that cell activity is compromised by changes to the cells' physiochemical or mechanical environment that occur during high speed exercise and with increasing age.

1.8. Changes to Physiochemical Environment

Due to the viscoelastic nature of tendon, some of the energy stored by the SDFT is released as heat, rather than being returned as kinetic energy (Ker 1981), which causes the temperature in the tendon core to increase, and temperatures of 45 °C have been recorded in the core of the SDFT *in vivo* during gallop exercise (Wilson and Goodship 1994).

Although exposing most cell types to temperatures above the normal physiological range would result in cell death, tenocytes from the SDFT cultured *in vitro* in suspension have been shown to be heat resistant up to 48 °C for a period of 10 minutes (Birch *et al.,* 1997b) (Figure 1-14). However, cells may be more sensitive to heating *in situ* where they are able to communicate via their gap junctions (Burrows *et al.,* 2009). Other studies have shown that above 45 °C equine tenocytes produce more pro-inflammatory cytokines, which will increase the production of matrix degrading enzymes (Hosaka *et al.,* 2006). No studies have measured temperature in the CDET *in vivo* but as this tendon experiences low strains and has a relatively small cross sectional area (mean CSA of 28 ± 7 mm^2 in the CDET compared to 96 ± 35 mm^2 in the SDFT (Batson *et al.,* 2003)) it is unlikely high temperatures would be reached. The poor blood supply in tendon contributes to the increase in temperature as the heat is dissipated relatively slowly, exposing the cells to high temperature for a longer period of time. The poor blood supply may also result in low oxygen levels in the tendon core which may further compromise cell activity. Tenocytes are capable of oxidative energy metabolism (Birch *et al.,* 1997a), which suggests their synthetic and degradative activity may be compromised by low oxygen levels.
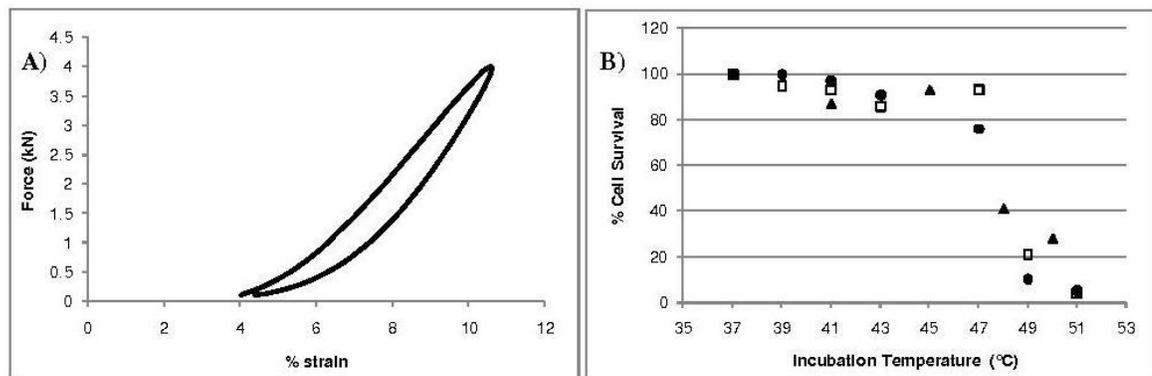
Figure 1-14: A) Hysteresis loop during cyclical loading and unloading of tendon. The energy lost is represented by the area within the loop. **B)** Equine tendon fibroblast survival following exposure to hyperthermia (10 min.). Different symbols represent fibroblasts grown from different horses. Data replotted from Birch *et al.* (1997b).

1.9. Changes to Mechanical Environment

The mechanical deformation experienced by the cells within the SDFT depends on the coupling between the cell and extracellular matrix and this in turn affects cell activity. It has for example been shown that the strain experienced by individual cells is not the same as that applied to tendon fascicles (Screen *et al.,* 2004). It is well established that cells are

able to respond to changes in their mechanical environment by altering synthesis and degradation of matrix components. This response was first documented in bone cells, which increase or decrease bone mass according to the amount of load placed on the bone. Frost proposed that osteoblasts have a pre-set sensitivity to deformation such that if strain-induced signals are above or below a certain level, termed the 'mechanostat set-point', the cells will remodel the matrix accordingly (Frost 1987). Recently, it has been shown that tendon cells have a similar ability to remodel the matrix according to the strain they experience (Lavagnino and Arnoczky 2005). Cells sense matrix deformation by mechanotransduction, which converts a mechanical signal into a biological response. Receptors on the cell surface will detect deformation, and this information will be passed to the nucleus via the internal cytoskeleton, where the appropriate response will be initiated (Ingber 1997). Recent studies have shown that there are large strain distributions within the tendon matrix, particularly compressive and shear strains which, due to complex local deformations, can, in some areas, exceed the overall strain applied to the tendon (Cheng and Screen 2007; Screen and Evans 2009).

While physiological strains may be expected to result in tendon adaptation unusually high strains may result in a damage response. *In vitro* studies have shown that exposing tenocytes to high strains causes them to alter their production of specific proteins. Exposing human tenocytes to 3.5% cyclical strain at a frequency of 1 Hz for 2 hours caused increased expression of MMP-3, which degrades proteoglycans, and interleukin 1-Beta (IL1-β), a cytokine that is able to induce expression of MMPs (Tsuzaki *et al.,* 2003). Cyclically stretching human tendon cells from 4-12% at a frequency of 0.5 Hz caused an increased production of prostaglandin E_2, which is an inflammatory mediator of tendinopathy (Wang *et al.,* 2003). It has also been found that loading rabbit tenocytes at 5% cyclical strain with a frequency of 0.33 Hz causes increased expression of MMP-1 and MMP-3. However, this upregulation only occurred with the addition of IL1-β (Archambault *et al.,* 2002). The strains used in these studies may not appear to be high relative to the strains seen by the SDFT in a galloping horse however it is difficult to recreate *in vitro* the strains experienced by cells *in vivo*. Visualising cells in fascicles exposed to 8% strain has shown that the local strain experienced by most cells does not appear to exceed 2% (Screen *et al.,* 2004), although there may be areas within the matrix where strains are amplified (Screen and Evans 2009). Cells in these *in vitro* studies are therefore being exposed to strains equivalent

to those that might be experienced *in vivo* by tenocytes in a high strain energy storing tendon such as the SDFT. However, cells in the SDFT may have a high mechanostat set-point, such that they have adapted to perceive high levels of strain as 'normal'. SDFT tenocytes may therefore only exhibit a degradative response when exposed to extremely high strains, generated as a result of micro-damage to the matrix or in regions of high stress concentrations within the tendon.

Alternatively, it has been proposed that it is the under-stimulation of cells rather than over-stimulation that causes the upregulation of matrix degrading enzymes. Micro-damage results in the unloading of the damaged fibrils (Lavagnino *et al.,* 2006), and fibril disruption will alter the cell-matrix interactions, which may result in the localised stress-shielding of some cells. Stress deprivation causes increased expression of the collagenase MMP-13, alterations in cell morphology and pericellular environment (Arnoczky *et al.,* 2008b), and increased apoptosis (Egerbacher *et al.,* 2007). It has also been reported that stress deprivation alters the cells' response to loading; cells in stress deprived tendons that were then cyclically loaded were found to express significantly higher levels of MMP-13 mRNA than cells that had not been stress deprived (Arnoczky *et al.,* 2008b). This suggests that under-stimulation causes the cells to alter their 'set point', so they may be unable to respond appropriately to mechanical stimuli and so will not repair any micro-damage. The cells may also actively degrade the matrix, which may accelerate the rate of micro-damage accumulation.

1.10. Abnormal Loading Events

Micro-damage has been shown to occur *in vitro* after a single loading event at 80% of failure strain (Lavagnino *et al.,* 2006). It is possible that during repetitive loading (i.e. during high speed exercise) one abnormal loading cycle may result in strains of a high enough magnitude to induce isolated micro-damage without clinical injury (Arnoczky *et al.,* 2008a). This abnormal loading may be as a result of muscle fatigue, changes in the neuromuscular response or exercise on an uneven surface (Kai *et al.,* 1999). This suggests that tendon injury is more likely to occur towards the end of a race, when racing over longer distances or over fences, and this is supported by studies that have found increased tendon injury in these situations (Peloso *et al.,* 1994; Pinchbeck *et al.,* 2004; Takahashi *et al.,* 2004; Williams *et al.,* 2001). Recently, it has been proposed that the SDFT may be at increased risk of injury due to fatigue of the deep digital flexor muscle. During high speed

locomotion, the DDFT stores little energy, but acts to stabilise the metacarpophalangeal joint during hyperextension. However, its corresponding muscle has a high percentage of fast twitch muscle fibres, and so is more susceptible to fatigue. If this occurs, the metacarpophalangeal joint will be de-stabilised and so the SDFT will be subjected to higher strains which may result in fibril micro-damage (Butcher *et al.,* 2007). These findings indicate that as the SDFT experiences high strains which are close to failure strains recorded *in vitro*, very small increases in strain may be sufficient to cause micro-damage and lead to clinical injury.

1.11. Injury Prevention and Treatment

1.11.1. Prevention

One of the most effective ways of preventing injury to musculoskeletal tissues is to increase tissue strength by undertaking a training programme which results in a tissue that is able to resist higher forces. However, this assumes that the cells within the tendon are able to remodel the matrix and it has been found that, unlike other skeletal tissues such as bone and muscle, mature tendon has a limited ability to undergo hypertrophy in response to exercise. However, mechanical loading is essential for development of tendons; foals that were confined in a stall from the ages of 1 week to 5 months were found to have smaller and weaker SDFTs than foals that were maintained at pasture (Cherdchutham *et al.,* 2001). Interestingly, foals kept at pasture also had stronger and more extensible tendons than foals that were housed in stalls and exposed to episodes of high intensity exercise daily, suggesting that longer periods of low intensity exercise may be required for the development of optimal tendon mechanical properties (Cherdchutham *et al.,* 2001). Kasashima and colleagues (2002) measured the changes in tendon CSA of foals aged 2-15 months; the CSA of the immature SDFT in foals exposed to high intensity exercise was found to increase to a greater extent when compared to foals that were allowed pasture exercise. However, a more recent study reported that exposing immature horses to additional exercise from birth had no effect on tendon matrix composition or mechanical properties (Kasashima *et al.,* 2008). Tendons appear to reach maturity at a relatively early age; in a study of 2 year old horses (Birch *et al.,* 1999b) neither the SDFT nor the DDFT underwent hypertrophy in response to high-intensity exercise. However, the CSA of the CDET increased significantly in the horses on the high-intensity training program. The

study indicated that the tendons reach maturity at different times; the SDFT reached maturity by the age of 2, whereas the DDFT and CDET reach maturity between the ages of 2 and 3. It is possible that pasture exercise is sufficient for the tendons to develop optimal mechanical properties (Kasashima *et al.,* 2008), and additional exercise does not increase the CSA of the mature SDFT as this would result in increased stiffness which would decrease the energy storing capacity of the tendon (Birch *et al.,* 1999b). As it does not appear possible to improve tendon mechanical properties by exposing the horse to different exercise intensities, it is important to develop training programmes that allow optimal adaptation of other musculoskeletal tissues without resulting in tendon overstrain injury.

1.11.2. Current Treatments

The earliest reported treatment for tendon injury is cautery, where heat or acid is applied to the skin adjacent to the injured tendon. This treatment is recorded in the earliest printed work on veterinary anatomy (Artis veterinariae; 500 AD), and is still in use today, although its effectiveness and use is controversial. Tendon blood supply is generally poor, and so this treatment is proposed to work by increasing circulation to the injured tendon. The use of cautery is supported by much anecdotal evidence, but the only long term experimental study concluded that it is no more effective than the conservative treatment consisting of a period of rest followed by an incrementally increasing exercise protocol (Silver *et al.,* 1983). In the last decade, several new treatments for equine tendon injury have been developed; these include extracorporeal shock wave therapy (ESWT) and injection of autologous bone marrow derived mesenchymal stromal cells (MSCs) or platelet rich plasma (PRP). However, these therapies are often used clinically before rigorous testing has been undertaken, and so their efficacy is not known.

ESWT involves applying high energy pressure pulses to a focused area and has been used to treat tendon injuries in the horse and human (Gerdesmeyer *et al.,* 2003; Lin 2005). Shock waves generate pressure fluctuations within the tissue which are thought to initiate a healing response within the tendon by increasing the metabolic activity of the resident tenocyte population (Lin 2005). Clinical studies suggest that ESWT increases the rate of lesion healing in equine tendons as assessed ultrasonographically (McClure and Weinberger 2003) and may also have an analgesic effect (Dahlberg *et al.,* 2006). However in a model using ESWT to treat collagenase-induced lesions in the SDFT it was found that

application of shock waves did not affect the ultrasonographic or histological parameters of tendon lesions compared to controls, but did result in increased neo-vascularisation (Kersh *et al.,* 2006). Evaluation of the effects of ESWT on properties of normal tendon has found that ESWT results in increased GAG and protein synthesis in the short-term (3 h after treatment) (Bosch *et al.,* 2007) however 6 weeks after treatment the matrix appears less organised compared to untreated tendons, and there was also increased gene expression for type I collagen (Bosch *et al.,* 2009a), which is a feature of tendon injury (Riley 2008). These results suggest that EWST should be used with caution in the treatment of tendon injuries, as any beneficial effects are yet to be fully elucidated, and there may be detrimental effects to surrounding healthy tendon tissue.

Another technique that has recently been developed to treat tendon injury is the injection of autologous mesenchymal stromal cells (MSCs) into the lesion. This treatment assumes that the resident tenocytes are incapable of repairing the tendon lesion and so uses bone marrow as a source of MSCs, which are isolated and expanded in culture and then injected into the injured tendon under ultrasonographic guidance (Smith and Webbon 2005). Clinical studies have found that horses treated with MSC injection had lower re-injury rates than those reported for horses treated using conventional methods (Smith 2008). Injection of MSCs into SDFT lesions in racehorses resulted in improved healing compared to controls treated conventionally as assessed by ultrasonography and successful return to racing in nine out of eleven cases (Pacini *et al.,* 2007). Autologous MSC implantation has also been reported to result in morphology closer to that of uninjured tendons than controls treated with saline injection (Young *et al.,* 2009a). Collagenase-induced lesions treated with MSCs had more normal histological parameters than the controls, but there was no difference in mechanical properties, gene expression levels, DNA and collagen content between MSC treated and control tendons (Schnabel *et al.,* 2009). While this appears to be a promising treatment, further work needs to be undertaken to determine the precise effect of MSC injection on lesion healing, both in the short and long term.

A treatment currently in use as an alternative to autologous MSC implantation is the injection of PRP into the site of injury. The reason for using PRP is that when activated, the platelets release several growth factors thought to enhance matrix repair by increasing tenocyte activity. *In vitro* studies have found that SDFT tenocytes cultured in PRP-rich

media express higher levels of genes coding for collagen, without an increase in gene expression for matrix degrading enzymes (McCarrel and Fortier 2009; Schnabel *et al.,* 2007). Injection of PRP into experimentally induced lesions resulted in increased metabolic activity and increased organisation of repair tissue compared to controls (Bosch *et al.,* 2009b; Bosch *et al.,* 2010).

Although there have been significant advances in treatment of tendon injuries in recent years, there is as yet no treatment available that is able to restore the tendon to its previous levels of functionality so that re-injury does not occur. The mode of action of the majority of treatments currently in use for tendinopathy is to increase cell activity within the lesion, either by the implantation of additional cells or through the use of methods designed to increase metabolic activity of the cells resident within the tendon. The effects of injecting substances such as MSCs and PRP have yet to be fully determined, and although they appear to increase the rate of repair to some extent, long recovery periods are still required and it is not know if the level of repair achieved is any greater of that achieved when managing tendon injury conservatively. It is difficult to assess the repair process when little is known about tenocyte phenotype, activity and matrix turnover in healthy tendon; how these parameters differ between functionally distinct tendons and if they are affected by increasing age.

1.12. Conclusions

High strains experienced by the energy storing human Achilles tendon and equine SDFT undoubtedly contribute to the high incidence of injury to these tendons. The findings of epidemiological studies support this as the forelimbs, which carry 60% of the horse weight when standing and even more during locomotion, are more susceptible to overload injury, and activities which result in particularly high strains such as galloping and jumping at speed increase the risk of injury. However the presence of degenerative matrix changes and the increased incidence with increasing age in both humans and horses suggest that injury is not due to a simple mechanical overload. Furthermore some human and equine athletes compete at the highest level and never suffer from tendon injury. There is a large variation in the strength and stiffness of the SDFT between individual horses which does not correlate with body weight or age; it is not known at present whether this relates to injury susceptibility. The ability to turnover matrix components is likely to be important but as yet

it is not clear why the capacity to do this should be less in the injury prone SDFT that the rarely injured CDET; and further work is required to elucidate any differences in cell phenotype between the functionally distinct tendons. An improved understanding of matrix turnover and ageing processes in tendon will provide opportunities to modify management of athletes' training programmes to prevent injury and also to improve the current treatment options available for tendinopathies.

CHAPTER TWO

2. Thesis Aims and Objectives

2.1. General Hypothesis

This thesis tests the hypothesis that tenocyte metabolism is programmed by the strains the cells experience *in vivo* meaning that functionally distinct high strain energy storing tendons have a lower rate of matrix turnover than low strain positional tendons and that this declines further with increasing age.

2.2. Aims and Objectives

The aim of this thesis is to test the hypothesis above by addressing the following objectives:

1. To measure the age of extracellular matrix molecules in functionally distinct tendons in the equine forelimb and determine how this differs between tendons and changes with increasing horse age.
2. To measure the ability of the tenocytes to synthesise structural components of the matrix and matrix degrading enzymes and determine if this differs between tendons and alters with increasing horse age.
3. To quantify markers of extracellular matrix turnover at the protein level and determine if levels differ between tendons or with increasing horse age.
4. To determine if tenocytes from functionally distinct tendons maintain their phenotype *in vitro* and if they are able to alter their phenotype if placed in a different strain environment from that which they experience *in vivo*.

Experimental design

General Methods (Chapter 3)

Tendons from the equine forelimb were harvested from horses chosen with a wide age range (4 - 30 years). Four tendons were collected from the right forelimb of each horse and were separated into two groups according to their functions; the high strain energy storing tendons (superficial digital flexor tendon (SDFT) and suspensory ligament (SL)) and the low strain positional tendons (common digital extensor tendon (CDET) and deep digital flexor tendon (DDFT)). The mid-metacarpal region was harvested from each tendon and processed for analysis as described in subsequent chapters.

Objective 1:   Matrix Age and Composition (Chapter 4)

The work in this chapter tests the hypothesis that the matrix composition and age of the SDFT and SL would be similar as these tendons have a similar function *in vivo*, as would the composition and age of the CDET and DDFT. Furthermore, it was hypothesised that the accumulation of markers of matrix age would be more rapid in the SDFT and SL than in the CDET and DDFT, indicating an 'older' matrix in the high strain energy storing tendons compared to the low strain positional tendons. Matrix composition was assessed in the forelimb tendons by measuring the collagen, GAG, water and DNA content, and assessing levels of pyridinoline crosslinks. Molecular matrix age was determined by measuring the accumulation of the advanced glycation end product pentosidine and D-aspartic acid; these have been previously used as markers of matrix age as they accumulate over time in proteins with a low rate of turnover. To determine if the rate of turnover differed between the collagenous and non-collagenous matrix proteins, the matrix of the SDFT and CDET samples was separated into the collagenous and non-collagenous fraction and the half-life of the proteins in each fraction was estimated by measuring the accumulation of D-aspartic acid.

Objective 2:   Potential for Matrix Synthesis and Degradation (Chapter 5)

The work in this chapter tests the hypothesis that cells in the low strain positional tendons have a greater potential for synthesis of structural matrix proteins and degradative enzymes than their counterparts in the high strain energy storing tendons. It was further hypothesised that the potential for matrix protein production and matrix degradation would decrease with increasing horse age in all tendons, representing a decrease in overall cell activity. In order to assess the tenocytes' ability to synthesise and degrade matrix components, mRNA was extracted from the tendon samples and the expression of genes coding for collagenous and non-collagenous matrix proteins and the enzymes that degrade these proteins was measured using real time reverse-transcription polymerase chain reaction (RT-PCR). To determine the potential for matrix degradation at the protein level zymography and a fluorometric assay were used to assess activity of specific matrix degrading enzymes in a semi-quantitative manner in tissue from the SDFT and CDET.

Objective 3: Turnover of the Collagenous Matrix (Chapter 6)

The work in this chapter tests the hypothesis that collagen turnover in high strain energy storing tendons is lower than that in low strain positional tendons and that the rate of collagen turnover decreases with increasing horse age in both tendon types. The rate of collagen turnover that occurs in the functionally distinct SDFT and CDET was assessed using several different techniques. The rate of collagen synthesis was determined by measuring the amount of the amino terminal pro-peptide of type I collagen (PINP) which is cleaved from the procollagen molecule during collagen synthesis. Collagen degradation was assessed by measuring the concentration of protein fragments produced when collagen undergoes enzymatic degradation; the cross-linked carboxy terminal telopeptide of type I collagen (ICTP) and the ¾ neoepitope (C1,2C) which are generated when collagen is cleaved by collagenases.

Objective 4: Cell Phenotype Conditioning (Chapter 7)

The work in this chapter tests the hypothesis that tendon cell phenotype is programmed by the *in vivo* strain environment. Specifically, it was hypothesised that tendon specific phenotype is due to the different *in vivo* cell environment and therefore culturing cells in monolayer in the absence of mechanical load and under the same conditions would result in significant alterations in cell phenotype and differences between SDFT and CDET tenocytes would be lost. Further, it was hypothesised that when exposed to strains they would not normally experience *in vivo*, the tenocytes will alter their phenotype; i.e. exposing tenocytes to low strains will cause them to exhibit a phenotype usually associated with cells from the CDET and *vice versa*. Initial experiments were carried out to determine the effect of culturing tenocytes from the SDFT and CDET in 2D and 3D culture systems on tenocyte phenotype. Tenocyte phenotype plasticity was assessed by loading medial accessory extensor tendons (MAET) in an *in vitro* system. MAETs were harvested from the medial border of the CDET and cyclically loaded at either low or high strains for a period of 20 hours. Cell phenotype was assessed by measuring the expression of genes coding for key matrix proteins and corresponding degradative enzymes and markers associated with a tenocyte phenotype using RT-PCR.

CHAPTER THREE

3.   General Methods

This chapter outlines the criteria for selecting horses for the study and protocols used for sample collection into RNAlater® and for snap freezing and processing snap frozen tendon tissue to a lyophilised ground state. The freeze dried tissue was used in the subsequent experimental chapters for biochemical analysis and the tissue collected into RNAlater® for gene expression studies.

3.1. Horse Selection

The distal region of forelimbs (dissected free at the level of the carpo-metacarpal joint) from skeletally mature horses (n = 32) aged between 4 and 30 (14.72 ± 1.39; median = 14) euthanased for reasons other than tendon injury were collected from a commercial equine abattoir (LJ Potter (SW) Ltd, Taunton, Somerset). Age was determined by examination of passports and confirmed by dental examination. To ensure horse age did not cluster at a particular value the entire age range was divided into ranges spanning either two or three years, and forelimbs from three horses were collected for each of these age ranges. Where possible breed and exercise history were recorded and any horses with a history of tendon injury were excluded from the study.

3.2. Tendon Tissue Collection

3.2.1. Samples for Biochemical and Gene Expression Analysis (Chapters 4, 5 and 6)

The superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), suspensory ligament (SL) and common digital extensor tendon (CDET) were harvested from the right forelimb of 30 horses on the same day as euthanasia. In 2 cases further examination in the laboratory revealed abnormalities in the right forelimb and so the tendons from the left forelimb were collected; one horse had sustained an injury to the skin superficial to the SDFT and the other had a small amount of fibrous tissue on the surface of the SL. Any tendons that had macroscopic evidence of degeneration, which is characterised by thickening or discolouration of the central core (Webbon 1977) were discarded. The tendons were harvested according to a protocol to ensure samples were taken from a comparable region of tendon. The skin was removed from the level of the carpo-metacarpal joint to the proximal interphalangeal joint to expose the tendons (Figure 3-1). After the

tendons were dissected free from the limb any connective tissue was removed from their surface.
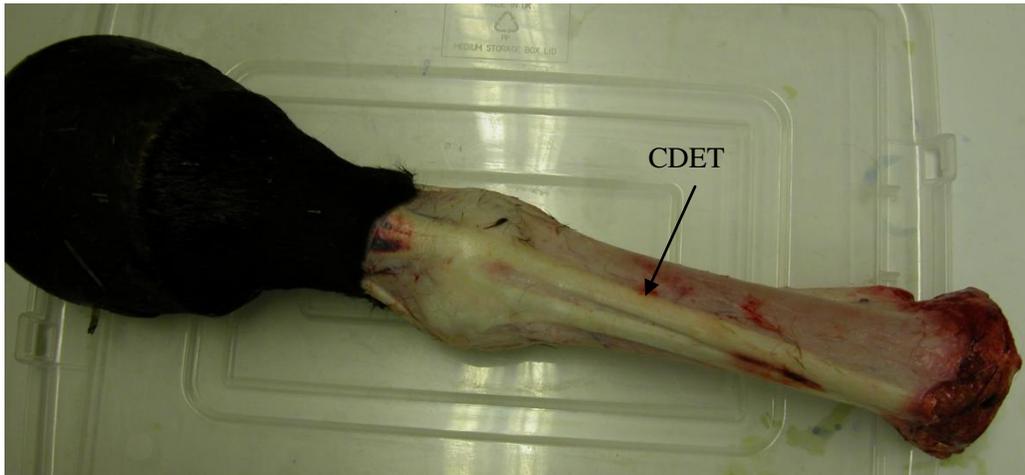
Figure 3-1: Dorsal view of the equine forelimb with skin removed to expose the tendons.

Before removal of the SDFT, the area where the communicating branch of the palmar nerve crosses the tendon was marked on the tendon (Figure 3-2). The communicating branch of the palmar nerve was used as an anatomical landmark to define the mid-metacarpal region of the tendon. A 0.5 cm section distal to the area marked was removed, the epitenon and peripheral tissue was dissected away and the remaining tendon tissue was placed in RNAlater® (Ambion, Applied Biosystems, Warrington, UK). A 2 cm section distal to this was removed and snap-frozen in hexane (Figure 3-2) cooled on dry ice.
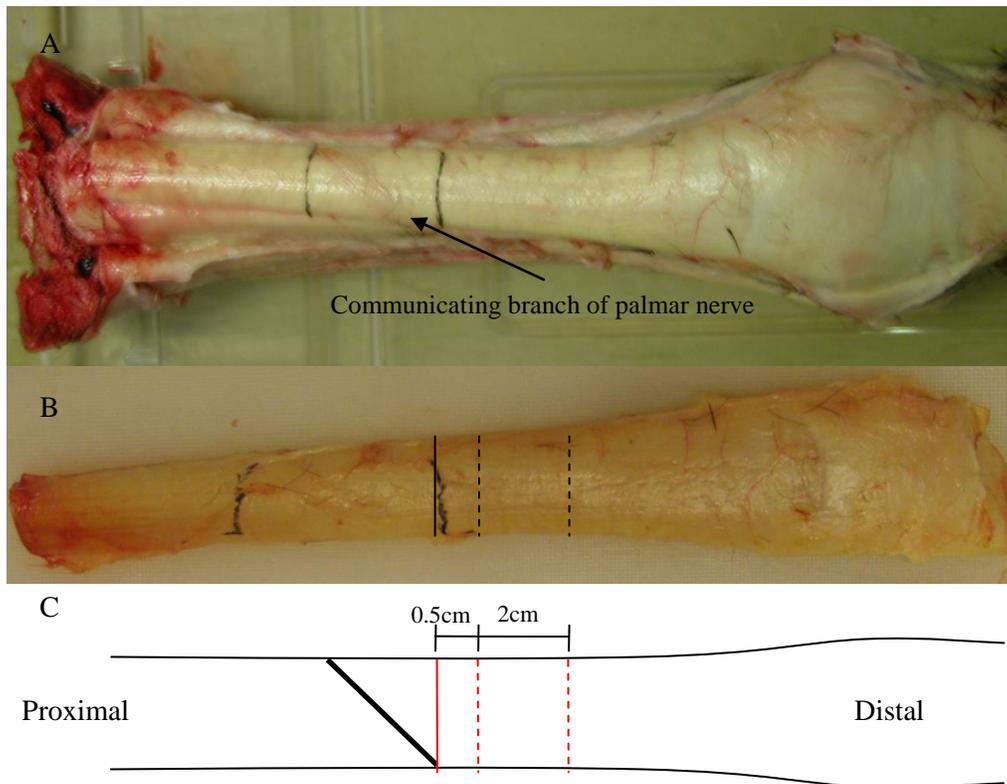
Figure 3-2: Tissue collection from SDFT. A- The location of the communicating branch of the palmar nerve was marked *in situ*. B- The SDFT was removed at the level of the metacarpo-phalangeal joint. C- The proximal section was placed in RNAlater and the distal section was snap frozen for matrix analysis.

The DDFT was removed at the level of the metacarpo-phalangeal (MCP) joint. The distance between the insertion of the accessory ligament and the widest part of the distal end was measured and the half way point was marked. A section 1 cm either side of this mark was removed and snap-frozen. A 0.5 cm section was removed proximally, processed as described for the SDFT and placed in RNAlater® (Figure 3-3).

C

0.5cm 2cm

Proximal

Distal

Midpoint between insertion of accessory ligament and widest part of distal end
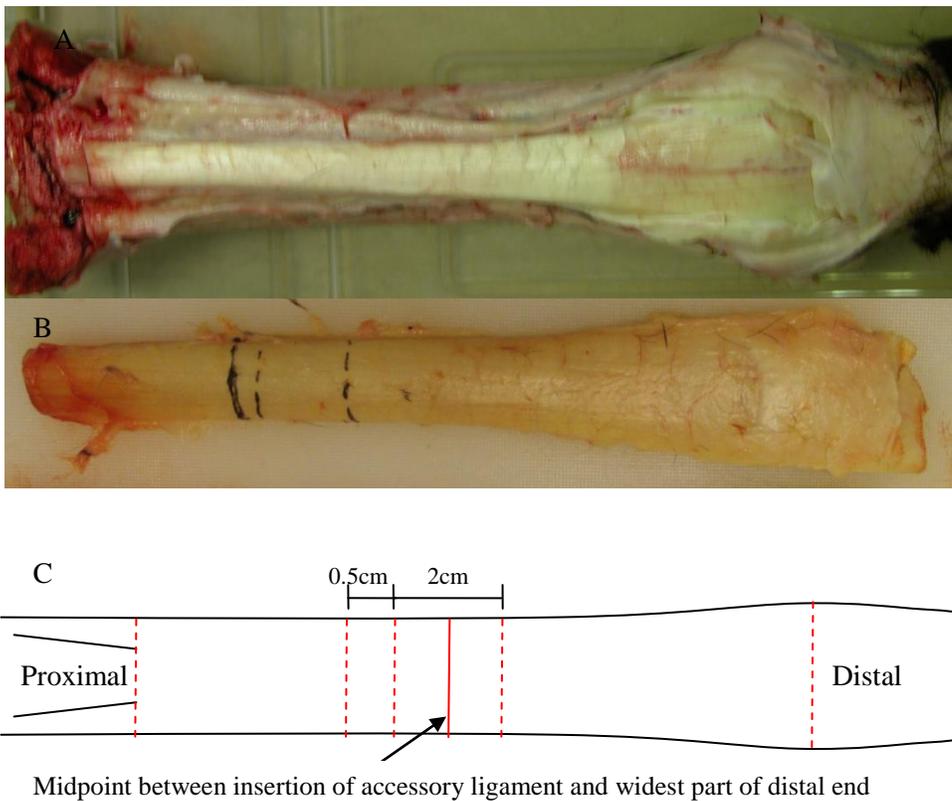
Figure 3-3: Tissue collection from the DDFT. A- The location of the DDFT *in situ*. B- The DDFT was removed at the level of the metacarpo-phalangeal joint and the midpoint between the insertion of the accessory ligament and widest part of the distal end was marked on the tendon. C- The proximal section was placed in RNAlater and the distal section was snap frozen for biochemical analysis.

The distal end of the SL was removed at the level of the MCP joint and the proximal end was removed as close to the origin as possible. The distance was measured from the proximal end to the point of bifurcation and the half way point was marked. Tissue was collected as for the DDFT (Figure 3-4).
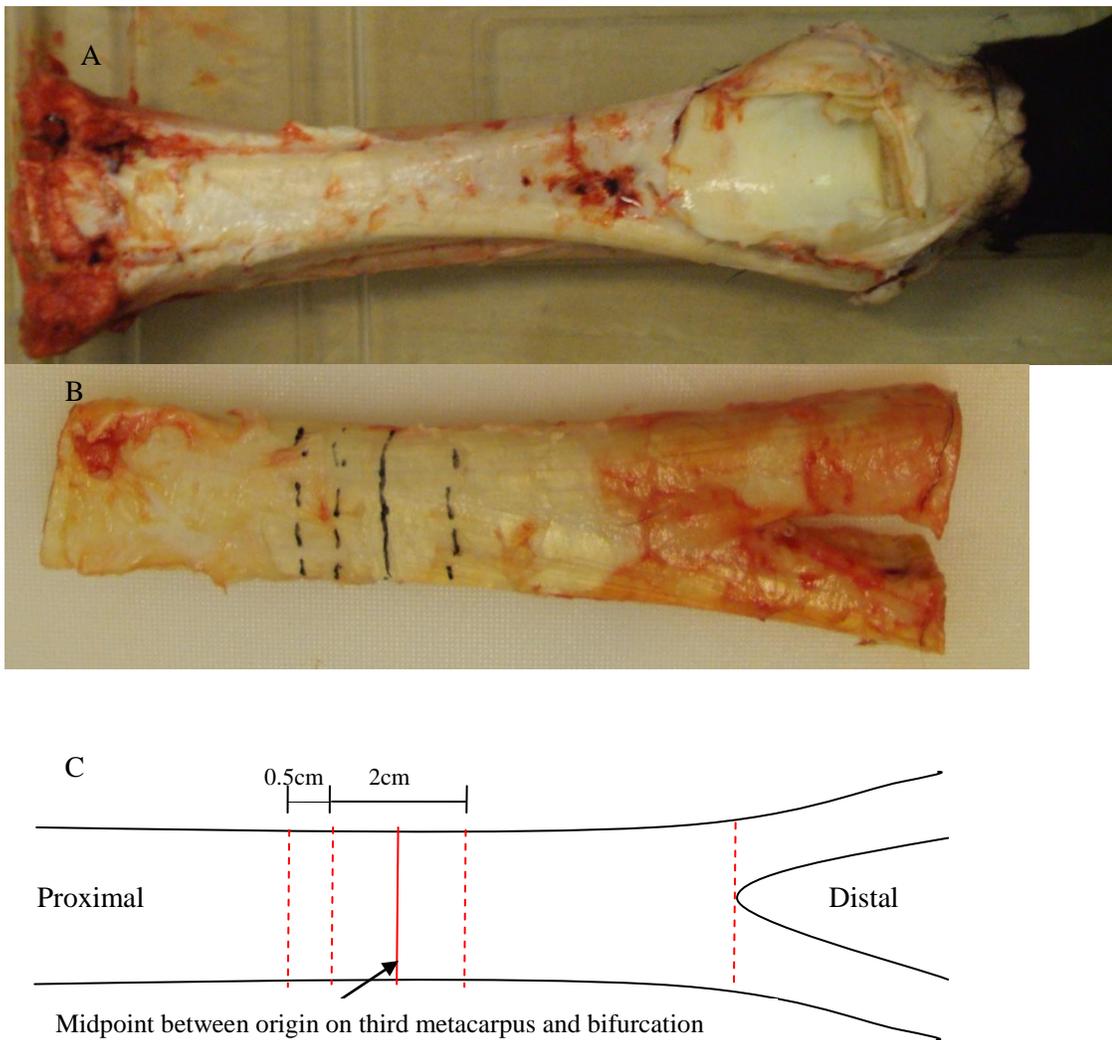
Figure 3-4: Tissue collection from the SL. A - The location of the SL *in situ*. B - The SL was removed at the level of the metacarpo-phalangeal joint and as close to the origin on the third metacarpal as possible. The midpoint between the origin and the bifurcation was marked on the tendon. C - The proximal section was placed in RNAlater and the distal section was snap frozen for biochemical analysis.

Before the CDET was removed, the length of the third metacarpal bone was measured and half of this distance was marked on the tendon. Tissue was collected as for the DDFT but as the CDET has a much smaller cross sectional area than the other tendons a section was cut 1.5 cm either side of the area marked and a section 1 cm in length was placed in RNAlater® (Figure 3-5).
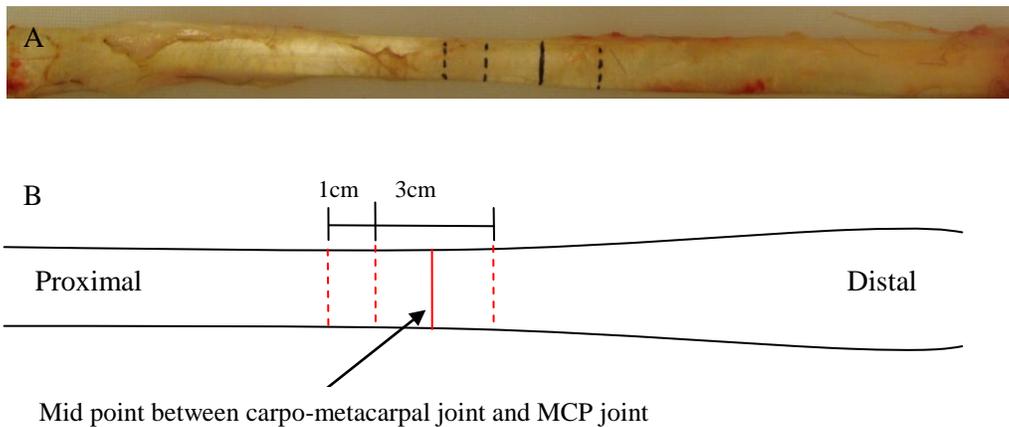
Figure 3-5: Tissue collection from the CDET. A - The midpoint betweed the carpo-metacarpal joint and the MCP joint was marked and CDET was removed at the level of the MCP. B - The proximal section was placed in RNAlater and the distal section was snap frozen for biochemical analysis. For the location of the CDET *in situ*, see Figure 3-1.

The samples suspended in RNAlater® were stored at 4 °C overnight to allow thorough penetration of the tissue before being stored at -20 °C. The snap-frozen samples were individually wrapped in cling-film and tinfoil and stored at -80 °C.

3.3. Tendon Tissue Preparation

When required, the snap-frozen samples were thawed slightly so the epitenon and surrounding connective tissue could be removed. The remaining tendon tissue was chopped, accurately weighed to within 0.1 mg and re-frozen at -80 ℃. Tendon tissue samples were then freeze-dried overnight until a constant weight was reached. The lyophilised samples were reweighed and the water content was calculated and expressed as a percentage of the wet weight. The samples were stored at -80 ℃ before being reduced to a fine powder by the use of a mikro-dismembrator (Sartorius, Germany) at 3000 rpm for 2 minutes. The powdered samples were stored at -80 ℃ until required for analysis.

3.4. Reagents

Except where stated, all reagents used in the experiments described in the subsequent chapters of this thesis were obtained from Sigma-Aldrich Company Ltd., Dorset UK, and VWR International Ltd., Leicestershire, UK.

CHAPTER FOUR

4. Molecular Markers of Matrix Age Identify a Difference in Matrix Half-life between Functionally Distinct Tendons

4.1. Introduction

Little is known about the age of matrix molecules in living tendon and whether matrix age differs between tendons with physiologically distinct roles, and the effect this has on tendon mechanical properties. Tendons require high mechanical strength as they transfer force between muscle and bone and therefore must be able to resist high forces, which can reach up to 90 MPa in some tendons (Biewener 1998; Brown *et al.,* 2003; Ker 2002). The ability to resist forces is provided mainly by collagen which makes up approximately 80% of the tendon dry weight. Tendons such as the human Achilles, and equine superficial digital flexor tendon (SDFT) and suspensory ligament (SL) also store and return energy during locomotion, increasing efficiency by as much as 36% during galloping exercise (Biewener 1998). Energy storing tendons need to be more extensible under physiological loads than positional tendons such as the human anterior tibialis tendon and equine deep digital flexor tendon (DDFT) and common digital extensor tendon (CDET). Previous studies have identified a difference in the material properties between functionally distinct tendons; the SDFT is composed of less stiff material than the CDET (Batson *et al.,* 2003). This is accompanied by differences in matrix composition; energy storing tendons have a greater proteoglycan content than positional tendons (Batson *et al.,* 2003) which may allow energy storing tendons to be more extensible by allowing greater sliding movement between the collagen fibrils (Scott 2003).

Tendon structure and mechanical properties must be maintained for efficient function, and this is achieved by balancing matrix synthesis and degradation. It was previously thought that, after maturity, the tenocytes resident within the tendon matrix became metabolically inactive such that the collagen comprising the matrix was not actively repaired and replaced during the course of a lifetime (Ballou and Thompson 1956; Neuberger *et al.,* 1951). However, it has recently been demonstrated that collagen in patella tendons is actively turned over (Miller *et al.,* 2005), and acute exercise results in increased synthesis of collagen type I in Achilles tendons (Langberg *et al.,* 1999; Langberg *et al.,* 2001). Specific tendons such as the equine SDFT and human Achilles are prone to pathological changes and the incidence of tendon injury increases with increasing age in both horses and humans

(Jarvinen *et al.,* 2005; Perkins *et al.,* 2005), suggesting there is an imbalance in matrix synthesis and degradation in these tendons resulting from a decreased ability to renew the matrix.

4.1.1. Matrix Composition

The matrix composition of tendons in the equine forelimb has been assessed previously and changes occurring with age, as well as differences occurring between tendons have been identified (Batson *et al.,* 2003; Birch *et al.,* 1999a; Birch *et al.,* 2002; Lin *et al.,* 2005b). The mature SDFT has higher glycosaminoglycan (GAG) content, water content and cellularity than the CDET (Batson *et al.,* 2003); these differences in composition result in differences in mechanical properties which are required for the efficient function of the SDFT and CDET. Differences in matrix composition have also been identified between the SDFT and DDFT (Birch *et al.,* 1999a), although these are not as great as the differences found between the SDFT and the CDET. The DDFT has lower cellularity and percentage of type III collagen relative to type I, and higher GAG content and percentage of large diameter collagen fibrils than the SDFT. Differences in collagen crosslink profile have also been reported, with lower levels of hydroxlysyl-pyridinoline crosslinks in the DDFT than in the SDFT (Birch *et al.,* 1999a). Matrix composition is also altered during injury, with increases in cellularity, type III collagen, GAG and water content as common features in SDFT degeneration (Birch *et al.,* 1998). Similar changes have been reported in human tendinopathy; Achilles tendon lesions have a higher water content, and increased levels of denatured collagen (de Mos *et al.,* 2007) than normal tendon samples.

4.1.2. Matrix Age

Molecular matrix age can be defined as the length of time a protein has been present in the matrix. This can be assessed in various tissues by measuring the age related accumulation of chemical modifications such as crosslinking and amino acid racemization that occur in tissues with a relatively low rate of metabolism. Matrix age gives an indication of the rate at which tenocytes are able to degrade damaged matrix and synthesise matrix components to repair any micro-damage, which is important to maintain tendon mechanical integrity and prevent the accumulation of micro-damage.

4.1.2.1. Crosslinks

Crosslinks that occur between collagen molecules are formed by two different mechanisms; an enzymatic-controlled process that occurs during growth and development and a spontaneous non-enzymatic reaction between collagen molecules and sugars present in the tissue that occurs during ageing (see Chapter 1) (Bailey *et al.,* 1998). In tendon, the pyridinolines are the major crosslinks that are formed by the action of the enzyme lysyl oxidase. Some enzymatic and non-enzymatic crosslinks fluoresce naturally, so crosslink content can be estimated by measuring tissue fluorescence; previous work has shown that the SDFT has higher levels of tissue fluorescence than the CDET (Birch *et al.,* 2008b), indicating greater matrix turnover in the CDET. This is surprising as it would be expected that there would be a higher rate of matrix turnover in the energy storing SDFT because it operates with a much lower safety margin than the CDET. However, previous work has also identified a difference in lysyl oxidase generated crosslink profile between the SDFT and CDET (Birch *et al.,* 2006) which may account for the lower levels of tissue fluorescence recorded in the CDET.

Accumulation of the glycated crosslink pentosidine has been used to assess matrix age and turnover in both human and equine tendon previously; pentosidine has been shown to increase linearly with subject age in human tendons that are rarely injured (Bank *et al.,* 1999). However, in the injury prone supraspinatus tendon pentosidine does not continue to accumulate in older aged individuals, and levels decrease in degenerated tendons (Bank *et al.,* 1999). Pentosidine levels are also lower in degenerated Achilles tendons than in uninjured tendon (de Mos *et al.,* 2007), suggesting that remodelling of the matrix occurs in tendon pathology in an attempt to repair the lesion. Assessment of the equine SDFT found that pentosidine increased with age in the sesamoidal region of mature tendon, but not in the mid-metacarpal region, where the majority of injuries occur (Lin *et al.,* 2005b). However, pentosidine concentration in immature tendons was found to increase with age in both the sesamoidal and mid-metacarpal regions, and reached significantly higher levels in the mid-metacarpal region (Lin *et al.,* 2005a). No previous studies have assessed matrix age in the functionally distinct tendons in the equine forelimb using accumulation of pentosidine as a probe.

4.1.2.2. Amino Acid Racemization

Another spontaneously occurring age related modification to the matrix is the racemization of amino acids. All amino acids are incorporated into proteins in the L-(levorotatory) form due to the stereo-specificity of enzymes required for protein synthesis (Shah *et al.,* 1999), but over time they spontaneously convert to the D-(dextrorotatory) form. This is a very slow process in all amino acids, but occurs at a relatively rapid rate in aspartic acid (asp), and so the ratio of D to L-Asp in any tissue gives an indication of matrix age. Racemization results in a change in the optical conformation of the aspartic acid residue via the formation of a succinamide intermediate (McCudden and Kraus 2006). Both L-Asp and L-asparagine can be dehydrated to form L-succinamide, which then undergoes racemization and isomerisation to form D-Asp (McCudden and Kraus 2006) (Figure 4-1).
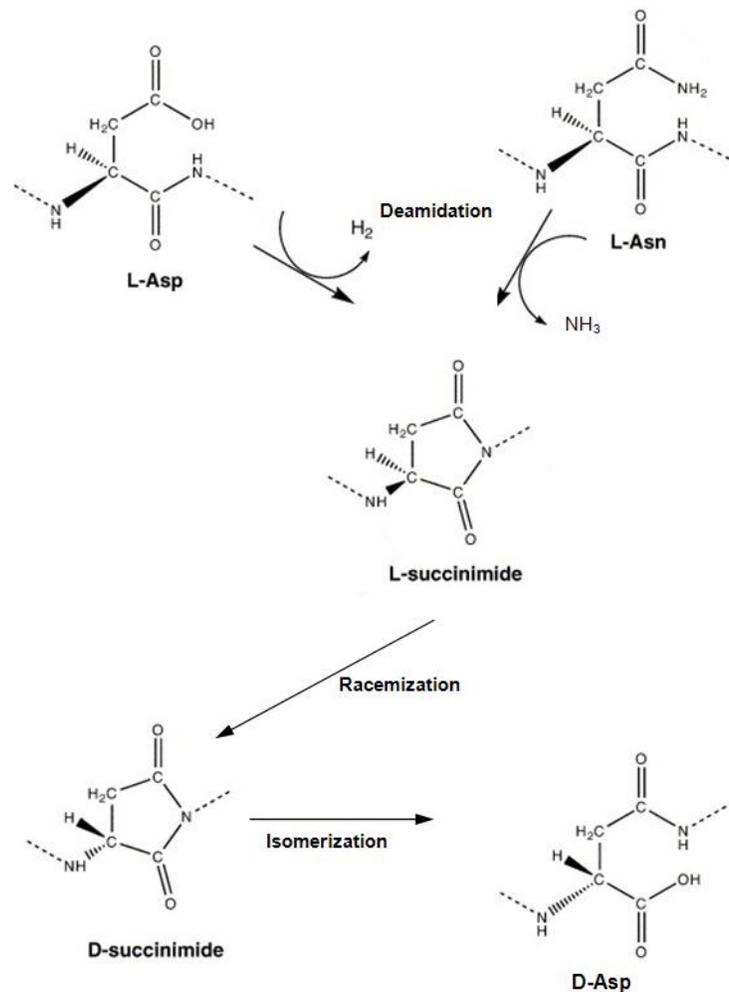
Figure 4-1: Conversion of L-Asp and L-Asn to D-Asp via the formation of a succinamide intermediate and subsequent racemization and isomerisation. Adapted from McCudden and Kraus (2006).

Amino acid racemization was originally studied in an attempt to determine the presence of extraterrestrial life, based on the hypothesis that amino acids that had been synthesised biologically would have an excess of either D- or L- forms (Bada and McDonald 1996). Aspartic acid racemization has also been proposed as an alternative to radiocarbon dating (Bada *et al.,* 1974) and as an age determinant in forensic science by comparison of the percent D-Asp in a sample of unknown age relative to that in a sample of known age (Ohtani and Yamamoto 1991). However, as the rate of racemization is dependent on the rate of protein turnover as well as subject age, only tissues that do not turnover over the course of a lifetime, such as dentin, can be used as a determinant of age. Recently, aspartic acid racemization has been used as a marker of matrix age in a variety of tissues and has been proposed as a biomarker of musculoskeletal diseases which are often characterised by alterations in protein turnover rates (McCudden and Kraus 2006).

D-Asp has been shown to accumulate with age in collagen in various connective tissues, including bone (Pfeiffer *et al.,* 1995), articular and intervertebral disc cartilage, and tendon in a similar manner to pentosidine (Maroudas *et al.,* 1992; Riley *et al.,* 2002; Sivan *et al.,* 2008; Verzijl *et al.,* 2000b). Lower levels have been measured in degenerated intervertebral discs (Sivan *et al.,* 2008) and tendons that are prone to pathological changes (Riley *et al.,* 2002) indicating an increased rate of protein turnover with disease. Proteoglycans are also susceptible to racemization; accumulation of D-Asp has been shown to occur in articular cartilage aggrecan (Maroudas *et al.,* 1998) and intervertebral disc aggrecan (Sivan *et al.,* 2006b). D-Asp accumulation can be used to calculate protein half-life using the rate of D-Asp accumulation in dentin (as turnover of collagen in dentin over the course of a lifetime is negligible) as the rate constant of racemization (Helfman and Bada 1976).

4.1.3. Aims and Hypothesis

The aims of this chapter were to define the matrix composition in functionally distinct tendons and to assess matrix age by measuring the accumulation of markers of matrix age. Based on previous findings, it was hypothesised that the matrix composition and age of the SDFT and SL would be similar as these tendons have a similar function *in vivo*, as would the composition and age of the CDET and DDFT. Furthermore, it was hypothesised that the accumulation of markers of matrix age would be more rapid in the SDFT and SL than in

the CDET and DDFT, indicating an 'older' matrix in the high strain energy storing tendons compared to the low strain positional tendons.

4.2.   Materials and Methods

Lyophilised tendon tissue from the SDFT, DDFT, SL and CDET of 32 horses collected and processed as described in chapter 3 was used for these studies.

4.2.1. Papain Digestion

A small amount (20-40 mg) of powdered tissue was accurately weighed out and digested with papain, which breaks down matrix proteins and cell walls. Papain buffer (2 ml; papain (2 units/ml) in sterile phosphate buffered saline (PBS) plus 5 mM cysteine.HCl, 5 mM EDTA.Na$_2$, pH 6.0) was added to each sample and the samples were digested for 24 hours at 60 °C (Birch *et al.,* 1998). The samples were vortex mixed several times during digestion to ensure all tissue was solubilised. A sample containing no tissue was included with each batch to act as a blank for subsequent assays.

4.2.2. Assessment of Matrix Composition

4.2.2.1. Tendon DNA Content

Immediately after papain digestion the DNA content of the tendons was measured using the bisbenzimidazole dye Hoechst 33258, which binds in the groove of the DNA double helix, and the method of Kim *et al.* (1988). Aliquots of papain digested tissue (150 µl) were diluted to 3 ml with dye buffer (0.1 µg/ml Hoechst 33258, 10 mM Tris-HCl, 1 mM EDTA.Na$_2$, 0.1 mM NaCl, pH 7.4) and fluorescence was measured using a fluorometer (Perkin Elmer, LS-50B; excitation wavelength: 348 nm, emission wavelength 457 nm) in the presence and absence of the dye to account for background tissue fluorescence. DNA concentration was calculated by comparison to a standard curve constructed using calf thymus DNA standards from 0-0.5 µg/ml dye. The fluorescence of the papain buffer was taken into account by measuring fluorescence in the absence of tissue and subtracting this value from the fluorescence of the samples. DNA content was expressed as µg per mg dry weight tendon tissue.

4.2.2.2.   Tendon Collagen Content

Collagen content was calculated by measuring the concentration of the imino acid hydroxyproline, which was assumed to make up 14% of collagen dry weight (Birch *et al.,*

1998). Aliquots (100 µl) of papain digested tissue were hydrolysed in 6 M hydrochloric acid (HCl), dried and re-dissolved in 10 ml deionised water. Hydroxyproline content was assessed using a method adapted from Bergman and Loxley (1963) and Bannister and Burns (1970) where hydroxyproline is oxidised by chloroamine T and coupled with dimethylaminobenzaldehyde (DMBA) which results in a coloured product which can be visualised at 550 nm using a spectrophotometer. Samples (1 ml) were mixed with 1 ml diluent (2 parts propan-2-ol to 1 part D.I. water), 1 ml of oxidant was added (0.42 g chloroamine T, 5 ml D.I. water, 25 ml stock buffer) and the samples were left to stand for 20 minutes. Colour reagent (1 ml; 3 g DMBA, 4.5 ml 70% percholoric acid, 25 ml propan-2-ol) was then added and the samples were left for 14 minutes before heating (70 ℃, 20 minutes). Absorbance was read on a spectrophotometer (Shimadzu, UV-160A) after allowing the samples to cool for 10 minutes. Concentration was calculated by comparison to a standard curve prepared using L-hydroxyproline standards from 0-2.5 µg/ml (final concentration). The absorbance of the papain buffer was taken into account by measuring absorbance in the absence of tissue and subtracting this value from the samples. Collagen content was expressed as a percentage of dry weight tendon tissue.

4.2.2.3. Tendon Glycosaminoglycan Content

Sulphated glycosaminoglycan (GAG) content was measured in the papain digested tissue using dimethylmethylene blue dye (DMB), which binds sulphated GAGs and forms a complex that absorbs light at 525 nm (Farndale *et al.,* 1986). Aliquots (100 µl) of papain digested tendon were mixed with 3 ml dye (1 l D.I. water containing 16 mg DMB, 3.04 g glycine, 2.37 g NaCl, 95 ml 0.1 M HCl). The absorbance of the resulting solution was measured using a spectrophotometer (Shimadzu, UV-160A) and GAG concentration was calculated by comparison to a standard curve prepared with bovine trachea chondroitin sulphate which was diluted in dye to give concentrations from 0-3.2 µg/ml (final concentration). The absorbance of the papain buffer was taken into account by measuring absorbance in the absence of tissue and subtracting this value from the samples. GAG concentration was expressed as µg per mg dry weight tissue.

4.2.3. Matrix Age

4.2.3.1. High Performance Liquid Chromatography

Tendon crosslink content and percent D-asp were measured using high performance liquid chromatography (HPLC). This technique is able to separate and quantify compounds with very similar chemical compositions. The equipment consists of mobile and stationary phases, and compounds are separated according to their affinity with these phases. The mobile phase is normally a mixture of organic and aqueous solutions, and the stationary phase is composed of silica or carbon and forms the lining of the column (Figure 4-2).
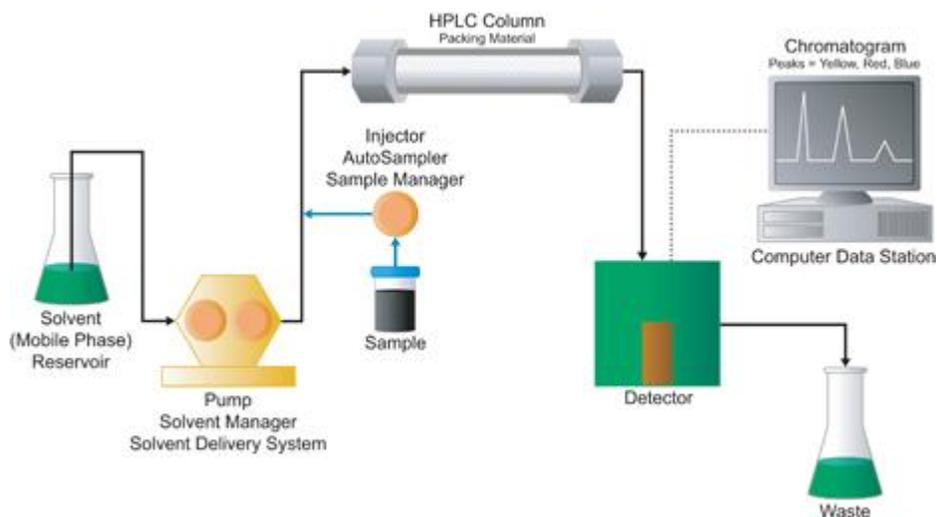
Figure 4-2: Diagram showing HPLC setup. From www.waters.com.

The sample is mixed with the mobile phase and is pumped through the column under high pressure (approximately 2000 psi). The rate at which the compounds present in the sample are eluted from the column depends on their affinity with the mobile and stationary phases; for example if the mobile phase is aqueous, hydrophilic compounds will be eluted from the column relatively rapidly whereas hydrophobic compounds will bind transiently to the column lining and will therefore take longer to travel through the column. The retention time of compounds can be altered by changing the mobile phases using a gradient program. Once separated, the concentration of various compounds can be determined by measuring their fluorescence at specific wavelengths using a fluorometer. The sample is exposed to a beam of light with a wavelength that causes the molecules in the compound to absorb photons, resulting in excitation of the molecules to a higher energy state. Once excited, the molecules then lose energy and return to their ground state, emitting photons in the process.

81

The excitation and emission wavelengths depend upon the structure of the molecules; therefore assessing fluorescence at different wavelengths enables the concentration of specific compounds within a sample to be determined. The fluorescence of each compound is plotted as a chromatogram and peak areas of unknown samples are compared to the peak area of a standard of known concentration. Some compounds, such as enzymatic and non-enzymatic crosslinks fluoresce naturally due to their molecular structure, which consists of a number of planar aromatic rings. However other compounds, including aspartic acid residues have to be derivatized with a compound that fluoresces so they can be quantified.

4.2.3.2. Quantification of Pyridinoline and Pentosidine Crosslinks

Hydroxylysyl-pyridinoline, lysyl-pyridinoline and pentosidine crosslinks were quantified using a single HPLC run. Approximately 15 mg powdered lyophilised tissue from the SDFT, DDFT, SL and CDET samples was hydrolysed in 3 ml 6 M HCl at 110 ºC for 24 h, dried and re-dissolved in 500 µl deionised water with 1% trifluoroacetic acid (TFA). The samples were filtered (0.2 µm filter) and 50 µl of sample was injected into the HPLC system (Series 200, PerkinElmer Life Sciences). The crosslinks were eluted using a Hypercarb column (150 × 4.6 mm, 7 µm internal diameter, Thermo Scientific) and a method adapted from Bailey *et al.* (1995). Separation of the crosslinks was achieved by using a 5 minute isocratic phase with 5% tetrahydrofuran (B) in deionised water (A), followed by a gradient from 5 to 20% B in 30 minutes, at a flow rate of 1 ml/min (Figure 4-3). Methods were set up using TotalChrom™ (Version 6.2.1, PerkinElmer). Mobile phases A and B both contained 0.5% TFA (v/v). Fluorescence was detected using a fluorometer (PerkinElmer). From 0-20 minutes the excitation wavelength was 295 nm and the emission wavelength was 405 nm; this was to detect the pyridinoline crosslinks. From 20-35 minutes the excitation wavelength was 335 nm and the emission wavelength was 385 nm; this was to detect pentosidine. To prevent column contamination, the column was washed with 70% B for 5 minutes after each sample had been separated.
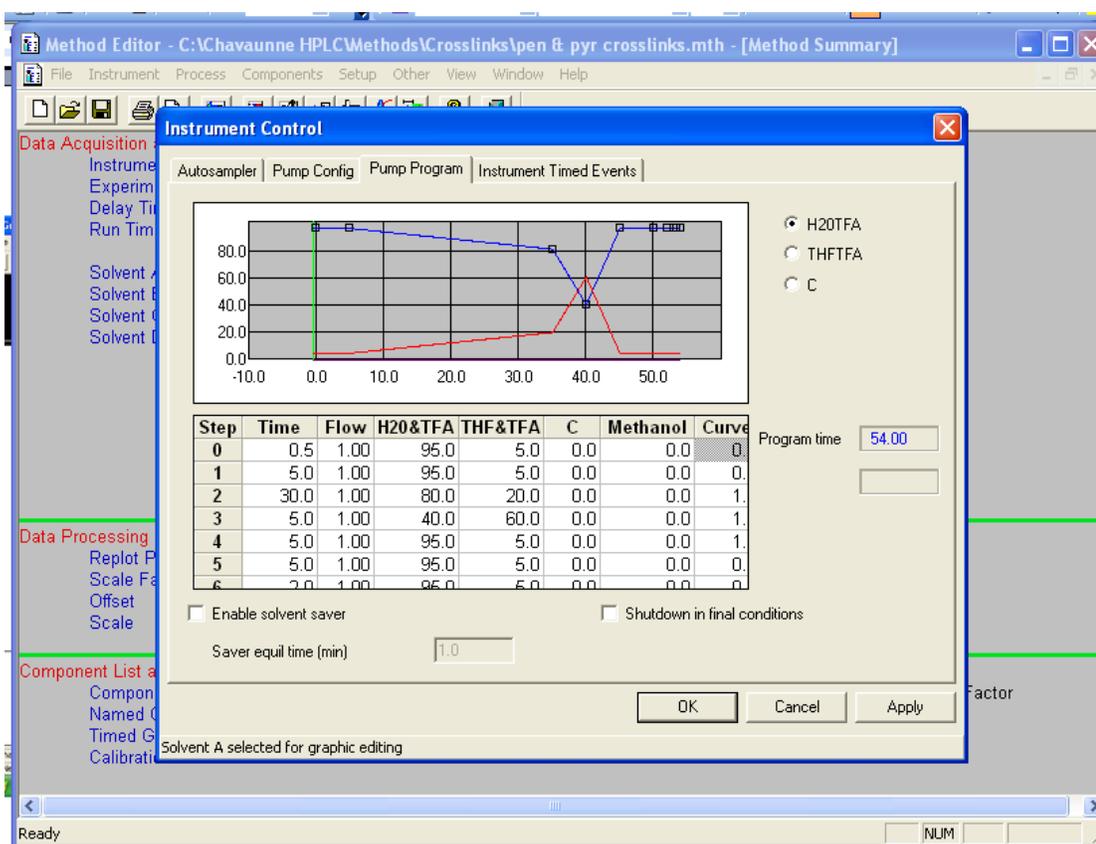
Figure 4-3: Screenshot showing gradient program set up in TotalChrom™. The blue line represents the percentage of mobile phase A and the red line represents the percentage of mobile phase B. The total run time, including the column wash, was 54 minutes.

Pentosidine concentration was calculated by measuring the fluorescence of known concentrations of pentosidine (12.5 pmoles in 50 µl injection volume) (kindly provided by Mr Nick Avery). Pyridinoline concentration was calculated using a commercially available standard (Metra Pyd/Dpd HPLC Calibrator, Quidel Inc, USA supplied by Technoclone Ltd, Surrey, UK) containing a mixture of lysyl-pyridinoline (348.5 pmoles) and hydroxylysyl-pyridinoline (722.5 pmoles) in an injection volume of 50 µl. Pyridoxamine (200 pmoles) was used as an external standard to detect any changes in run-to-run retention time. Hydroxylysyl-pyridinoline standards eluted in 2 peaks, corresponding to 2 isomers, at 6.4 and 7 minutes and lysyl-pyridinoline standards eluted in 2 peaks at 10 and 11.2 minutes (Figure 4-4). Pentosidine standards eluted at 34.0 minutes (Figure 4-5).
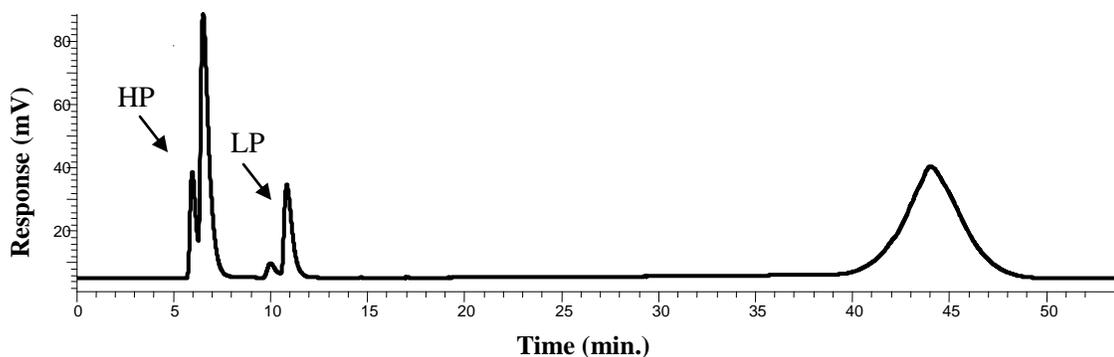
Figure 4-4: Chromatogram showing retention time of HP and LP standard solution.
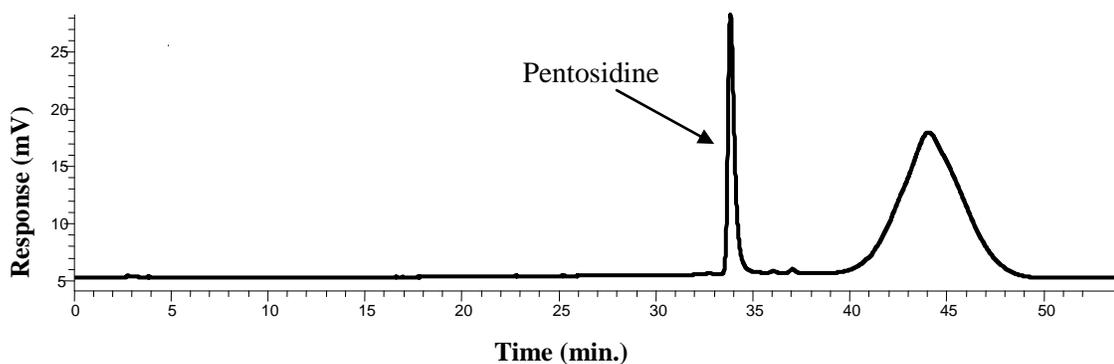
Figure 4-5: Chromatogram showing retention time of pentosidine standard

Standards were diluted serially in D.I. water containing 1% TFA to determine if the fluorometric response was linear in the expected range of crosslinks concentration in the samples. The fluorometric response of the pyridinoline standards was found to increase linearly with standard concentration for both HP and LP (Figure 4-6) and for pentosidine (Figure 4-7).
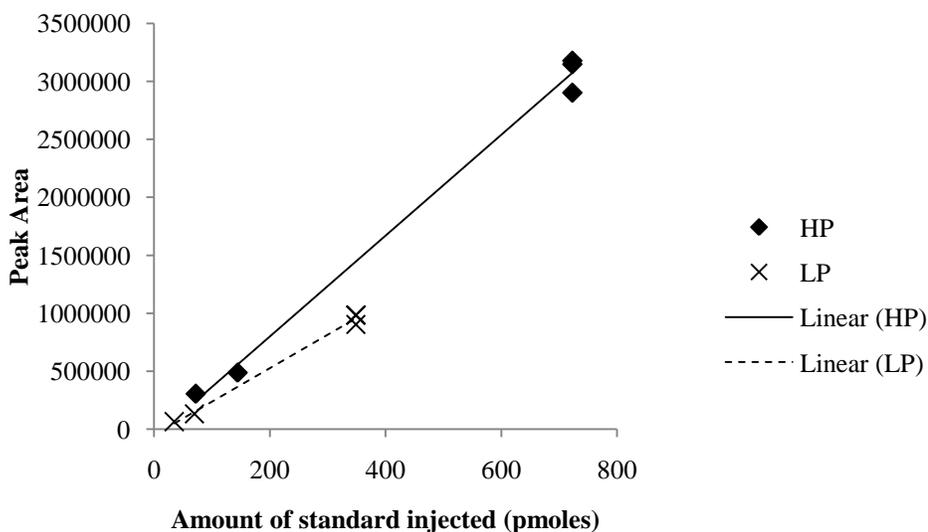
Figure 4-6: Amount of HP and LP standard solution (pmoles injected onto column) plotted against fluorometric response (peak area). The fluorometric response of both HP and LP standards showed an excellent correlation with the concentration injected (r=0.99).
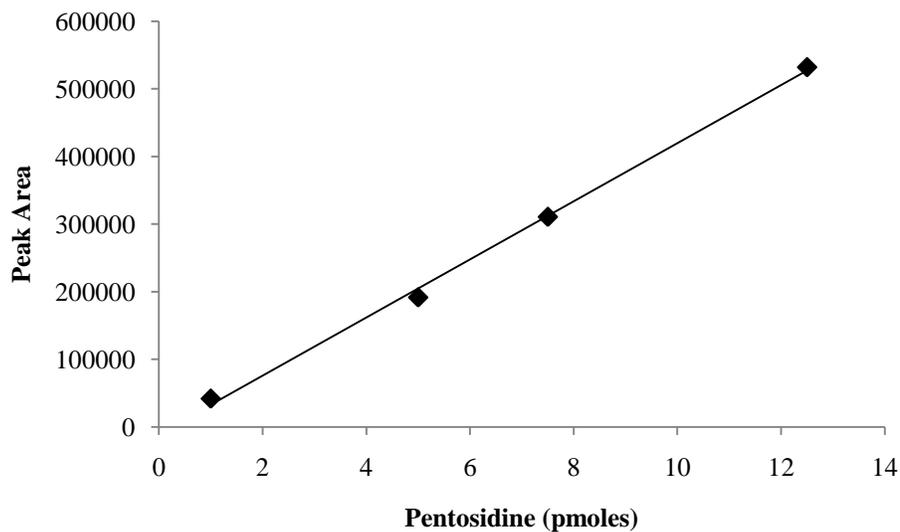
Figure 4-7: Amount of pentosidine standard (pmoles injected onto column) plotted against fluorometric response (peak area). There is a very strong correlation between pentosidine concentration and peak area (r=0.99).

Sample concentration was calculated by comparing the sample peak area for each crosslink with that of the appropriate standard. Peak area was calculated automatically using TotalChrom™ software (Figure 4-8).
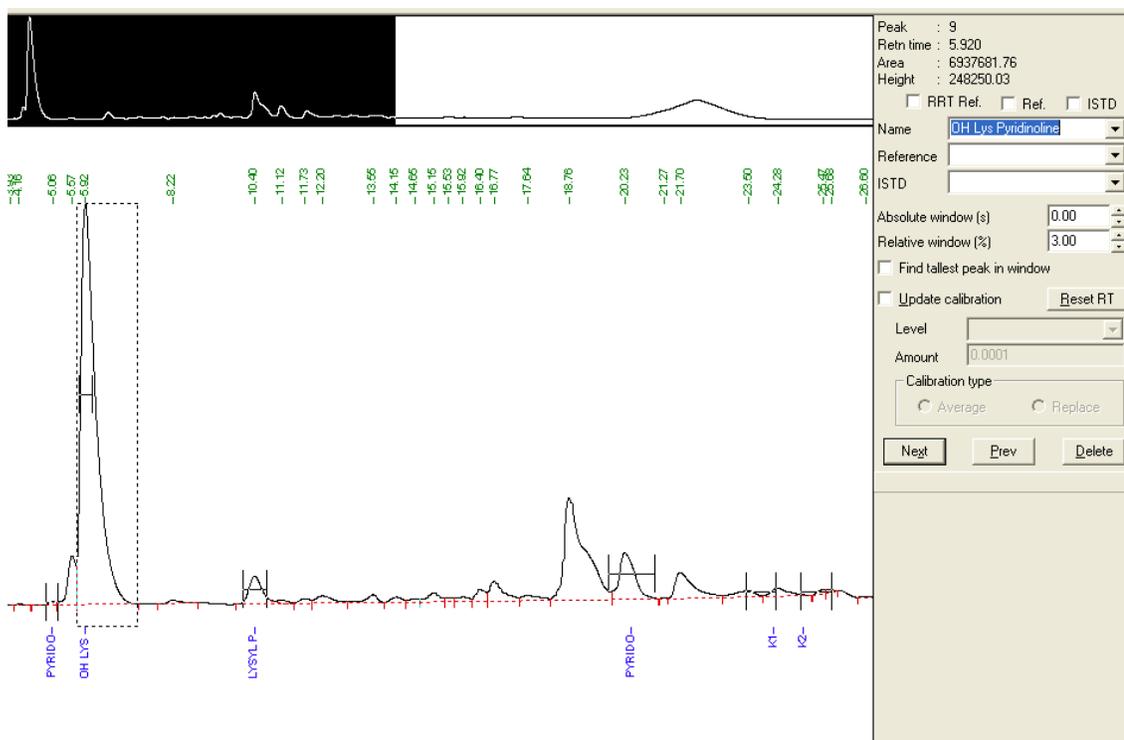
Figure 4-8: Screenshot showing crosslink peak area as determined by TotalChrom™ software.

Variation in the fluorometric response of the system was accounted for by measuring the fluorescence of the pentosidine standard at the beginning, middle and end of each batch of samples, and correcting for any change in fluorescence. Crosslink concentration was expressed as mM/M collagen, assuming the molecular weight of collagen to be 300 000. Collagen content was assessed in diluted (500 fold) aliquots (100 µl) of the hydrolysed tissue for approximately half the tendon samples using the method described above. However, pentosidine concentration did not differ when calculated using the collagen content of the papain digested samples or that of the crosslink hydrolysates and there was a strong correlation (r=0.99) between the two calculations of pentosidine concentration (Figure 4-9), so pentosidine concentration was calculated using the collagen content of the papain digested tissue for the remainder of the samples.
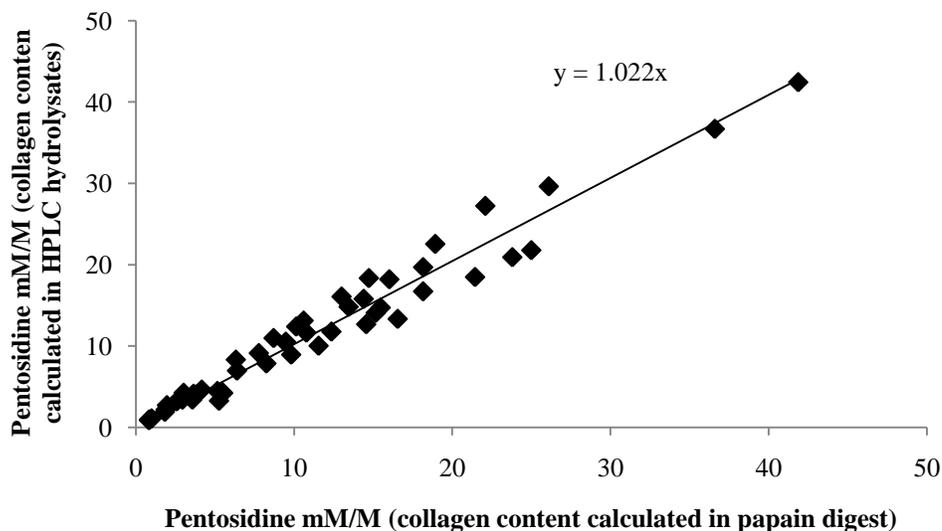
Figure 4-9: Graph showing pentosidine concentration calculated using collagen content of hydrolysed or papain digested tissue (r=0.99)

4.2.3.3. Measurement of Aspartate Racemization

The percent of L- and D-Aspartate was also measured in tissue from the tendons in the equine forelimb using HPLC. Aliquots (100 µl) of papain digested tissue were hydrolysed in 1 ml 6 M HCl at 110 ºC for 24 h, dried and re-dissolved in 10 ml 0.1 M HCl. Aspartic acid racemers do not fluoresce naturally and so the samples were derivatized with o-phthaldialdehyde-N-acetyl-L-cysteine (OPA-NAC) solution according to Yekkala *et al.* (2007). OPA-NAC reagent was prepared by dissolving 5.5 mg OPA in 420 µl methanol before adding 13.4 mg NAC. The volume was made up to 10 ml by the addition of 0.4 M sodium borate buffer adjusted to pH 9.4 with 1 M sodium hydroxide. Standards and samples (100 µl) were mixed with 200 µl OPA-NAC and allowed to stand for 5 minutes. The reaction was quenched by the addition of 200 µl of 0.3 M sodium phosphate buffer (pH 7.50), and the mixture was left for 5 minutes before 50 µl was injected into the HPLC system. Derivatization was performed by an autosampler (PerkinElmer) to ensure that the timings of the derivatization and quenching reactions did not change between samples.

The column used to achieve the separation was a Nova-Pak C8 column (150×3.9 mm, 4 µm particle size, Waters), as it has been shown to have special selectivity for the derivatized racemers of aspartic acid (Yekkala *et al.,* 2007). Separation was achieved using a gradient program according to Yekkala *et al.* (2007). Mobile phase A was 10% methanol and 90% 30 mM sodium phosphate buffer (pH 7.5), mobile phase B was 90% methanol and

10% 30 mM sodium phosphate buffer (pH 7.5). A gradient program was run as follows; 0-8 min 0-100%B, 8-13 min 100% B, 13-15 min 100-0% B and 15-35 min 0% B (Figure 4-10). Fluorescence was measured at an excitation wavelength of 337 nm and an emission wavelength of 442 nm.
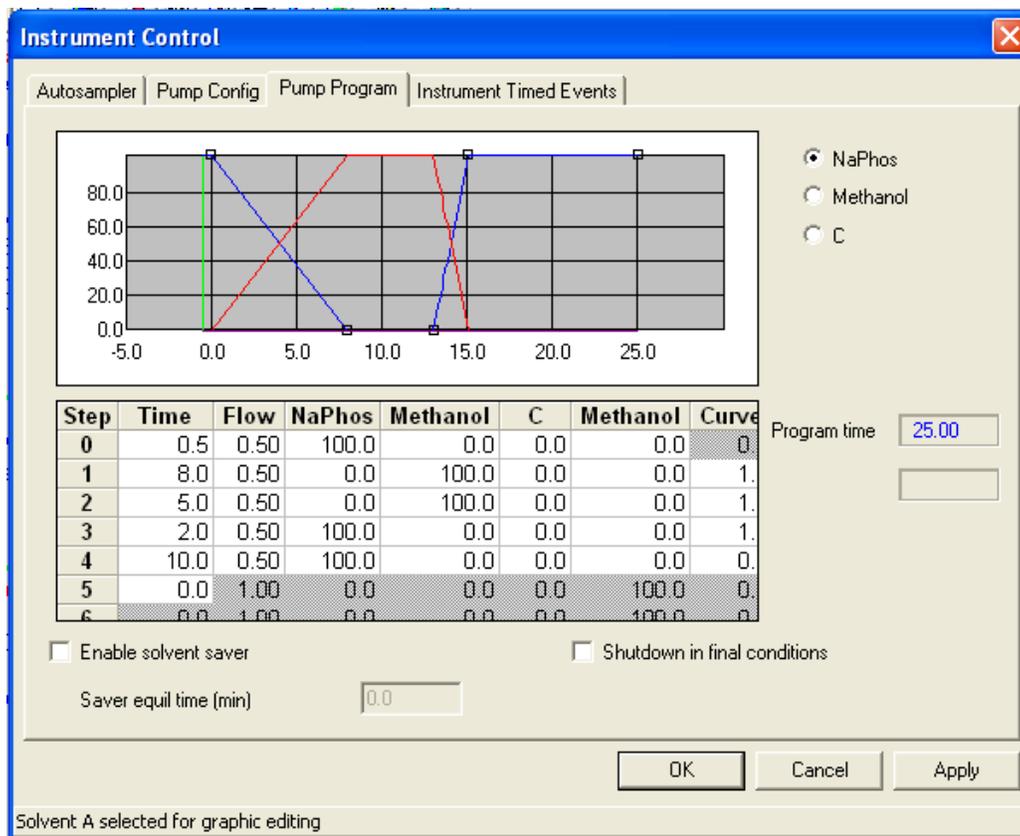
Figure 4-10: Screenshot showing gradient set up for separation of D- and L-Asp using TotalChrom™ software. The blue line represents mobile phase A and the red line represents mobile phase B.

Percentage of D- and L-Asp was calculated by expressing the peak area of D-Asp in each sample as a percentage of the peak area of L-Asp and correcting for the difference in fluorescence. Peak areas were measured using TotalChrom™ software (Figure 4-11).
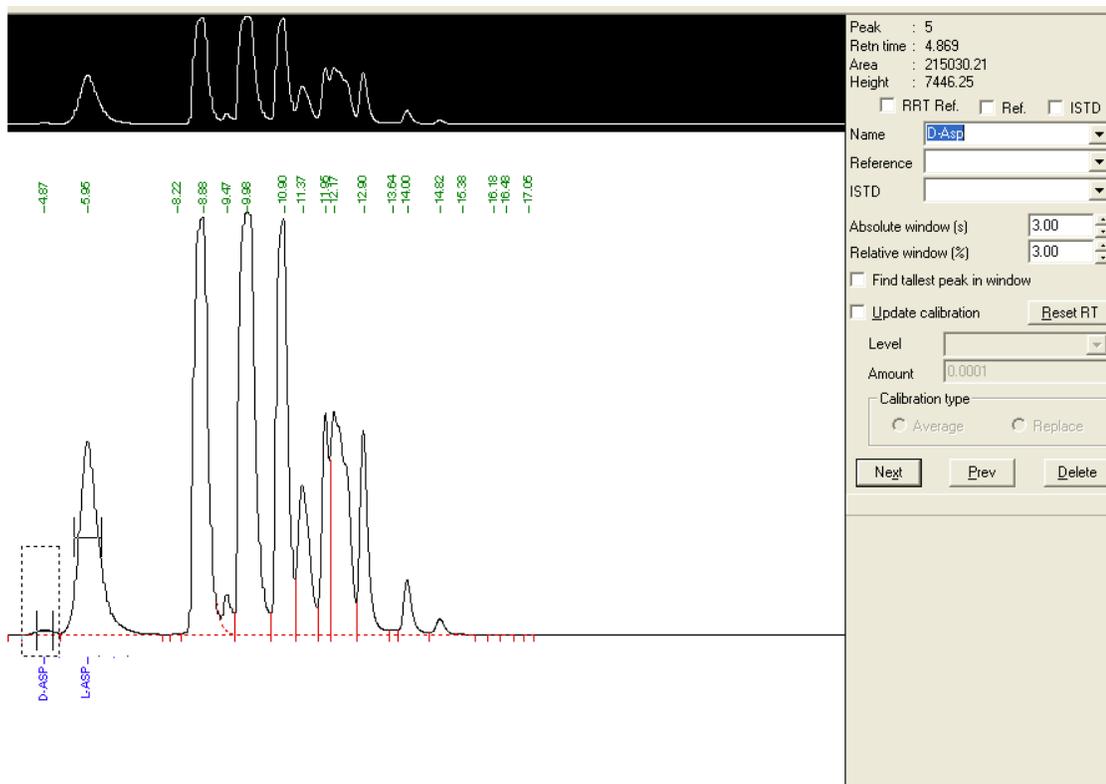
Figure 4-11: Screenshot showing D- and L-Asp peak area as determined by TotalChrom™.

To assess the validity of the HPLC technique (Yekkala *et al.,* 2007) in our laboratory, the following tests were carried out before the samples were analysed:

1. To ensure D- and L-Asp could be separated from each other and from other amino acids, a mixture containing 1 nmoles of D-and L-Asp (Sigma), and 1.25 nmoles of other amino acids (Thermo Scientific) in a 50 µl injection volume was injected into the HPLC system (Series 200, Perkin-Elmer) and separated according to the method outlined above.

   D-Asp eluted at 5.3 minutes and L-Asp eluted at 6.7 minutes; both peaks showed clear separation from the other amino acids which eluted after 9 minutes (Figure 4-12).
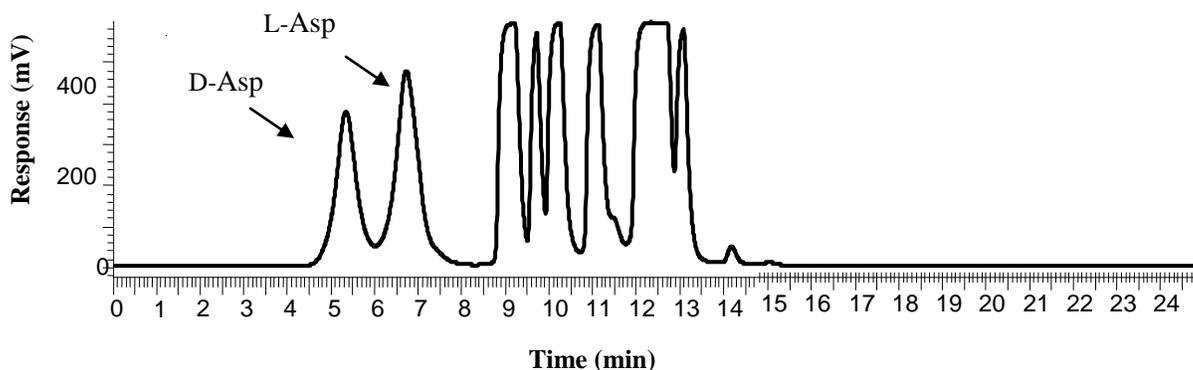
Figure 4-12: Trace showing separation of D-Asp (retention time 5.3 min) and L-Asp (retention time 6.7 min) from other amino acids (after 9 min). Also note the difference in fluorescent response between the D- and L-Asp peaks.

2. To account for any D- or L-Asp present in the buffer or derivatization reagents 0.1 M HCl was derivatized and D- and L-Asp were measured.

   System peaks eluted with the D- and L-Asp derivatives (equivalent to 0.52 pmoles D-Asp and 6.82 pmoles L-Asp) and so the areas of these peaks were subtracted from the areas of the D- and L-Asp peaks before the ratio of D- to L-Asp was calculated.

3. Specific fluorescence of D- and L-Asp was calculated by injecting equal amounts (250 pmoles) of D- and L-Asp and determining the difference in peak area.

   When equal amounts of D- and L-Asp were injected, the ratio of D:L-Asp peak area was 1:1.29. This difference in specific fluorescence was corrected for by multiplying the percentage of D-Asp by 1.29.

4. The precision of the measurement of the fluorescent response was determined by calculating the percent relative standard deviation (RSD) of the peak ratio of D- and L-Asp from 6 measurements where 900 pmoles L-Asp and 100 pmoles D-Asp were injected into the HPLC system.

   The RSD was 0.69%, indicating low variability between runs.

5. The fluorescent response of increasing amounts of D-Asp was measured while L-Asp was kept constant at 900 pmoles to determine if the change in measured ratio of D- to L-Asp was linear from 0.1-11% (the expected range of D-Asp concentration in the samples).

Fluorescent response was found to be linear with increasing concentration of D-Asp (Figure 4-13).
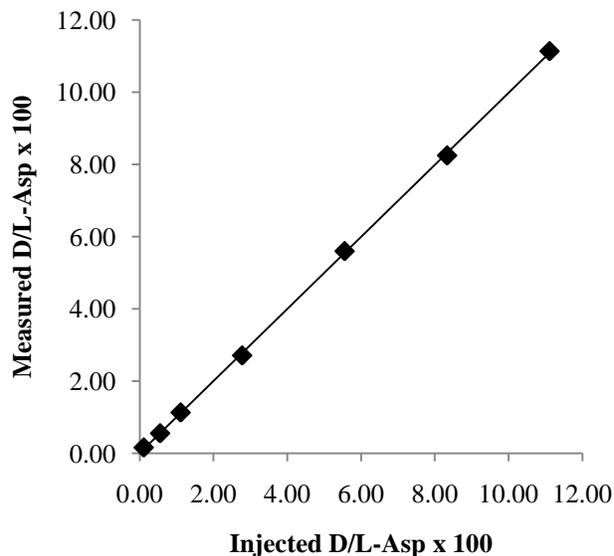
Figure 4-13: Ratio of D-Asp injected plotted against ratio of D-Asp measured (r=0.99).

6. To determine the smallest amount of D-Asp that could be detected in the presence of a large amount of L-Asp concentrations of D-Asp from 0.5 to 50 pmoles were injected while L-Asp was kept constant at 900 pmoles.

The smallest amount of D-Asp that could be detected was 1 pmole and the smallest amount that could be quantified accurately in the presence of 900 pmoles L-Asp was 5 pmoles (0.56%) (Figure 4-14). This value is lower than the percentage of D-Asp recorded in all the samples before correction.
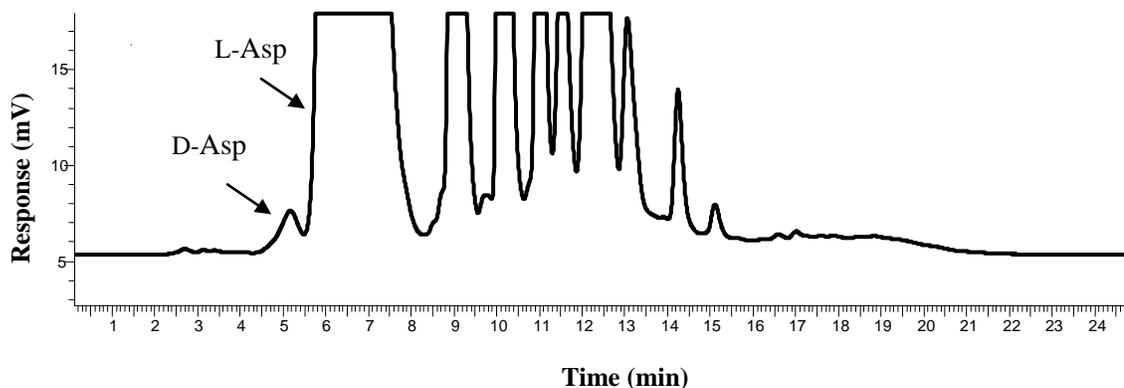
Figure 4-14: Trace with scale adjusted to show the smallest amount of D-Asp (5 pmoles) that could be quantified accurately in the presence of a greater than 100 fold excess of L-Asp (900 pmoles).

7. Heating is known to cause racemization, so this was accounted for by hydrolysing a known amount of L-Asp and measuring the amount of racemization that occurred during the hydrolysis step.

    Hydrolysis caused an average of 2.64% L-Asp to convert to D-Asp, once the system peaks had been subtracted so this percentage was subtracted from the % D-Asp values calculated for the samples.

8. To account for any D- or L-Asp present in the papain or guanidine hydrochloride (GuHCl) buffers a blank (buffer minus tissue) was included for each tissue treatment and D- and L-Asp content was measured.

    Small amounts of D- and L-Asp were detected in the papain and GuHCl extraction buffers, and so the corresponding peak area was subtracted from the D- and L-peak area for each sample before the ratio of D- to L-Asp was calculated.

Separation of Matrix Components

Aspartic acid is present in all proteins; it is not specific to collagen, and so to determine the age of the collagenous and non-collagenous matrix components these components were separated by GuHCl extraction before the percentage of D-Asp present was determined by HPLC. Approximately 20 mg powdered SDFT and CDET was suspended in 2 ml 4 M GuHCl (pH 5.8) with 0.1 M sodium acetate plus protease inhibitors (10 mM 6-aminohexanoic acid, 5 mM benzamidine-HCl, 1 mM phenylmethyl-sulfonyl fluoride, 10 mM EDTA and 5 mM N-ethylmaleimide). The extraction was carried out for 24 hours at 4 °C with agitation, the samples were then centrifuged (4500 g for 5 minutes) and the supernatant was removed. The collagenous pellets were washed extensively in D.I. water to remove the GuHCl and freeze dried and stored at -80 ºC. The supernatant was also stored at -80 ºC until required.

To ensure the GuHCl extraction successfully separated the collagenous and non-collagenous matrix, the GAG and collagen content of the pellet and supernatant from a small number of samples (n = 5) was assessed using the methods described above; GuHCl extraction successfully separated SDFT and CDET samples into collagenous and non-collagenous matrix components. The collagenous pellet contained 87% of the collagen

content of the whole matrix and 13% of the GAG content of the matrix. After filtration, the supernatant contained 75% of the total GAG content and 1% of the total collagen content of the matrix.

The collagenous pellet remaining after GuHCl extraction was papain digested and 100 µl of papain digest was hydrolysed and the aspartate racemization was measured using the methods described above. GuHCl was removed from the supernatant, which contained the proteoglycan components of the matrix, using centrifugal filter units (Microcon YM-3, 3000 molecular weight cut-off, Millipore) according to the manufacturer's instructions. Aliquots (20 µl) of the remaining sample were hydrolysed, dried and re-dissolved in 1 ml 0.1 M HCl before aspartate racemization was measured as described. Due to time constraints, aspartate racemization in the collagenous and non-collagenous matrix components was assessed in the SDFT and CDET, and not in the DDFT or SL.

Half-life Calculations

The accumulation of D-Asp in any tissue depends on the intrinsic rate constant of racemization, i.e. the amount of racemization that occurs when no tissue is turned over (k_i), and the protein turnover (k_T). As the amount of D-Asp formed *in vivo* is very small (D/L < 0.15), racemization is approximated as irreversible. The rate of accumulation of D-Asp over time can therefore be expressed as in Equation 1.

Equation 1: $d(C_{Asp}(D)/(D+L))/dt = k_i\, C_{Asp}(L)/(D+L) - k_T\, C_{Asp}(D)/(D+L)$

where C_{Asp} is the concentration of aspartic acid in the protein. Assuming C_{Asp} is constant over time and that D/(D+L) < 0.1 (Maroudas *et al.,* 1998), which it is *in vivo*, D/(D+L) ≈ D/L and L/(D+L) ≈ 1, Equation 1 simplifies to Equation 2.

Equation 2: $d(D/L)/dt = k_i - k_T(D/L)$

To calculate protein turnover, this equation can be rearranged into Equation 3.

Equation 3: $k_T = (k_i - (d(D/L)/dt))/(D/L)$

The accumulation rate (d(D/L)dt) in Equation 3 was approximated by fitting a linear curve to the measured data for the ratio D/L in each matrix component of tendon as a function of time.

The rate constant of racemization (k_i) is taken as the rate of accumulation of D-Asp in dentin; collagen in dentin is the most stable during the human lifetime and so negligible collagen turnover occurs in this tissue. The accumulation rate of D-Asp in dentin is 7.87 x 10^{-4}/year (Helfman and Bada 1976). However, the rate of racemization is temperature dependent; if it is assumed that the average temperature of dentin is 33 °C based on tooth surface temperature (Fanibunda 1986) and the average temperature of tendon is 37 °C (Wilson and Goodship 1994), then the value of k_i can be corrected for difference in temperature using Equation 4 (Bada 1984).

Equation 4: $k_i(T) = k_i(310) \times \exp(- ((E_a/R) \times (310 - T)/(T \times 310)))$

where T is temperature in Kelvin, E_a is the activation energy (33.4 x 10^3 as calculated by Bada *et al.* (1973)) and R is the gas constant (1.987). Using this equation, k_i at 37 °C was calculated to be 1.604 x 10^{-3}/year. This value for k_i was used to calculate k_T for the whole matrix and the collagenous component of the matrix. Previous work has found that non-collagenous proteins have a higher intrinsic rate of racemization; the k_i value was calculated to be 1.873 x 10^{-3}/year in articular cartilage aggrecan (Maroudas *et al.,* 1992). Intra-articular temperature is comparable to that of dentin (Becher *et al.,* 2008) and so when the difference in temperature between articular cartilage and tendon is accounted for using Equation 4 the k_i value for non-collagenous protein is 3.73 x 10^{-3}/year.

The rate of protein turnover was then used to calculate protein half-life ($t_{1/2}$) using Equation 5.

Equation 5: $t_{1/2} = \ln(2)/k_T$

4.2.3.4 . Tissue Fluorescence

Tissue fluorescence was measured as part of the DNA assay to account for any fluorescence that did not result from the DNA-binding dye. Tissue fluorescence is non-specific but has been shown previously to correlate with horse age and to be lower in the CDET than the SDFT (Birch *et al.,* 2008b). Tissue fluorescence is expressed as arbitrary units/mg collagen.

4.2.4. Statistical Analysis

Statistical significance was assessed using a linear mixed effects model (SPlus, Version 16, Insightful) with horse as a grouping factor to determine the effect of tendon type and increasing age on each variable measured. All data were found to be normally distributed when analysed using a Kolmongorov-Smirnoff test (Minitab, Version 15) and so correlation analysis was performed for each variable using Pearson's product moment correlation (SPSS, Version 14). Data are displayed as mean ± SEM.

4.3. Results

The concentration of matrix components and markers of matrix age are shown in Table 4-1 and the correlations of these components with increasing horse age are shown in Table 4-2.

	SDFT	DDFT	SL	CDET
Water (%)	64.91±0.29	63.60±0.26***	67.76±0.23***	58.78±0.20***
Collagen (%)	75.81±1.45	76.90±2.06	65.11±1.62**	80.38±1.26*
GAG (µg/mg)	10.40±0.86	11.86±0.71	13.30±0.94*	1.83±0.12***
DNA (µg/mg)	0.54±0.02	0.35±0.02***	0.69±0.03***	0.39±0.02***
Tissue Fl. (Arb. Units/mg)	58.22±3.43	55.51±2.77	52.98±2.67*	38.33±1.69***
Pentosidine (mM/M)	13.83±1.69	18.01±2.25*	17.33±2.06***	14.80±2.14
OH-lys Pyr (mM/M)	737.84±45.18	584.79±31.01***	598.67±40.98*	70.11±4.53***
Lys Pyr (mM/M)	101.51±19.11	90.72±16.87	100.23±17.13	84.33±16.33
D/L-Asp × 100 (total)	1.12±0.12	1.59±0.17*	1.16±0.15	1.18±0.12
D/L-Asp × 100 (collagen)	5.64±0.52	-	-	4.31±0.42**
D/L-Asp × 100 (non-coll.)	0.88±0.25	-	-	1.58±0.26**
Half-life (whole matrix)	7.85±0.82	11.94±1.31***	8.14±1.08	8.02±0.84
Half-life (collagen)	197.53±18.23	-	-	34.03±3.39***
Half-life (non-coll.)	2.18±0.41	-	-	3.51±0.51*

Table 4-1: Concentration of matrix components and markers of matrix age in equine forelimb tendons. Data are displayed as Mean ± SEM, n = 32; *Indicates significant difference from SDFT: *p<0.05; **p<0.005; ***p<0.001

	SDFT	DDFT	SL	CDET
Water (%)	0.403(0.022)	NS	NS	0.366(0.039)
Collagen (%)	NS	NS	NS	NS
GAG (µg/mg)	NS	NS	NS	-0.643(<0.001)
DNA (µg/mg)	NS	NS	NS	NS
Tissue Fl. (Arb. Units/mg)	0.883(<0.001)	0.873(<0.001)	0.785(<0.001)	0.820(<0.001)
Pentosidine (mM/M)	0.847(<0.001)	0.882(<0.001)	0.849(<0.001)	0.893(<0.001)
OH-lys Pyr (mM/M)	NS	NS	NS	NS
Lys Pyr (mM/M)	0.390(0.027)	NS	NS	0.481(0.005)
D/L-Asp × 100 (total)	0.694(<0.001)	0.567(0.001)	0.522(0.002)	0.637(<0.001)
D/L-Asp × 100 (collagen)	0.376(0.034)	-	-	NS
D/L-Asp × 100 (non-coll.)	NS	-	-	0.465(0.008)
Half-life (whole matrix)	0.687(<0.001)	0.568(0.001)	0.525(0.002)	0.643(<0.001)
Half-life (collagen)	0.376(0.034)	-	-	NS
Half-life (non-coll.)	NS	-	-	0.458(0.008)

Table 4-2: Correlation coefficient (r) of concentration of matrix components and markers of matrix age with increasing horse age (n = 32); p-value is shown in brackets; NS = not significant.

4.3.1. Matrix Composition

4.3.1.1. Water Content

Water content was similar to previously reported values (Batson *et al.,* 2003; Birch *et al.,* 1998) and was significantly different (p<0.001) between the four tendons (Figure 4-15). The SL had the highest percentage water, followed by the SDFT, then the DDFT. The CDET had the lowest water content. Water content was not correlated with age in any tendon.
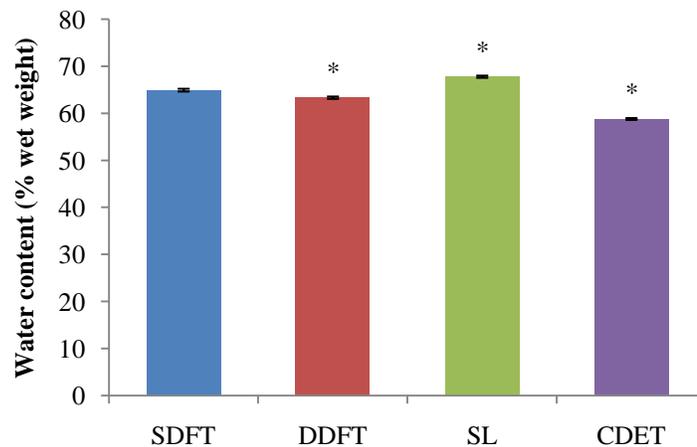
Figure 4-15: Tendon water content (mean ± SEM). n = 32 * indicates significant difference relative to the SDFT.

4.3.1.2. DNA Content

DNA content of SL was higher (p<0.001) than in the other tendons, and DNA content was higher (p<0.001) in the SDFT than in the DDFT and CDET (Figure 4-16), indicating that the SL and SDFT have a higher cellularity than DDFT and CDET. DNA content was not correlated with age in any of the tendons.
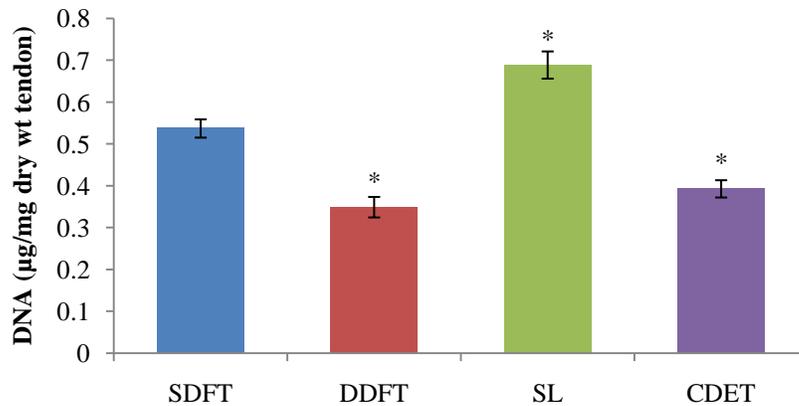
Figure 4-16: DNA content of SDFT, DDFT, SL & CDET (mean ± SEM). n = 32. * Denotes significant difference relative to the SDFT

4.3.1.3. Sulphated Glycosaminoglycan Content

The GAG content was significantly lower (p<0.001) in the CDET than in the other tendons. GAG content was significantly higher (p=0.001) in the SL than in the SDFT and decreased significantly (r=-0.643. p<0.001) with age in the CDET (Figure 4-17), but showed no relationship with age in the other tendons.
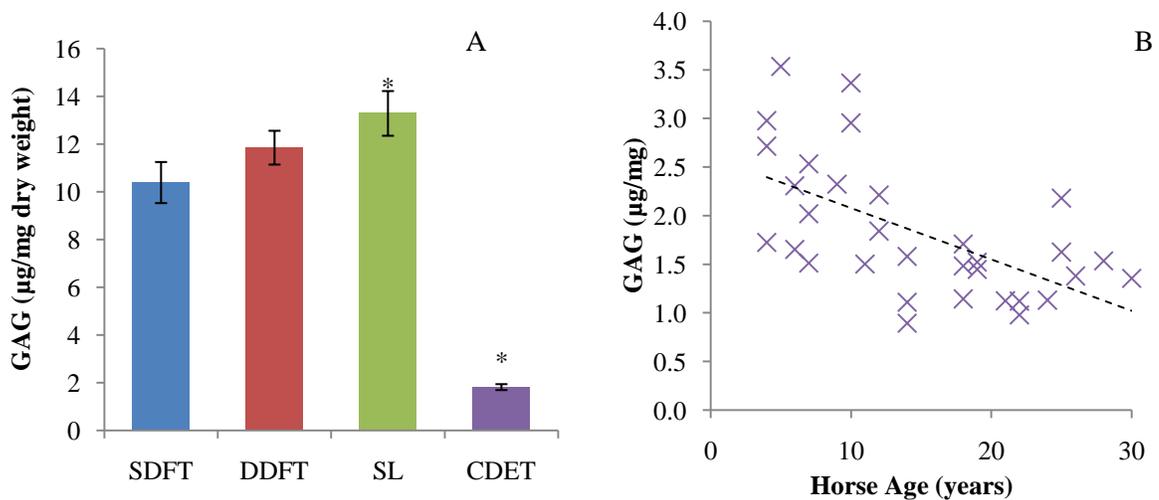
Figure 4-17: Tendon GAG content (mean ± SEM) n = 32 (A). *Indicates significant difference relative to the SDFT. Scatterplot showing decreased GAG content (B) in the CDET with increasing age (r=-0.643).

4.3.1.4. Collagen Content

The collagen content of the SL was significantly lower (p<0.001) than in the other tendons and was significantly higher (p≤0.029) in the CDET than in the SDFT (Figure 4-18). Collagen content was not correlated with age in any tendon.
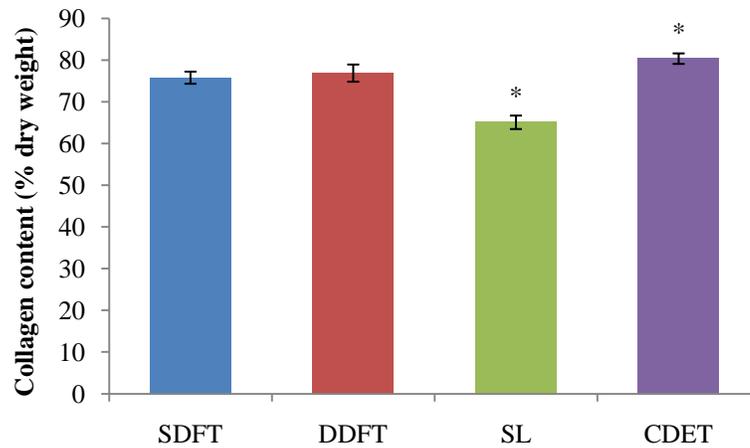
Figure 4-18: Tendon collagen content (mean ± SEM) n = 32. * indicates significant difference relative to the SDFT.

4.3.2. Matrix Age

4.3.2.1. Tissue Fluorescence

In agreement with previous findings (Birch *et al.,* 2008b), tissue fluorescence of the CDET was lower (p<0.001) than in the other tendons (Figure 4-19). Tissue fluorescence increased significantly (p<0.001) with age in all tendons (Figure 4-20).
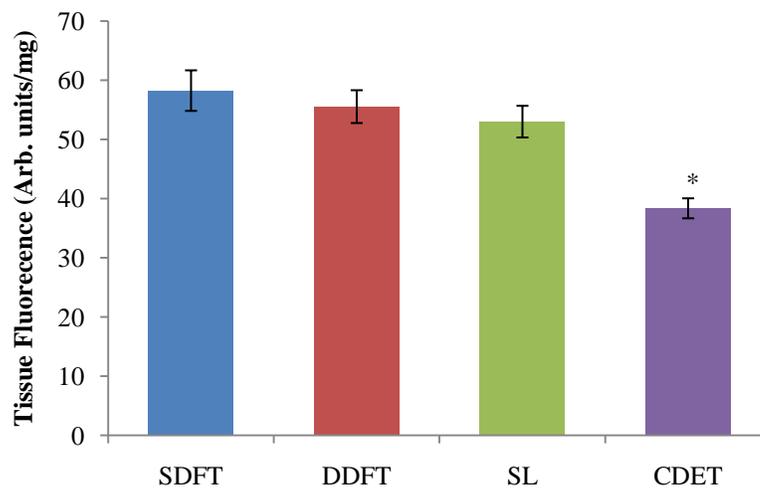
Figure 4-19: Tendon tissue fluorescence (mean ± SEM). n = 32 * indicates difference relative to the SDFT.
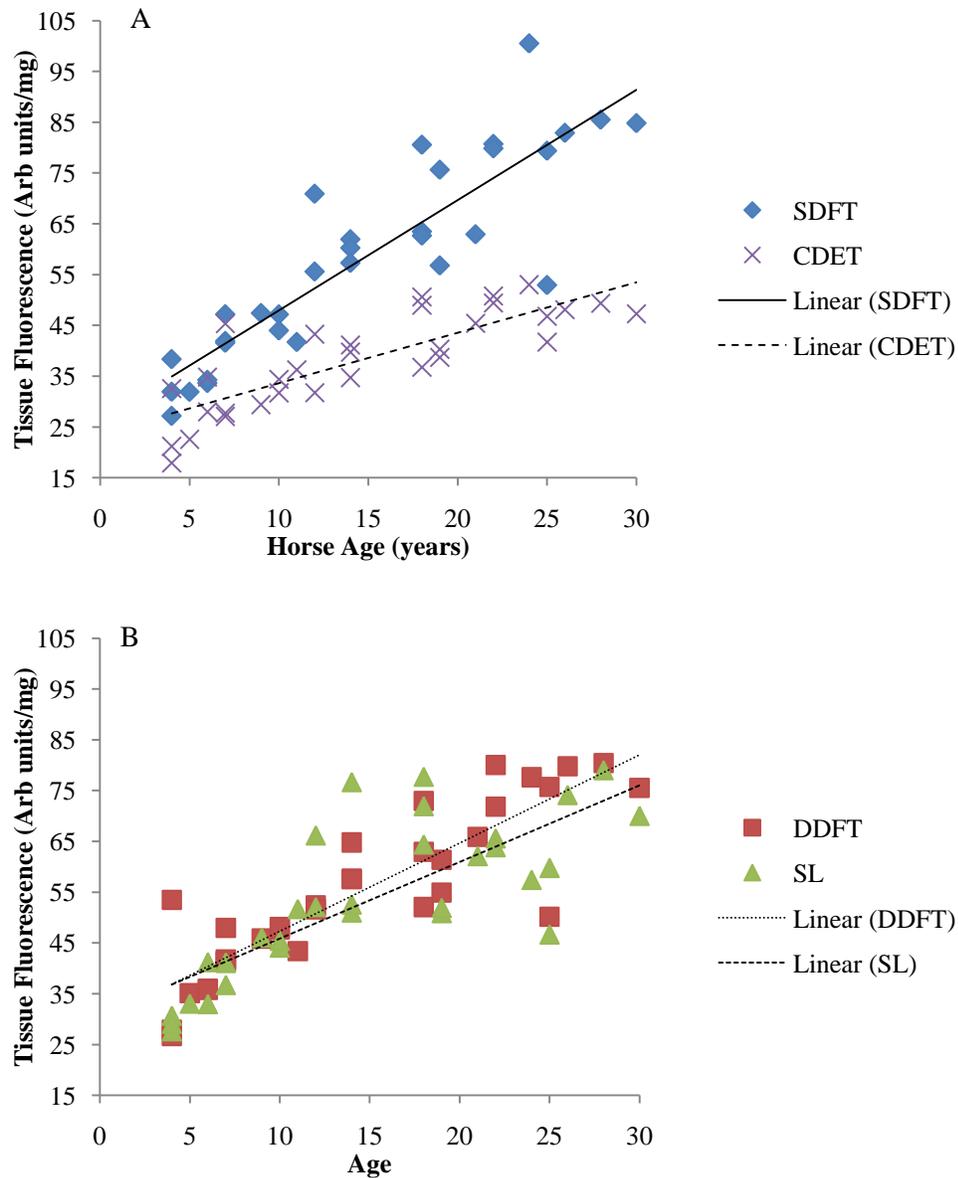
Figure 4-20: Tissue fluorescence of the SDFT & CDET (A), and DDFT & SL (B) as a function of horse age. Tissue fluorescence increased linearly with horse age in all tendons (SDFT, r=0.88, p<0.001; DDFT, r=0.87, p<0.001; SL, r=0.79, p<0.001; CDET, r=0.82, p<0.001).

4.3.2.2. Pentosidine and Pyridinoline Crosslinks

Pentosidine and pyridinoline crosslinks were detected in all tendon samples, a typical chromatogram showing separation of crosslinks in an SDFT sample is shown in Figure 4-21. The DDFT and SL had similar crosslink profiles to the SDFT, whereas the CDET exhibited a distinct crosslink profile (Figure 4-22).
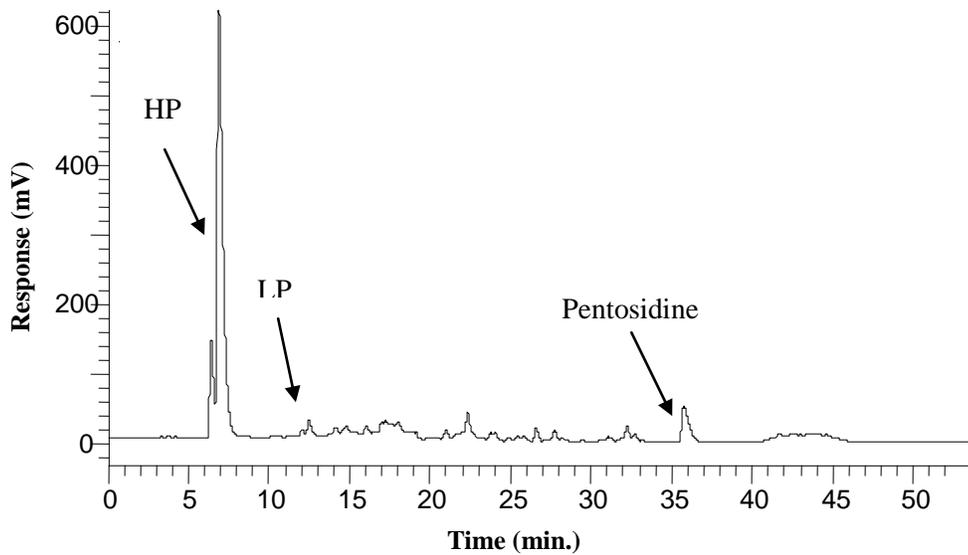
Figure 4-21: Typical chromatogram showing separation of hydroxylysyl-pyridinoline (HP) lysyl-pyridinoline (LP) and pentosidine crosslinks from a sample of hydrolysed SDFT.
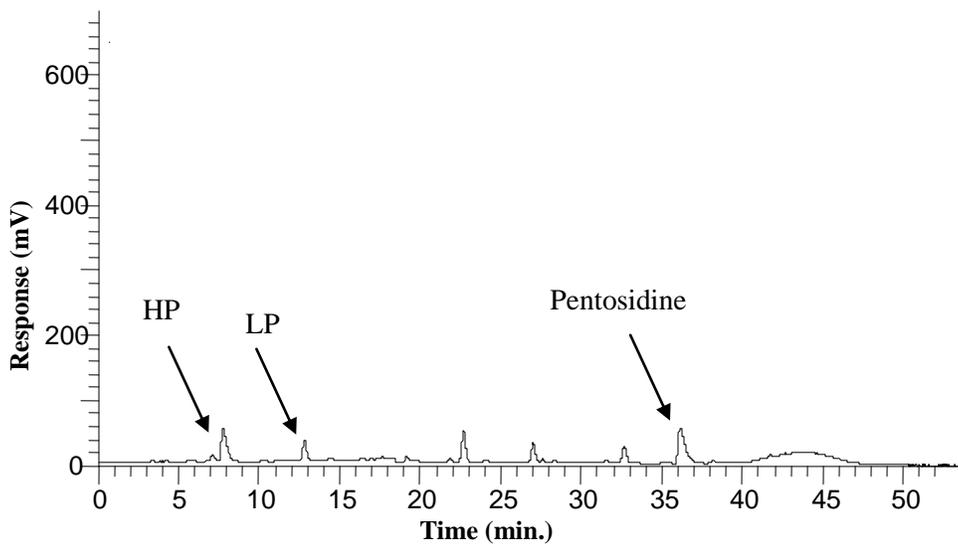
Figure 4-22: Chromatogram showing separation of hydroxylysyl-pyridinoline (HP) lysyl-pyridinoline (LP) and pentosidine in the CDET. Note the difference in crosslink profile when compared to that of the SDFT shown in Figure 4-21.

HP crosslinks were present at significantly higher concentrations in the SDFT than in the other tendons (p≤0.0017), with levels approximately 10-fold lower in the CDET (Figure 4-23). HP concentration did not change significantly with increasing horse age.
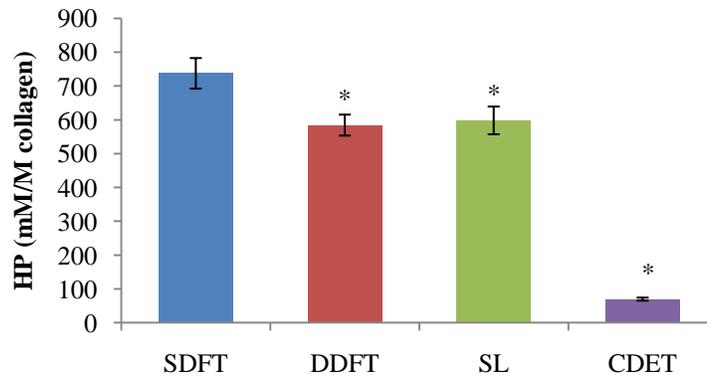
Figure 4-23: Tendon HP content (mean ± SEM) n = 32. * indicates significant difference relative to SDFT.

LP crosslinks were present at lower levels than HP in all tendons. In contrast, LP crosslink concentration was not significantly different between tendon types, and increased significantly with horse age in the SDFT and CDET (p≤0.027) (Figure 4-24).
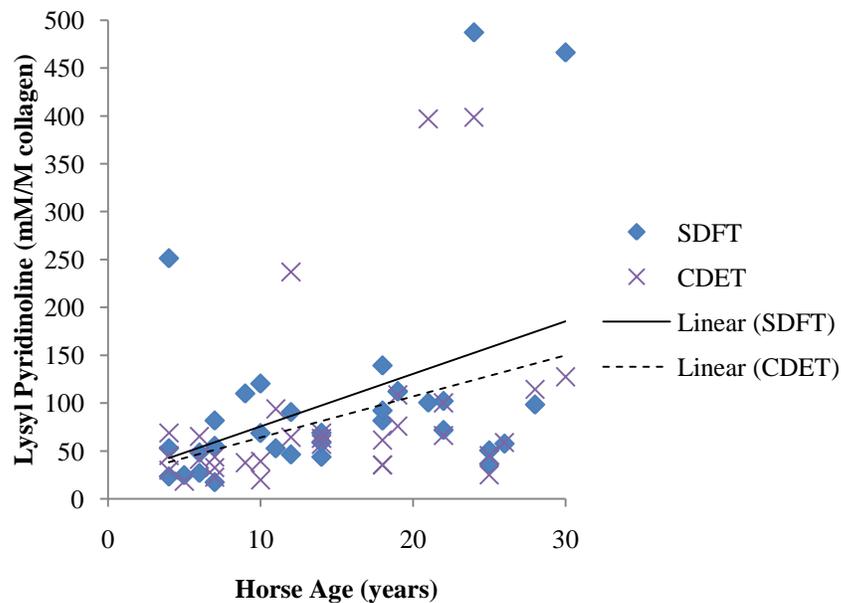
Figure 4-24: Concentration of lysyl-pyridinoline in the SDFT and CDET as a function of horse age. LP concentration increased significantly with horse age in the SDFT (r=0.39, p=0.03) and CDET (r=0.48, p=0.005).

Pentosidine content was significantly higher (p≤0.003) in the DDFT and SL than in the SDFT and CDET (Figure 4-25). Pentosidine concentration increased significantly (p<0.001) with age in all tendons (Figure 4-26).
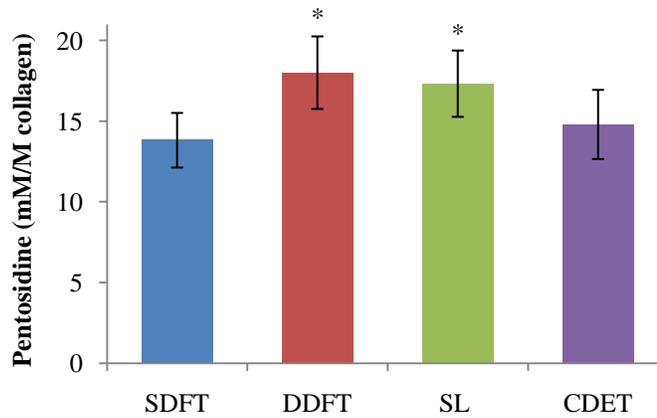
Figure 4-25: Tendon pentosidine content (mean ± SEM) n = 32. * indicates significant difference relative to the SDFT.
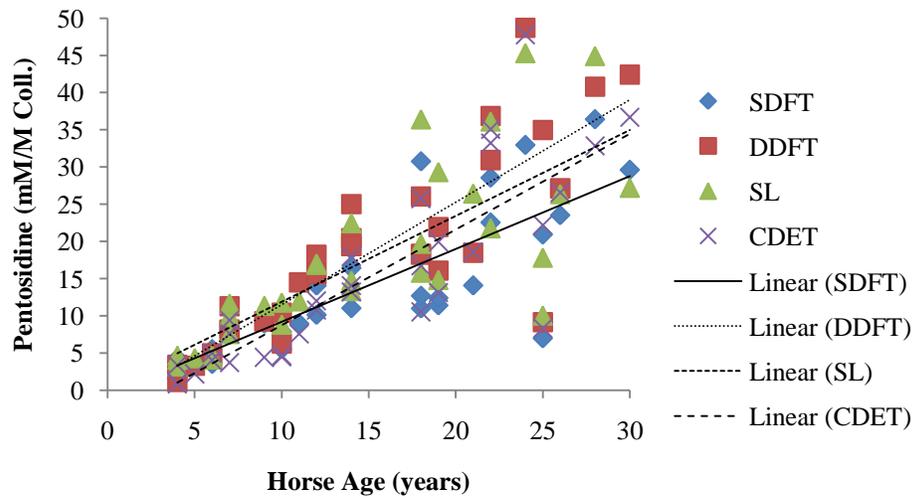
Figure 4-26: Pentosidine content of SDFT, DDFT, SL & CDET as a function of horse age. Pentosidine content increased significantly with horse age in all tendons (SDFT, r=0.85, p<0.001; DDFT, r=0.88 p<0.001; SL, r=0.85, p<0.001; CDET r=0.89, p<0.001).

4.3.2.3. Matrix Half-life

The accumulation rates of D-Asp used to calculate matrix half-life are shown in Table 4-3.

Fraction	Accumulation rate of D-Asp ($\times 10^{-4}$/year)			
	SDFT	**DDFT**	**SL**	**CDET**
Whole Tendon	5.87	6.82	6.16	5.82
Collagenous	14.06	-	-	7.27
Non-Collagenous	2.57	-	-	6.58

Table 4-3: Accumulation rates of D-Asp per year in the SDFT and CDET ((d(D/L)dt) in Equation 1).

Whole Matrix

Percent D-aspartate was significantly higher (p≤0.037) in the DDFT than in the other tendons. As with pentosidine, the percentage of D-aspartate increased significantly (p<0.001) with age in all tendons (Figure 4-27).
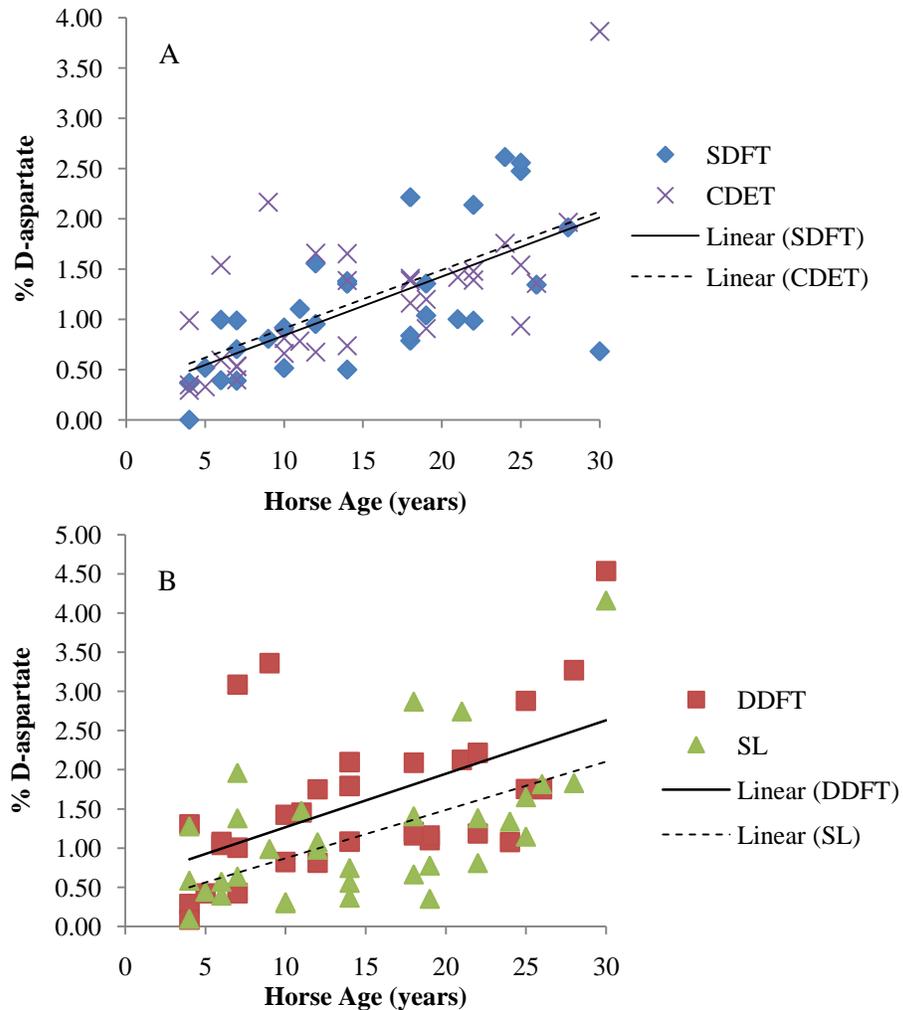
Figure 4-27: Percent D-aspartate in SDFT & CDET (A), and DDFT & SL (B) as a function of horse age. Percent D-Asp increased linearly with horse age in all tendons (SDFT, r=0.69, p<0.001; DDFT, r=0.57, p=0.001; SL, r=0.52, p=0.002; CDET, r=0.64, p<0.001).

Correspondingly, matrix half-life was greater in the DDFT than in the other tendons (p≤0.0001) (Figure 4-28) and increased significantly with horse age in all tendons (Figure 4-29).
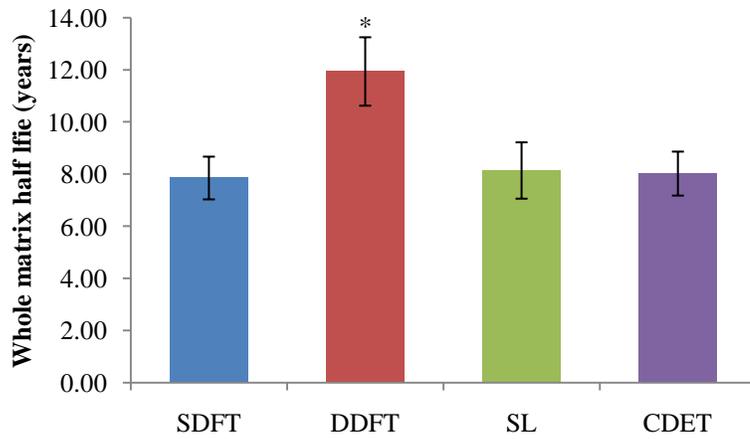
Figure 4-28: Whole matrix half-life (mean ± SEM) n = 32. * indicates significant difference relative to the SDFT.
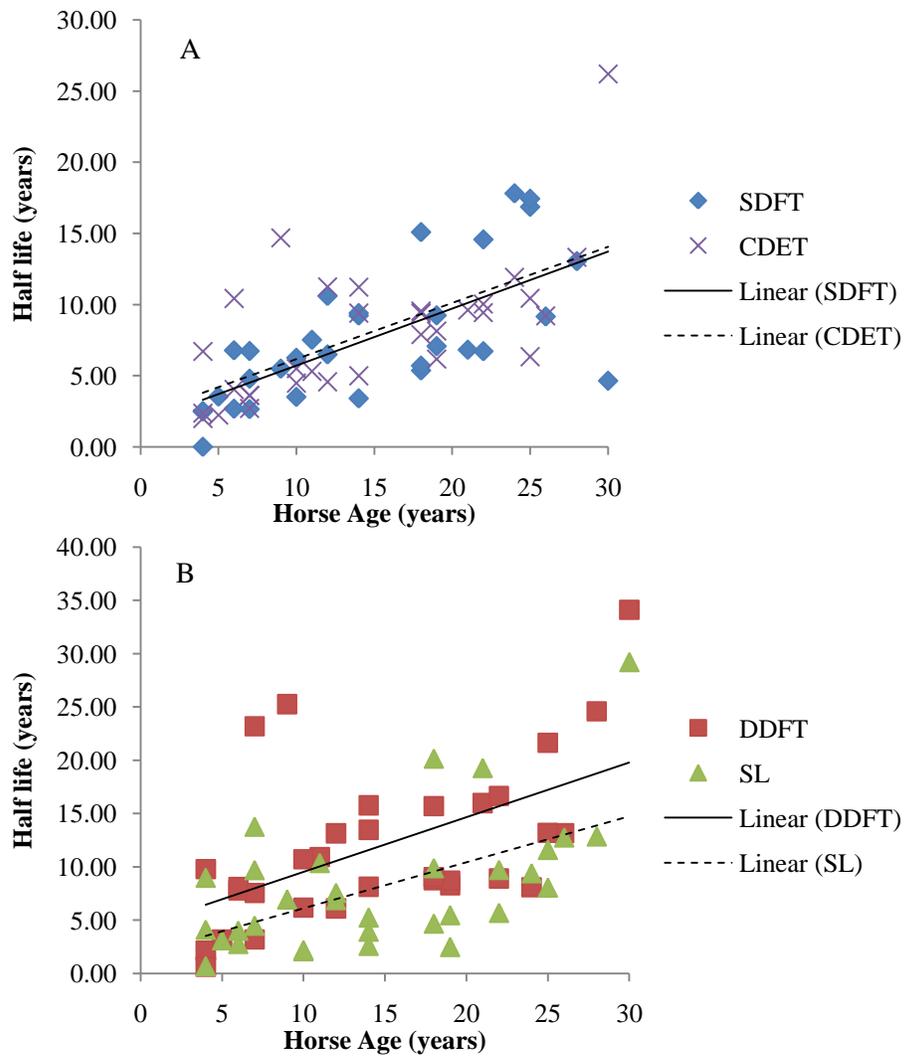
Figure 4-29: Whole tendon half-life in the SDFT & CDET (A) and DDFT & SL (B) as a function of horse age. The half-life of the whole matrix increased significantly with horse age in all tendons (SDFT, r=0.69, p<0.001; DDFT, r=0.57, p=0.001; SL, r=0.53, p=0.002; CDET, r=0.64, p<0.001).

Percent D-Asp showed a good correlation with pentosidine concentration in all tendons (p≤0.005) (Figure 4-30).
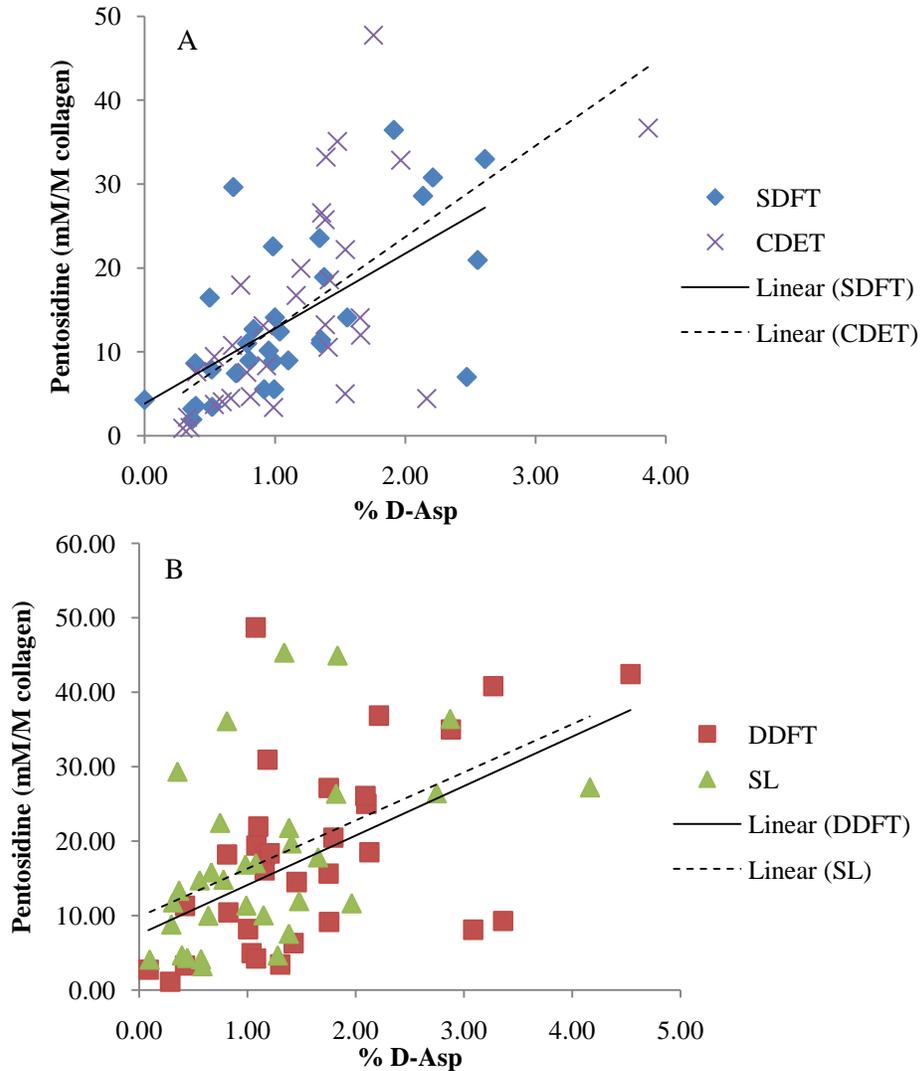
Figure 4-30: Correlation of pentosidine with % D-Asp in the SDFT & CDET (A), and DDFT & SL (B). Pentosidine concentration was significantly positively correlated with D-Asp in all tendons (SDFT, r=0.65, p<0.0001; DDFT, r=0.52, p=0.002; SL, r=0.49, p=0.005; CDET, r=0.63, p<0.0001).

Half-life of Collagenous Matrix Components

After separation of the matrix into collagenous and non-collagenous fractions, collagen half-life was significantly (p<0.001) higher in the SDFT (197.53 (±18.23) years) than the CDET (34.03 (±3.39) years) (Figure 4-31) and increased significantly with age in the SDFT (p=0.03) but not in the CDET (Figure 4-32).
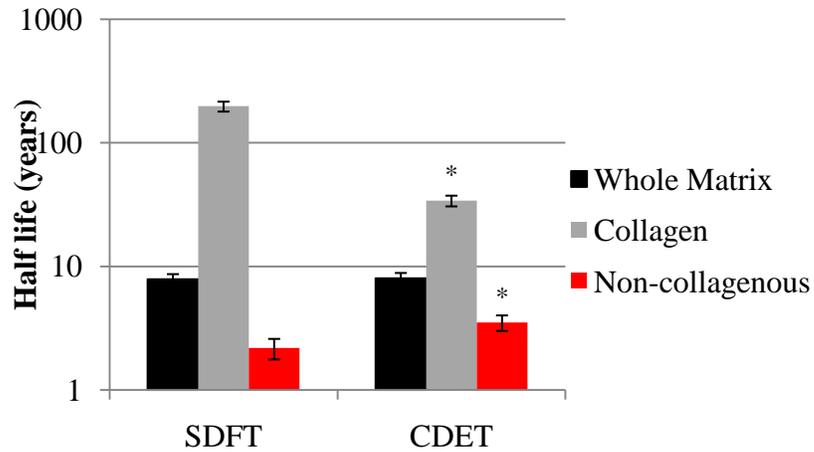
Figure 4-31: Half-life of matrix components in the SDFT and CDET (mean ± SEM) n = 32. * indicates a significant difference relative to the SDFT. Data are displayed on a Log_{10} scale.
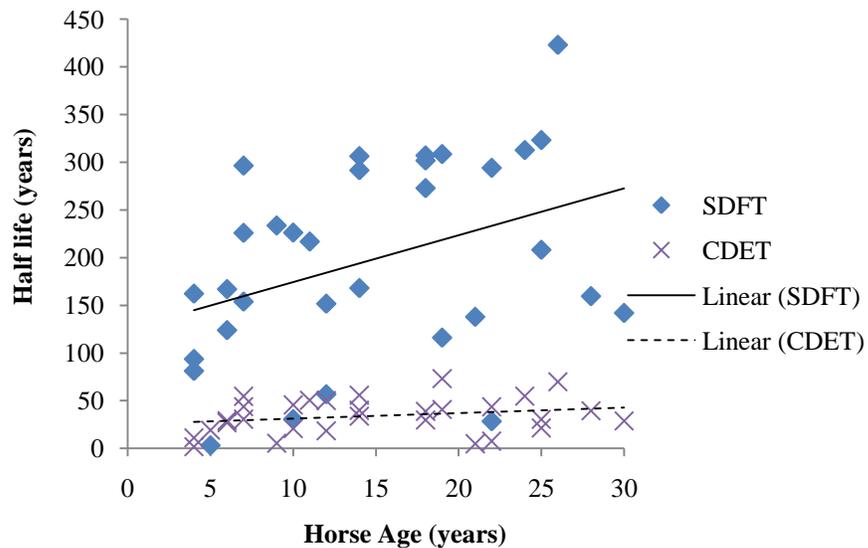
Figure 4-32: Half-life of the collagenous matrix of the SDFT and CDET as a function of horse age. Collagen half-life increased significantly with age in the SDFT (r=0.38, p=0.03) but not in the CDET.

Half-life of Non-collagenous Matrix Components

The half-life of the non-collagenous component was considerably lower than that of the collagen component for both tendon types. As with collagen, the non-collagenous proteins showed a significant difference in half-life within the matrix but in this case the SDFT (2.18 (±0.41) years) showed a lower residence time than the CDET (3.51 (±0.51) years) (p=0.04) (Figure 4-31). Non-collagenous matrix half-life increased significantly with age in the CDET (p=0.008) but not in the SDFT (Figure 4-33).
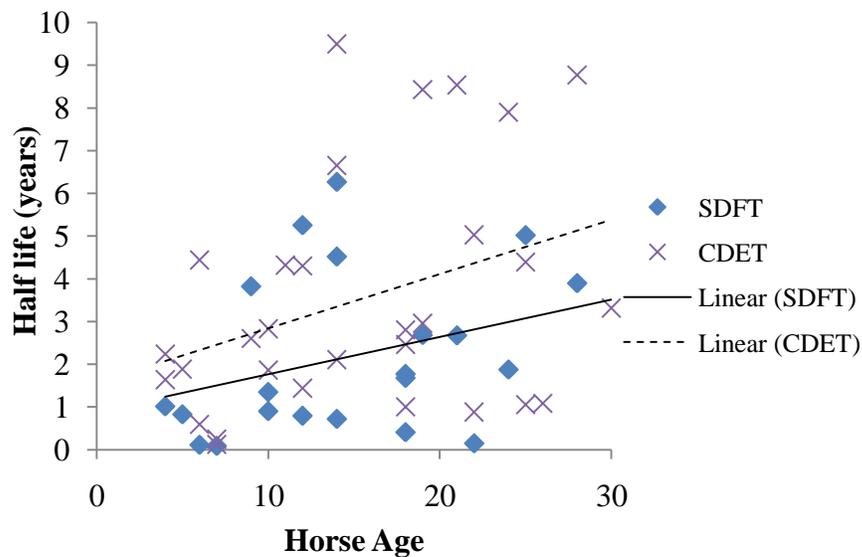
Figure 4-33: Half-life of the non-collagenous matrix proteins in the SDFT and CDET as a function of horse age. Non-collagenous matrix half-life increased significantly with age in the CDET (r=0.46, p=0.008) but was not correlated with age in the SDFT.

4.4. Discussion

The results of this work confirm that there is considerable variation in the matrix composition between the different tendon types. Furthermore the results show that the rate of turnover differs between tendon types and changes with horse age. It is widely accepted that tendon pathology is a degenerative condition resulting in an accumulation of micro-damage and decreased mechanical competence however the nature and progression of the micro-damage is not clear. Matrix age and corresponding rate of turnover gives an indication of the ability of the tenocytes to maintain the matrix and is therefore of particular relevance to the accumulation of micro-damage to collagenous and non-collagenous matrix components with ageing in injury prone tendons such as the SDFT. A logical assumption would be that high strain tendons which work with low safety margins such as the SDFT would require a greater capacity to repair micro-damage and have a matrix which appears relatively younger than low strain tendons such as the CDET. However, previous work based on the measurement of tissue fluorescence suggested that the opposite scenario was true (Birch *et al.,* 2008b) and so this chapter tested the hypothesis that the matrix of high strain energy storing tendons would be 'older' than the matrix of low strain positional tendons.

4.4.1. Age of Tendon Matrix when Assessed as a Whole

Small amounts of D-Asp and pentosidine indicate relatively rapid protein turnover and short residence time (Stabler *et al.,* 2009), while higher levels of D-Asp and pentosidine indicate that protein turnover is slower. Our results show that both D-Asp and pentosidine accumulate with age in the tendons in the equine forelimb, suggesting a relatively low rate of protein turnover in all tendons assessed. No previous studies have calculated protein half-life in tendon using aspartate racemization however the D/L ratios have been measured in human supraspinatus and biceps tendon tissue and the levels recorded (approximately 1%) were similar to those obtained in this study (Riley *et al.,* 2002) without separation into collagen and non-collagenous components. Levels of pentosidine are also similar to those reported previously in human tendon (Bank *et al.,* 1999). In contrast to previous work (Lin *et al.,* 2005b), a significant increase was identified in pentosidine concentration with horse age in the mid-metacarpal region of the SDFT. The levels reported in this chapter are approximately 15 fold higher than those reported by Lin *et al*. (2005b); it is possible that the detection method used here is more sensitive than that used previously and was able to detect small changes in tendon pentosidine content with increasing age.

However, when the matrix was studied as a whole, no differences in matrix age were identified between the functionally distinct SDFT and CDET; this is surprising as previous results suggest a lower rate of matrix turnover in the injury prone SDFT than in the positional CDET, based on measurements of tissue fluorescence (Birch *et al.,* 2008b). In agreement with previous results, a difference in tissue fluorescence between tendons was also identified. However, both enzymatic and lysyl oxidase crosslinks fluoresce naturally, and so a difference in crosslink profile could also account for the lower levels of fluorescence in the CDET. Indeed, levels of hydroxylysyl-pyridinoline were significantly lower in the CDET. The CDET has been shown previously to contain a significant number of histidinohydroxylysinonorleucine crosslinks which are not present in the SDFT (Birch *et al.,* 2006) and do not fluoresce naturally so are not detected by the methods used in this thesis. Contrary to the hypothesis, this study was not able to group the DDFT or SL with either the SDFT or CDET based on matrix composition or whole matrix age. No difference was identified in matrix age in the SL compared to the SDFT or CDET but composition of this tendon was significantly different from that of the SDFT. The half-life data and pentosidine levels presented in this chapter indicate that the rate of matrix turnover in the

DDFT is lower than in the other tendons; this tendon is not thought to act as an energy store, and is not exposed to high strains (Platt *et al.,* 1994) and so a low rate of turnover may not be detrimental to its function, which is thought to be mainly to flex the distal phalangeal joint during late swing (Butcher *et al.,* 2009).

4.4.2. Age of Collagenous and Non-Collagenous Matrix Proteins

Separation of the matrix of the functionally distinct SDFT and CDET into its collagenous and non-collagenous components reveals a difference in protein half-life that is not evident when the matrix is assessed as a whole. The results presented in this chapter suggest a half-life of 198 and 34 years for the SDFT and CDET collagenous matrix respectively and 2.2 and 3.5 years respectively for the non-collagenous matrix proteins. These results support the original hypothesis, showing that the age of the collagenous matrix is greater in the high strain energy storing SDFT than in the low strain positional CDET. The relatively low rate of turnover of the collagenous matrix in the injury prone SDFT may predispose this tendon to microdamage accumulation. In contrast, it appears that microdamage in the CDET would be repaired relatively rapidly due to the greater rate of collagen turnover in this tendon. However, turnover of the non-collagenous matrix proteins appears to occur more rapidly in the SDFT than in the CDET.

Collagen half-life has been calculated in other tissues; Sivan *et al.* (2008) reported a half-life of 95 to 215 years in intervertebral disc collagen depending on donor age whereas Verzijl *et al.* (2000b) reported 117 years in articular cartilage. Non-collagenous protein half-life has also been calculated in other tissues, with values of 3 to 25 years reported for aggrecan fractions in articular cartilage (Maroudas *et al.,* 1998), and 6 to 26 years for intervertebral disc aggrecan (Sivan *et al.,* 2006b). The half-life of collagenous and non-collagenous matrix components calculated in these studies are in the same region as the values reported here. It would be of value to assess the half-life of the collagenous and non-collagenous proteins in the DDFT and SL to determine if either tendon shows similarities to the SDFT or CDET in terms of matrix age.

4.4.3. Factors Affecting Half-life Calculations and the Rate of Pentosidine Accumulation

The half-life value of almost 200 years for the collagenous component of the SDFT reported in this chapter suggests that only 0.25% of the collagen is renewed each year. Therefore, in biological terms, turnover is insignificant and the collagen is essentially inert. The absolute values however reported in this chapter and by others rely on assumptions made in the calculations, as the accumulation of D-Asp is affected by several factors, including temperature, pH and protein structure (McCudden and Kraus 2006); increasing temperature by 4 °C causes the rate constant of racemization to double. The calculations were based on a tendon temperature of 37 ºC and assumed that the pH in dentin and tendon were not significantly different. Tendons in the equine forelimb are likely to be exposed to a wide range of temperatures; during exercise the SDFT has been shown to experience temperatures as high as 45 °C (Wilson and Goodship 1994), and this temperature variation may alter the rate constant of racemization. The difference in the calculated half-life is significant; for example a 1 °C increase would change the half-life of the collagenous matrix in the SDFT from 198 years to 77 years while an increase to 45 °C would decrease the half-life to 8 years (Figure 4-34). However the half-life of the majority of tendon collagen is likely to be closer to the higher values calculated. Regardless of absolute values, the data presented in this chapter show a clear difference between tendon types in relation to collagen and non-collagenous matrix turnover which were not appreciated when the whole tissue was analysed. The half-life of the collagenous matrix in the SDFT is approximately five times greater than in the CDET supporting the hypothesis.
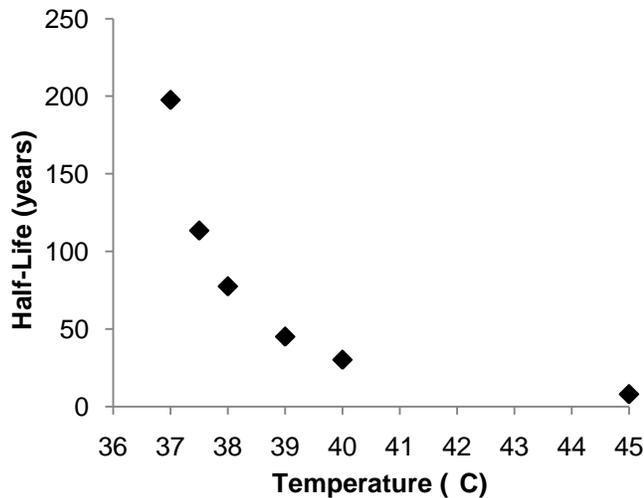
Figure 4-34: Effect of temperature on predicted average collagen half-life in the SDFT. An exponential equation was found to give the best fit (r=0.99).

The rate at which pentosidine accumulates is also affected by several factors, including the availability of sugars in the matrix, protein structure and temperature. Tendon blood supply is generally poor, especially in the core region, and therefore accumulation of AGEs may be limited by low levels of sugars within tendon. Glycation has been shown to occur at much lower levels in collagen fibrils than in monomeric collagen *in vitro* (Slatter *et al.,* 2008). It is not clear why pentosidine levels are similar between the SDFT and CDET when measurement of D-Asp in the collagenous and non-collagenous matrix components showed clear differences between these functionally distinct tendons; this is an area which requires further investigation.

Differences in matrix age may not have been apparent when the age of the entire matrix was assessed as neither pentosidine nor D-Asp are specific to collagen; pentosidine has also been shown to accumulate with age in aggrecan from cartilage (Pokharna and Pottenger 1997; Verzijl *et al.,* 2001) and intervertebral discs (Sivan *et al.,* 2006b). However, proteoglycans are present at low levels within tendon and are turned over more rapidly than the collagenous matrix; therefore the levels of pentosidine within the non-collagenous matrix are likely to be very low. D-Aspartate also accumulates with age in non-collagenous proteins in dentin (Masters 1983) and in cartilage aggrecan (Maroudas *et al.,* 1998; Sivan *et al.,* 2006b). The rate constant of D-Asp accumulation is approximately 2.5 fold greater for aggrecan than for collagen (Maroudas *et al.,* 1992), therefore although proteoglycan content

in tendon is relatively low, the proteoglycans present may contain a disproportionately high number of D-Asp residues.

4.4.4. Why are Matrix Proteins Turned Over at Different Rates in the SDFT and CDET?

A lower rate of collagen turnover in the high strain SDFT compared to the low strain CDET appears counterintuitive, and there are several possible explanations for this. The difference may reflect a compromise of the synthetic ability of tenocytes within the SDFT due to physiochemical factors such as nutrient availability, oxygen tension or increased temperature. However, other investigators have suggested that collagenous structures which require very high mechanical strength are protected from re-modelling which may weaken the structure (Laurent 1987). In a comparable manner, the collagenous matrix in the SDFT may not be turned over in an attempt to preserve its structural integrity and keep it within the narrow range of strength and stiffness required for optimal energy storage. Alternatively, differences in the way that the tendons are loaded during high speed locomotion may result in a predisposition to collagen damage over proteoglycan damage in the CDET. This possibility is supported by the lower half-life of the non-collagenous component of the SDFT compared to the CDET and previous work of others showing a high rate of proteoglycan turnover in flexor tendon (Rees *et al.,* 2000). The viscoelastic behaviour of tendon is complex and depends on the rate of deformation. Studies in rat tail tendon have shown that at small strain rates the response is dominated by the elastic component whereas at high strain rates the response is dominated by viscosity which is largely provided by the proteoglycan matrix (Puxkandl *et al.,* 2002). In addition to higher strains, the SDFT is loaded during the stance phase of gallop and is subjected to a faster rate of loading than the CDET. Further, proteoglycans within tendon matrix are thought to contribute towards strain transfer between collagen fibrils (Scott 2003). The SDFT is exposed to strains up to six times greater than the CDET and so if proteoglycans do play a role in strain transfer, the proteoglycans in the matrix of the SDFT are more likely to be damaged, and therefore require repair, than those in the CDET. Assessment of matrix composition showed that the SDFT has a GAG content approximately six fold greater than the GAG content of the CDET. In addition, the GAG content in the CDET (but not the SDFT) decreased significantly with increasing horse age, providing further evidence to suggest proteoglycans have a more important role in energy storing tendons. Therefore

turnover of the non-collagenous matrix is greater in the SDFT than in the low strain CDET as damage is more likely to occur to proteoglycans in this tendon due to the high strains it experiences.

Although this work separates collagen and non-collagenous components of the matrix there may, in addition, be pools of collagen which differ in their turnover rate. The extraction technique used does not retain all of the collagen in the insoluble fraction; a small amount of soluble collagen (less than 13%) is extracted from the matrix which is likely to represent a more labile pool of collagen. However the insoluble collagen does also appear to be slowly replaced giving support to other studies which have detected collagen synthesis and degradation in tendon (Langberg *et al.,* 1999; Langberg *et al.,* 2001; Miller *et al.,* 2005) but are not able to distinguish between pools of collagen that are turned over at different rates.

4.4.5. Effect of Age Related Modifications on Tendon Mechanical Properties

Age related modifications to tendon collagen, as identified in this chapter, are of biological significance as they are likely to change the mechanical behaviour of the tendon. Glycation of collagen is likely to have a much more significant effect on mechanical properties than aspartic acid racemization. Although the accumulation of D-aspartic acid is a useful marker of tissue age, it is present in very low concentrations within the matrix and therefore it is unlikely to alter tendon mechanical properties significantly. Extensive glycation may however alter tendon mechanical properties; *in vitro* studies have shown that glycation of rabbit Achilles tendon increases the stiffness and strength of the tendon (Reddy *et al.,* 2002) and it is well documented that AGEs are strongly associated with diabetic complications (Monnier *et al.,* 2008) such as stiffening of collagenous tissues. However previous work has not found an increase in tendon stiffness with increasing horse age (Birch 2007); the reasons for this are yet to be determined.

4.4.6. Spread of Data

The data presented in this chapter show a degree of variation between individuals, even once age has been accounted for; especially in the case of percent D-Asp and pentosidine. It is possible that low values could be an early sign of tendon degeneration, however all tendons included in the study appeared macroscopically normal. Increased water content is

indicative of early tendon injury; none of the tendons in this study exhibited a particularly high percentage of water. Furthermore, tendons that had high or low percent D-Asp or pentosidine had water contents that were in the middle of the range reported, suggesting that the tendons analysed in this study were not injured. In addition, previous studies have reported considerable variations in tendon strength and stiffness between individual horses that is not correlated with horse weight or exercise history (Birch 2007), it would be of value to determine if there is any correlation between tendon mechanical properties and the markers of matrix age studied in this chapter.

In this chapter a difference has been identified in the turnover of the collagenous and non-collagenous matrix components in functionally distinct tendons in the equine forelimb. The collagenous matrix in the high strain energy storing SDFT is turned over at a much slower rate than in the low strain positional CDET; while this may protect the tendon under normal circumstances, if micro-damage does occur it is more likely to accumulate in the SDFT. Furthermore, increased half-life of collagen in aged SDFTs may be a factor that contributes to the increased incidence of injury with ageing in this tendon. In contrast, turnover of the non-collagenous matrix occurs more rapidly in the SDFT than in the CDET, supporting the hypothesis that proteoglycans contribute significantly to force transfer at high strain levels and rates. The long half-life of the collagen molecules in the SDFT also results in age related modifications such as racemization and glycation, which may further alter tendon mechanical properties. The high strains the SDFT experiences combined with the low rate of collagenous matrix turnover and associated alteration in mechanical properties with increasing horse age would suggest that gross failure of this tendon would be unavoidable. However, many horses compete at the highest level and never suffer from a tendon injury. The data presented in this chapter show considerable variation between horses and previous studies have also found a wide variation in material properties of tendons from different horses which are not related to horse size or exercise history (Birch 2007) indicating that some individuals may be more at risk of tendon damage than others. The low rate of collagen turnover in the high strain SDFT may result from lower cell activity in terms of collagen metabolism and may have important implications for matrix renewal and repair. The increased collagen half-life in the SDFT in older horses suggests that cell activity may decline further with ageing. It is therefore important to assess cell activity in these functionally distinct tendons to determine if this is the case.

4.5. Conclusions

- Matrix composition differs between functionally distinct tendons in the equine forelimb.

- The prioritisation of collagenous and non-collagenous matrix protein turnover differs between the functionally distinct SDFT and CDET.

- Collagenous matrix proteins are metabolised more rapidly in the low strain positional CDET than in the high strain energy storing SDFT.

- Turnover of non-collagenous matrix proteins occurs more rapidly in the SDFT than in the CDET.

- Ageing results in an increase in the half-life of collagen in the SDFT and an increase in the half-life of non-collagenous protein in the CDET.

CHAPTER FIVE

5. Potential for Matrix Synthesis and Degradation Differs between Functionally Distinct Tendons

5.1. Introduction

The composition of tendon matrix is governed by the cellular component of tendon and the rate of matrix turnover is determined by cell activity. The variation in matrix composition between the different tendon types suggests that there is a difference in the pattern of gene expression by the resident tenocytes. In addition, the age of matrix components differs between the high strain energy storing superficial digital flexor tendon (SDFT) and low strain common digital extensor tendon (CDET) in the equine forelimb, as shown by the data presented in the previous chapter of this thesis, suggesting different levels of gene expression activity between the cells in these functionally distinct tendons.

Despite a greater cellularity in the SDFT than the CDET, the collagen appears to be older in this tendon suggesting that cells in the SDFT are less active than their counterparts in the CDET in terms of collagen synthesis and degradation. Correspondingly, previous work has shown that cells from the CDET express higher levels of genes coding for collagen type I and III, and collagen degrading enzymes than cells in the SDFT (Birch *et al.,* 2008b). In contrast, non-collagenous matrix half-life is greater in the CDET than in the SDFT, suggesting that turnover of the non-collagenous matrix proteins occurs more rapidly in the SDFT than in the CDET. Differences in the turnover of the collagenous and non-collagenous matrix proteins between functionally distinct tendons are likely to be due to differences in the way in which these tendons are loaded; the SDFT experiences greater strain levels and rates than the CDET. It has been shown that at high strain rates the response is dominated by viscosity, which is provided mainly by the proteoglycanous components of the matrix, rather than elasticity, which is provided mainly by the collagenous component of the matrix (Puxkandl *et al.,* 2002). It would therefore be logical to expect cells in the SDFT to produce more message for non-collagenous matrix proteins; however previous work has not identified a difference in expression of the non-collagenous proteins decorin and collagen oligomeric matrix protein (COMP) between the SDFT and CDET (Birch *et al.,* 2008b). The potential for matrix synthesis and degradation has not been investigated previously in the other tendons in the equine forelimb; the suspensory ligament (SL) acts as an energy store in a similar manner to the SDFT and is thought to

experience relatively high strains. In contrast, the deep digital flexor tendon (DDFT), like the CDET, is mainly positional in function; acting to stabilise the metacarpophalangeal joint during extension of the forelimb (Butcher *et al.,* 2007) rather than playing a role in energy storage and is not subjected to high strains.

It is well documented that cells are able to alter their phenotype according to the amount of deformation they experience (Ingber 1997), cells are thought to have a pre-set sensitivity to deformation such that if strain-induced signals are above or below a certain level, termed the 'mechanostat set-point', the cells will remodel the matrix accordingly (Frost 1987). It would appear that a certain level of mechanical load is required to maintain normal tenocyte phenotype and function; stress deprivation causes increased expression of the collagenase matrix metalloproteinase-13 (MMP-13) and a loss of cell-matrix interactions (Arnoczky *et al.,* 2008b). High strains also result in perturbations to tenocyte phenotype, causing increased expression of MMP-1 and -3 (Archambault *et al.,* 2002; Sun *et al.,* 2008; Tsuzaki *et al.,* 2003). Differences in the amounts and rates of strain experienced by tenocytes within the forelimb tendons is therefore likely to be an important determinant of gene expression and cell phenotype in functionally distinct tendons.

It is also possible that ageing has an effect on cell phenotype; collagenous matrix half-life was found to increase with horse age in the SDFT (see chapter 4), suggesting a decrease in collagen turnover in aged tendons. Further, it has previously been suggested that overall cell activity within tendon declines with ageing (Smith *et al.,* 2002a). This may have important consequences in terms of initiation and progression of tendon degeneration. Epidemiological studies have established that the incidence of tendinopathy is increased in aged individuals (Clayton and Court-Brown 2008; Kasashima *et al.,* 2004). However, previous work was not able to identify any alterations in collagen gene expression with increasing age (Birch *et al.,* 2008b). Further work is therefore required to identify the underlying causes of age related tendinopathy, which is characterised by a gradual decrease in mechanical integrity caused by accumulation of micro-damage to the extracellular matrix rather than an acute overstrain injury (Riley 2008). Micro-damage accumulation indicates that there is an inability of the cells to degrade and repair areas of damaged matrix. It is possible that overall gene expression decreases with increasing age, such that cells in aged tendons are less able to repair the matrix when micro-damage occurs and therefore overuse

injuries and clinically detectable degeneration are more likely to develop. The SDFT is injured far more frequently than the CDET (Ely *et al.,* 2004; Ely *et al.,* 2009); this tendon may be further predisposed to micro-damage accumulation due to its relatively low cell activity in terms of collagen turnover.

Tendinopathy, both in humans and horses, is associated with alterations in the expression of specific genes. Several studies have reported increased expression of degradative enzymes, including MMPs -1, -9, -13 and -23, and ADAM-12 in tendon lesions (de Mos *et al.,* 2007; Jones *et al.,* 2006; Karousou *et al.,* 2008; Nomura *et al.,* 2007). Increased collagen gene expression has also been reported in chronic Achilles tendinopathy (de Mos *et al.,* 2007; Ireland *et al.,* 2001) and experimentally induced lesions in the SDFT showed increased collagen type I and III expression during healing (Dahlgren *et al.,* 2005). However, there appears to be a decrease in the synthesis of enzymes that degrade the non-collagenous matrix components; studies have found that expression of MMP-3 and -10 decreases in Achilles tendinopathy (Ireland *et al.,* 2001; Jones *et al.,* 2006). Although the changes in gene expression that occur in tendon rupture and tendinopathy have been documented, there are little data available regarding differences in gene expression between tendons with specific functions. Further, it has not been established if any changes in gene expression occur with normal ageing in tendons that do not exhibit pathological changes.

5.1.1. Synthesis of Matrix Proteins

5.1.1.1. Transcription

In all tissues the synthesis of specific proteins is tightly controlled and occurs in several stages. When a protein is required, it is not synthesised directly from the DNA template, instead the DNA sequence is transcribed into messenger RNA (mRNA), and this is used as a template for protein synthesis. The DNA double helix is unwound by the enzyme helicase so a single nucleotide chain can be copied. The RNA polymerase enzyme binds to the DNA template; the binding sites are determined by promoters which are sequences located upstream to the start site for transcription and are recognised by transcription factors. RNA polymerase reads the DNA strand in the 3' to 5' direction, and transcribes a single strand of mRNA in the opposite direction. Synthesis of mRNA is terminated when the RNA polymerase encounters a stop codon (Stryer *et al.,* 1995).The mRNA then migrates from the nucleus into the cytoplasm, where it binds to ribosomes and is used as a template for

peptide synthesis in a process known as translation. By quantifying the amount of mRNA it is therefore possible to determine which genes are being expressed by cells in a specific tissue at a specific time. However, the amount of mRNA produced is very small, and so for quantification it needs to be amplified. Amplification of mRNA can be achieved using real time reverse transcriptase polymerase chain reaction (RT-PCR), which results in large enough copy numbers to enable accurate quantification of small amounts of mRNA.

RT-PCR

The first stage in the RT-PCR reaction is the reverse transcription of the mRNA to complementary DNA (cDNA); this step is required as RNA is not able to act as a template for polymerisation (Bustin 2000). This reaction is catalysed *in vitro* by a reverse transcriptase enzyme such as Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) which synthesises cDNA from the mRNA strands using oligo-nucleotides. Once cDNA has been synthesised, it can then be amplified by PCR; the cDNA is incubated with a DNA polymerase and primer sequences that have been designed specifically for the genes of interest, plus a fluorescent dye such as SYBR® green, which does not fluoresce in solution, but does when bound to double stranded DNA. Therefore the increase in fluorescence over a certain number of heating cycles can be used to monitor the amount of DNA, and therefore corresponding mRNA, present in a sample (Bustin 2000). A PCR cycle consists of three stages; first the samples are heated to 95 °C for 15 seconds to separate the cDNA strands. The samples are then cooled to 54 °C to allow the gene specific primers to anneal to the cDNA strands. The solution is then heated to 72 °C, which allows the DNA polymerase to synthesise new DNA strands. Primer annealing is required for the DNA polymerase to be able to bind to the DNA strands, meaning that only cDNA coding for the gene of interest is amplified. The samples are then heated to 95 °C again to separate the newly synthesised DNA strands and allow further cDNA synthesis; repeating this for 20 cycles results in a million-fold amplification (Stryer *et al.,* 1995).

5.1.1.2. Translation

Translation of mRNA into proteins occurs in the rough endoplasmic reticulum; like transcription this process is subject to a high level of control and occurs in three stages. However, translation is much more complex than transcription and is mediated by more than one hundred molecules, including transfer RNAs, enzymes, protein factors and ribosomes (Stryer *et al.,* 1995). The first step in mRNA translation is initiation, which is

precisely coordinated and involves several eukaryotic initiation factors which form the 43S pre-initiation complex on the mRNA sequence that is undergoing translation. This complex joins with the 60S ribosomal subunit at the AUG start codon which results in the formation of the 80S initiation complex. Once this has occurred, elongation, which is mediated by the 80S ribosome, can begin. Elongation of the polypeptide chain requires the participation of translation elongation factors, which supply the ribosome with appropriate amino acids and translocate the ribosome along the mRNA. Termination of translation occurs when a stop codon is encountered; this final step is mediated by the eukaryotic release factor, which dissociates the ribosome from the mRNA and releases the ribosomal subunits (Tavernarakis 2008).

Protein synthesis can therefore be controlled both at the transcriptional and translation levels by targeting a variety of molecules required for these processes. At the transcriptional level, gene expression is controlled predominantly by the activity of transcription factors and microRNAs (Hobert 2008). Transcription factors are required for the initiation of transcription and their activity can be regulated by post translational modifications, including phosphorylation, and they can either positively or negatively regulate gene expression. In contrast, MicroRNAs generally modulate gene expression by repression. MicroRNAs are RNA sequences 19 to 25 nucleotides long which bind to the target mRNA sequence and therefore down-regulate expression of a specific gene (Pillai *et al.,* 2007). MicroRNAs are often partially complementary to many mRNA sequences and therefore one microRNA can target up to hundreds of genes (Chen *et al.,* 2009). Translation is mainly controlled at the initiation stage; phosphorylation of specific eukaryotic initiation factors decreases their ability to form the pre-initiation complex, a step which is essential for successful translation (Tavernarakis 2008).

Further control is achieved after protein synthesis by post-translational modifications; for example matrix degrading enzymes are synthesised as inactive pro-enzymes, in order to activate the enzyme the pro-peptide must be cleaved by other proteases present within the matrix (Suzuki *et al.,* 1990). Active matrix degrading enzymes can be inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the enzyme active site (Nagase *et al.,* 2006). The potential for matrix degradation can therefore be assessed at the protein level by measuring the concentration of specific MMPs present within the matrix; active

MMPs have a lower molecular weight than pro-MMPs, which allows separation and quantification of both pro- and active enzymes.

5.1.2. Aim and Hypothesis

The aim of this chapter was to assess the potential for matrix synthesis and degradation in functionally distinct equine tendon and to determine if this alters with ageing. This was achieved by measuring the expression at the transcriptional level of key matrix proteins and corresponding degradative enzymes using real time RT-PCR. Protein synthesis at the translational level was assessed by measuring the pro- and active forms of enzymes that are able to degrade specific components of the matrix. It is not possible to directly measure newly synthesised collagen and non-collagenous protein levels in tendon tissue against a background of long-lived matrix proteins. It was hypothesised that cell activity is programmed by the *in vivo* strain environment such that cells in low strain positional tendons (DDFT and CDET) will exhibit a greater capacity to turnover the collagenous matrix whereas cells in high strain energy storing tendons (SDFT and SL) will show a greater potential for non-collagenous matrix turnover. Furthermore, it was hypothesised that overall cell activity will decrease with increasing horse age in all tendons.

5.2. Materials and Methods

Lyophilised tendon tissue from the SDFT, DDFT, SL and CDET of 32 horses collected and processed as described in chapter 3 was used for the protein experiments outlined below and RNA was extracted from the tendon tissue stored in RNAlater® (see Chapter 3).

5.2.1. Measurement of Gene Expression

5.2.1.1. RNA extraction

Approximately 200 mg of tissue was finely chopped and frozen in liquid nitrogen before being powdered using a mikro-dismembrator (Braun Biotech International, Melsungen, Germany). The dismembrator equipment was cooled in liquid nitrogen immediately before use to ensure the tissue remained frozen during powdering. Each sample was shaken for 2 minutes at 2000 rpm. If the tendon had not been reduced to a powder it was re-frozen in liquid nitrogen and the process was repeated. The dismembrator equipment was washed with a disinfectant (Virkon, 1% solution) between samples to ensure no contamination occurred. The powdered tissue was placed in TRI reagent® solution (Ambion, Applied

Biosystems, Warrington, UK) at a concentration of approximately 100 mg/ml. TRI reagent® contains phenol and guanidine thiocyanate, which inhibit RNase activity. This was left to stand at room temperature for up to 1 hour before being stored at -80 °C, where it is stable for up to 1 month.

RNA extraction was achieved by using RNeasy mini kits (Qiagen Ltd., Crawley, UK). The samples stored in TRI reagent® were allowed to thaw completely, vortex mixed and centrifuged at 12 000 g for 10 minutes at 4 °C. The supernatant was removed into a clean tube and the remaining tissue was discarded. Chloroform (200 µl) was added to each tube and they were vortex mixed thoroughly before being left at room temperature for 10 minutes. The samples were then centrifuged at 12 000 g for 15 minutes at 4 °C. This separates the sample into 3 phases; the upper aqueous phase which contains the RNA, the interphase which contains the DNA and the organic phase which contains the proteins. Up to 375 µl of the aqueous phase was removed to a clean tube, and an equal amount of 70% ethanol was added (made up with 0.1% diethylpyrocarbonate treated (DEPC) water). Up to 700 µl of each sample was transferred to an RNeasy spin column in a collection tube and centrifuged at 8000 g for 15 seconds. This causes any RNA longer than 200 bases to bind to the column membrane, while contaminants are washed away. Any remaining DNA was removed by the inclusion of an on-column DNA digestion step using RNase-free DNase. The RNA was extracted according to the manufacturer's instructions (RNeasy mini handbook, Qiagen) and was eluted in 50 µl RNase free water and stored at -80 °C. RNA concentration was measured using a nano-drop® spectrophotometer (ND 3300, NanoDrop Technologies, Wilmington, USA). The spectrophotometer was cleaned before use and zeroed with RNase free water. 1 µl of RNA containing water was placed on the spectrophotometer, and RNA concentration was measured in ng/µl.

5.2.1.2. Reverse Transcription to cDNA

Reverse transcriptase was used to transcribe the RNA to cDNA. 1-2 µg RNA from each sample was made up to 11 µl with 0.1% DPEC water and 1 µl Oligo-dT primer was added to each sample to give a final volume of 12 µl. The samples were vortex mixed and centrifuged briefly before heating for 5 minutes at 70 °C and then immediately placed on ice. A master mix was made up containing 5 µl 5x reverse transcriptase buffer, 1.25 µl of dATP, dCTP, dTTP and dGTP (all 10 mM), 0.63 µl RNase inhibitor, 1 µl M-MLV reverse

transcriptase and 1.38 µl dH2O per sample. Master mix (13 µl) was added to each sample and they were vortex mixed and centrifuged briefly. The samples were heated for 60 minutes at 42 °C, then for a further 5 minutes at 95 °C. They were placed on ice before being stored at -20 °C.

5.2.1.3. Identification of Endogenous Control Genes

The most stably expressed genes were identified from a panel of eight potential endogenous control genes. The expression levels of gylceraldehyde 3-phosphate dehydrogenase (GapDH), 18S, TATA-box binding protein (TBP), succinate dehydrogenase A (SDHA), hypoxanthine phosphoribosyltransferase-1 (HPRT1), HIRA interacting protein-5 (HIRP5), mitochondrial ribosomal protein S7 (MRPS7) and β-actin (ACTB) were determined in the SDFT, DDFT, SL and CDET from 4 horses. To ensure the reference genes selected did not alter significantly with increasing age the tendon samples used to assess stability were selected from horses aged 4 to 24. This panel of genes was selected as they have been identified as some of the most stably expressed genes in a variety of tissues from different species (Ayers *et al.,* 2007; Bogaert *et al.,* 2006; Maccoux *et al.,* 2007; Taylor *et al.,* 2009). The two genes that had the least variation between samples were identified using GeNorm (Vandesompele *et al.,* 2002). GeNorm is available from www.medgen.ugent.be/~jvdesomp/geNorm and is a collection of macros for Microsoft Excel that determines the two most stably expressed genes. This method is based on the principle that the ratio of two ideal control genes should be equal in all samples, regardless of cell type or experimental conditions, so any variation in this ratio means the genes are not constantly expressed. A gene stability measure (M) is generated by calculating the pairwise variation of each gene with other control gene and excluding the gene with the highest M value in a stepwise manner, until the two most stably expressed genes with the lowest M values remain (Vandesompele *et al.,* 2002). HIRP5 and MRPS7 were found to be the most stably expressed genes in equine tendon from the panel of 8 genes assessed (Figure 5-1).
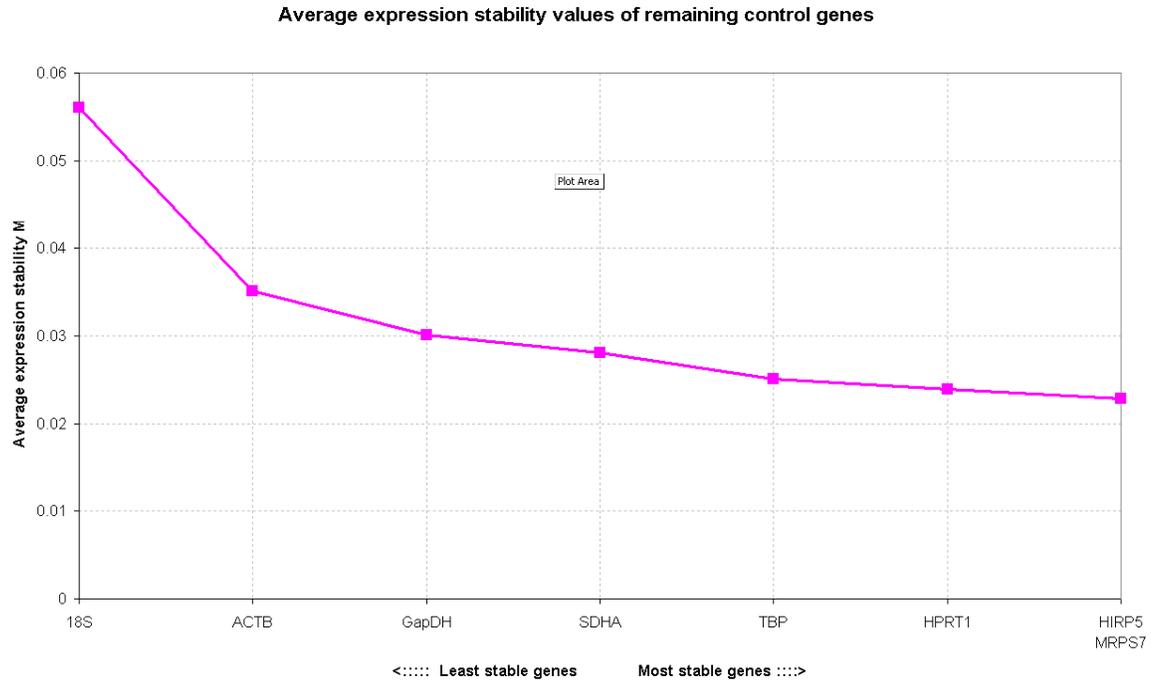
Figure 5-1: Graph showing expression stability values for control genes measured.

The pairwise variation between two sequential normalisation factors (V) is calculated to indicate how many control genes it is necessary to include to ensure the data is normalised accurately. Large V values indicate that the addition of another control gene has a significant effect and so should be included. Vandesompele *et al.* (2002) suggest that at least three internal control genes are used for normalisation, and propose a cut-off value for V of 0.15, below which there is no need to include any additional control genes. The variation between using two and using three control genes is 0.007 in equine tendon (Figure 5-2), so two control genes were used as the inclusion of another gene would have little effect on normalisation. A limited amount of cDNA was available for some samples and so using fewer control genes allowed the mRNA expression of more genes of interest to be quantified. HIRP5 and MRPS7 were used as endogenous control genes for all samples. As previous data were normalised to GapDH (Birch *et al.,* 2008b), expression of GapDH was measured alongside the reference genes to determine if normalising the data to GapDH had a significant effect on gene expression levels calculated. Expression levels of Col12A1, MMP-9, MMP-10, ADAM-12 and ADAMTS-2 were calculated relative to GapDH expression in approximately half the tendon samples and compared to the expression levels calculated when using MRPS7 and HIRP5 as reference genes.

127
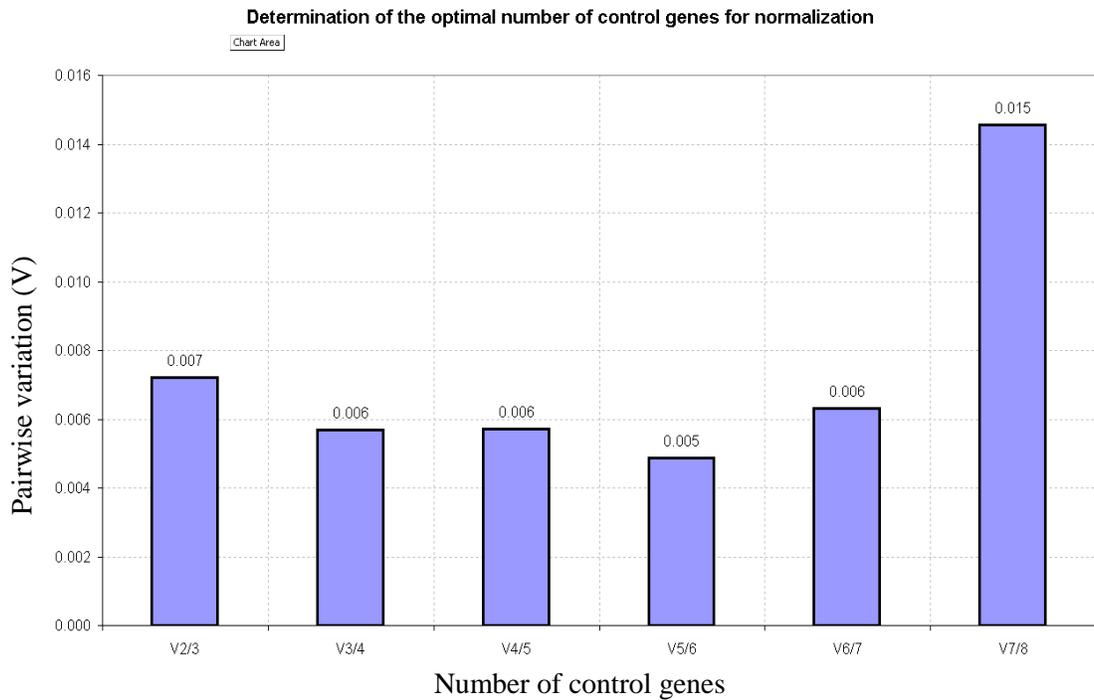
Figure 5-2: Determination of the optimal number of control genes by calculating the pairwise variation between sequential normalisation factors. Each bar represents the effect of including an additional control gene. These data show that the inclusion of additional control genes has little effect on the stability of the control genes.

5.2.1.4.   Primer design

Equine specific primers were designed using programs available online. The horse genome has now been sequenced and is available through Ensembl (www.ensembl.org/Equus_caballus/index.html). The gene of interest (e.g. TIMP-4) was searched for in Ensembl and the transcript sequence for the equine gene was obtained (Figure 5-3).

Transcript sequence	AATAGGGGCCAGGGGCTTCTCTCCTGGCTTTCCTCTTCATCTCACTGAGCCTGCCGGCCA GGAGGGTAGCTGCTCCTGCAGCCTCGGGTGGCCTGCAGCCAAAGGAGGGCGGAGCCCATC TGGGCGGGATTGGCCCTAGGTCCACCTCATAAAGCCTGGGGCGAGGGGCGCGACGCGTTC TGAAAGAGCCCTGGAGGGGCTGTTTGGTCCAACAGATCCCATAGGCTCAGTCGGGGCTCT GCAGTGTCATGCCCCGGAGCCCCCGGACAGCGCCGAGCTGGGCGCTGTTGCTGCGGCTGT TGGCACTGCTGCGGCCGCCAGGGCTGGGCGAGGCGTGCAGCTGCGCCCCCGCGCACCCCC AGCGGCACGTCTGCCACTCGGCGCTTGCAATCCGGGCCAAAATCTCCAGTGAGAAGGTAG TTCCTGCCAGTGCAAATCCTGCTGACACTCAAAAAATGATCCGGTATGAAATCAAACAAA TAAAGATGTTTAAAGGGTTTGAGAAAGTCAAGGATGTTCAGTATATCTATACACCTTTTG ATTCCTCCCTGTGTGGTGTGAAACTAGAAGCCAACAGCCAGAAGCAGTATCTCTTGACCG GTCAGGTCCTCAATGATGGGAAAGTCTTCATTCATCTGTGCAACTACATTGAGCCCTGGG AGAACTTGTCCTTTTTGCAGAGAGAAAGTCTGAATCATCACTACCTTCTGAACTGTGGCT GCCAAATCACCACCTGCTACACACTGCCCTGTACGATCTCGGCCCCGCAACGAGTGCCTCT GGACAGACTGGCTGTTGGAACGGAAGCTCTATGGGTACCAGGCCCAGCATTATGTCTGCC TGAAGCATGTTGATGGCACCTGCAGGTGGTACCAGGGCCGCCTGCCCCTCAGGAAGGAGT TTGTTGACATCATCCAGCCCTAGTAGGGACCAGTGACCACCACACTCCTTCAAGAGTCCT GAAGACCAAGCCAGTTCTCCTTCCCTGCAGACCTCTGGCTGTCACCATCTGCCTCATTGC TGCCACCCCATGGGAAGTACCAAGTAGACAGTCTGGCTAGCATTAGGGCAGGGATGGGGC ATG

Figure 5-3: Transcript sequence for equine TIMP-4 obtained from Ensembl. The alternating text colour indicates different exons.

Homologous sequences in other sequences were identified using a basic local alignment search tool (BLAST, www.ncbi.nlm.nih.gov/blast/Blast.cgi). The alignment between equine and human sequences was determined using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2) (Figure 5-4).

Figure 5-4: Alignment of human and equine sequences for TIMP-4. The stars indicate areas of sequence homology.

Information regarding intron size and location was obtained from Ensembl (Figure 5-5). To prevent amplification of any contaminating genomic DNA, primers were designed so that the sequence they amplified was intron spanning. If possible, the primers were designed around large introns close to the 3' end of the sequence where there was good alignment between human and equine sequences as this further reduces the chance of amplifying genomic DNA and increases the efficiency of the amplification.

No.	Exon / Intron	Chr	Strand	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence								..........tcccagtgccttccctctacttctagaaggcttaattgctcaggcaagga
1	ENSECAE00000070924	16	1	5,091,232	5,091,618	-	1	387	AATAGGGGCCAGGGGCTTCTCTCCTGGCTTTCCTCTTCATCTCACTGAGCCTGCCGGCCA GGAGGGTAGCTGCTCCTGCAGCCTCGGGTGGCCTGCAGCCAAAGGAGGGCGGAGCCCATC TGGGCGGGATTGGCCCTAGGTCCACCTCATAAAGCCTGGGGCGAGGGGCGCGACGCGTTC TGAAAGAGCCCTGGAGGGGCTGTTTGGTCCAACAGATCCCATAGGCTCAGTCGGGGCTCT GCAGTGTCATGCCCCGGAGCCCCCGGACAGCGCCGAGCTGGGCGCTGTTGCTGCGGCTGT TGGCACTGCTGCGGCCGCCAGGGCTGGGCGAGGCGTGCAGCTGCGCCCCGCGCACCCCC AGCGGCACGTCTGCCACTCGGCGCTTG
	Intron 1-2	16	1	5,091,619	5,092,780			1,162	gtgagtccggaggcccgtgaggtcc..........ttccctcttttctcttcctatgcag
2	ENSECAE00000070953	16	1	5,092,781	5,092,878	1	0	98	CAATCCGGGCCAAAATCTCCAGTGAGAAGGTAGTTCCTGCCAGTGCAAATCCTGCTGACA CTCAAAAAATGATCCGGTATGAAATCAAACAAATAAAG
	Intron 2-3	16	1	5,092,879	5,093,308			430	gtacatggggacaggacagggcgtt..........ctgcttttggtaattgcctttcag
3	ENSECAE00000071004	16	1	5,093,309	5,093,423	0	1	115	ATGTTTAAAGGGTTTGAGAAAGTCAAGGATGTTCAGTATATCTATACACCTTTTGATTCC TCCCTGTGTGGTGTGAAACTAGAAGCCAACAGCCAGAAGCAGTATCTCTTGACCG
	Intron 3-4	16	1	5,093,424	5,096,048			2,625	gtaagttaaagaccagcaagtggcc..........ggttctcttgcctctcccttgaag
4	ENSECAE00000071039	16	1	5,096,049	5,096,173	1	0	125	GTCAGGTCCTCAATGATGGGAAAGTCTTCATTCATCTGTGCAACTACATTGAGCCCTGGG AGAACTTGTCCTTTTTGCAGAGAGAAAGTCTGAATCATCACTACCTTCTGAACTGTGGCT GCCAA
	Intron 4-5	16	1	5,096,174	5,096,821			648	gtaagaaaatgtccatttccaaggt..........tgatgttgtgtggctctgactctag
5	ENSECAE00000071110	16	1	5,096,822	5,097,179	0	-	358	ATCACCACCTGCTACACACTGCCCTGTACGATCTCGGCCCGCAACGAGTGCCTCTGGACA GACTGGCTGTTGGAACGGAAGCTCTATGGGTACCAGGCCCAGCATTATGTCTGCCTGAAG CATGTTGATGGCACCTGCAGGTGGTACCAGGGCCGCCTGCCCCTCAGGAAGGAGTTTGTT GACATCATCCAGCCCTAGTAGGGACCAGTGACCACCACACTCCTTCAAGAGTCCTGAAGA CCAAGCCAGTTCTCCTTCCCTGCAGACCTCTGGCTGTCACCATCTGCCTCATTGCTGCCA CCCCATGGGAAGTACCAAGTAGACAGTCTGGCTAGCATTAGGGCAGGGATGGGGCATG
	3' downstream sequence								ttacagcttgtgtccaagaccccagtccagaacctgtcaagggctaggga..........

Figure 5-5: Intron information for equine TIMP-4.

Exon boundaries were marked with square brackets and the relevant portion of the sequence was copied to a primer design program (www.roche-applied-science.com/sis/rtpcr/upl; http://frodo.wi.mit.edu) (Figure 5-6). Primers were chosen according to the optimum properties outlined by Bustin *et al.* (2000). Ideally, primers should be 15-20 bases long, have a G/C content between 20 and 70%, the melting temperature (Tm) should not differ more than 2 °C between the forward and reverse primers and the maximum and minimum Tm should be 58 °C and 60 °C respectively. All primers used met these criteria. Forward and reverse primer sequences were ordered from Eurogentec (Seraing, Belgium).

Primer	Length	Position	Tm	%GC	Sequence
Left Primer	23	142 - 164	59	43	ccttttttgcagagagaaagtctg
Right Primer	20	200 - 219	60	60	gcagtgtgtagcaggtggtg

Amplicon (78 nt)

ccttttttgcagagagaaagtctgaatcatcactaccttctgaactgtggctgccaaatca
ccacctgctacacactgc

[Download pack insert] [PDF report] [Text report] [Order probes or set]

Transcript overview:
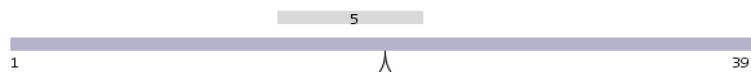
Detailed view:
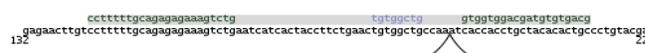
Figure 5-6: Primers for equine TIMP-4 designed by Roche's assay design centre.

For some genes, searching for the equine sequence in Ensembl did not produce any results (ADAM-8, ADAM-12, ADAMTS-3, ADAMTS-8, ADAMTS-12, MMP-7, MMP-10, MMP-19, MMP-25, TIMP-4) so the BLAST was used to identify sequences homologous to the human gene. If no corresponding equine sequences were found, the BLAST program from Ensembl was used to display regions of the equine genome that are homologous to the human gene sequence (www.ensembl.org/Equus_caballus/blastview) (Figure 5-7).
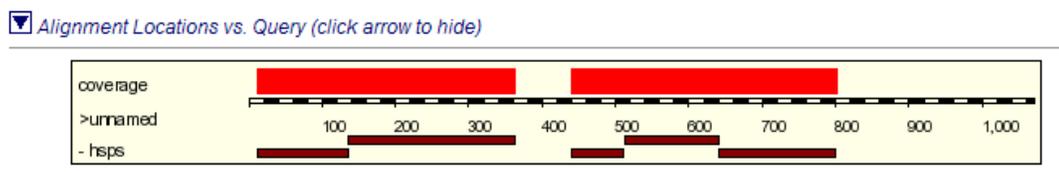
Figure 5-7: Alignment of equine sequences with human MMP-7

The alignment of the sequence with the greatest homology was checked against the human gene sequence using ClustalW2, and if the alignment was sufficient primers were designed from the equine sequence. Forward and reverse primer sequences are shown in Table 5-1.

Gene	Primer Sequence	
	Forward	Reverse
18S[*]	GGCGTCCCCCAACTTCTTA	GGGCATCACAGACCTGTTATTG
ACTB[α]	CCAGCACGATGAAGATCAAG	GTGGACAATGAGGCCAGAAT
ADAM-12	AGTTCGCCTCAGCGAGTG	GGAGGGTTTGGCTTACGG
ADAM-17	CGCATTCTCAAGTCTCCACA	TGAAAAGATGTGCTAGGCAGAC
ADAMTS-2	CCTATCCAGAAGACCTCGTCA	CAGCACAGCTTGTTGTAGCC
Aggrecan[§]	GAGGAGCAGGAGTTTGTCAACA	CCCTTCGATGGTCCTGTCAT
Biglycan[*]	TGAAGCTCAACTACCTGCGAATC	AGATGGAGTTCATTCAGGGTCTCT
Col 1A2[*]	GCACATGCCGTGACTTGAGA	CATCCATAGTGCATCCTTGATTAGG
Col 3A1[*]	ACGCAAGGCCGTGAGACTA	TGATCAGGACCACCAACATCA
Col 5A1[*]	TCCTTCAAAGTGTACTGCAACTTCA	GATTCTGGCCCCCTCAGACT
Col 12A1	GCGAGAGGGGTATTGGATCT	CTGGTGGACCTGTTCTGGAT
COMP[*]	GGTGCGGCTGCTATGGAA	CCAGCTCAGGGCCCTCAT
Decorin[*]	CATCCAGGTTGTCTACCTTCATAACA	CCAGGTGGGCAGAAGTCATT
Fibromodulin[*]	CAACCAGCTGCAGAAGATCC	GCAGAAGCTGCTGATGGAG
GapDH[*]	GCATCGTGGAGGGACTCA	GCCACATCTTCCCAGAGG
HIRP5[*]	TTGCATCTGGCTTACCCTTAG	CCACAACTTCATCATCTTCTTCA
HPRT1[α]	GGCAAAACAATGCAAACCTT	CAAGGGCATATCCTACGACAA
Lumican[*]	GTGTCAAGACAGTAAGGATTCAAAGC	CAATGCCAAGAGGAAAGTAAACG
MMP-1[§]	GGTGAAGGAAGGTCAAGTTCTGAT	AGTCTTCTACTTTGGAAAAGAGCTTCTC
MMP-3[§]	TCTTGCCGGTCAGCTTCATATAT	CCTATGGAAGGTGACTCCATGTG
MMP-9[‡]	TTGGACATGCACGACGTCTT	AAGCGGTCCTGGGAGAAGTAA
MMP-10	CCTGATGTCGGTCACTTCAC	GCATCTCTTGGCAAATCCTG
MMP-13[§]	CTGGAGCTGGGCACCTACTG	ATTTGCCTGAGTCATTATGAACAAGAT
MMP-23[*]	GGCGGCATCCACTTTGAC	CATACGTCTTCTTCCAGCTGTA
MRPS7[*]	CCGTGATCAGTAAATTCACCAACA	TCATGAGGGATCTGGCCAAT
Scleraxis[*]	TCTGCCTCAGCAACCAGAGA	TCCGAATCGCCGTCTTTC
SDHA[λ]	ACAGAGGAATGGTCTGGAATACTGA	GTGAGCACCACGTGACTCCTT
TBP[*]	TGCTGCTGTAATCATGAGGGTAA	TCCCGTGCACACCATTTTC
Tenascin[*]	GGGCGGCCTGGAAATG	CAGGCTCTAACTCCTGGATGATG
TIMP-3	CTGCAACTTCGTGGAGAGGT	GGCAGGTAGTAGCAGGATTTGA
TIMP-4	CCTTTTTGCAGAGAGAAAGTCTG	GCAGTGTGTAGCAGGTGGTG

Table 5-1: Forward and reverse primer sequences. Primers were designed by C.T. Thorpe, except where indicated: [*]Designed by S.E. Taylor; [‡]Designed by E.D. Barr; [§]Designed by P.D. Clegg [α]From: Bogaert *et al.* (2006); [λ]Designed by E. Humphrey.

Primer validation

Primer stocks were made up for each gene of interest by combining forward and reverse primers to give a concentration of 3 μM in 0.1% DEPC water. Complementary DNA was pooled from several samples to give a final volume of 100 μl, which is sufficient to validate 8 primers in a 96 well plate format. The cDNA was diluted 10-fold 5 times from an original 1 in 9 dilution. A master mix was made up for each gene containing 12.5 μl SYBR® green PCR master mix (Applied Biosystems), 2.5 μl primer stock and 5 μl dH$_2$O per well. SYBR® green PCR master mix contains SYBR Green 1 Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and optimized buffer components. A

96 well plate was made up according to the layout in Table 5-2, with 5 µl of the pooled

cDNA solution in each well. All measurements were made in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
B	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
C	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
D	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
E	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
F	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
G	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O
H	10000	10000	1000	1000	100	100	10	10	1	1	dH$_2$O	dH$_2$O

Table 5-2: Plate layout for primer validation. A to H are the primers that were validated (Plate 1: ADAM-8, -12 and -17; ADAMTS-2, -3, -8 and -12; Col-12A1. Plate 2: Col -14A1; MMP-2, -7, -10, -19, and -25; and TIMP-3 and -4). Each concentration of cDNA was measured in duplicate.

C_t values for each cDNA concentration were determined by RT-PCR. An absolute

quantification followed by a dissociation curve was performed using an ABI 7300 HT

system (Applied Biosystems). The cycling conditions were 10 min. polymerase activation

at 95 ºC followed by 40 cycles at 95 ºC for 15 sec. and 60 ºC for 60 sec. The C_t values for

each gene were plotted against the log of the relative concentration of cDNA. If there was

only 1 peak in the dissociation curve (Figure 5-8) and the gradient of the line describing the

relationship between concentration and C_t values was -3.32 ± 0.2 (Figure 5-9), the primer

was specific for the gene and had good amplification efficiency and therefore could be used

to measure expression levels accurately. More than one peak in the dissociation curve

indicates the primer is not gene specific. If these criteria were not met, the primers were re-

designed.
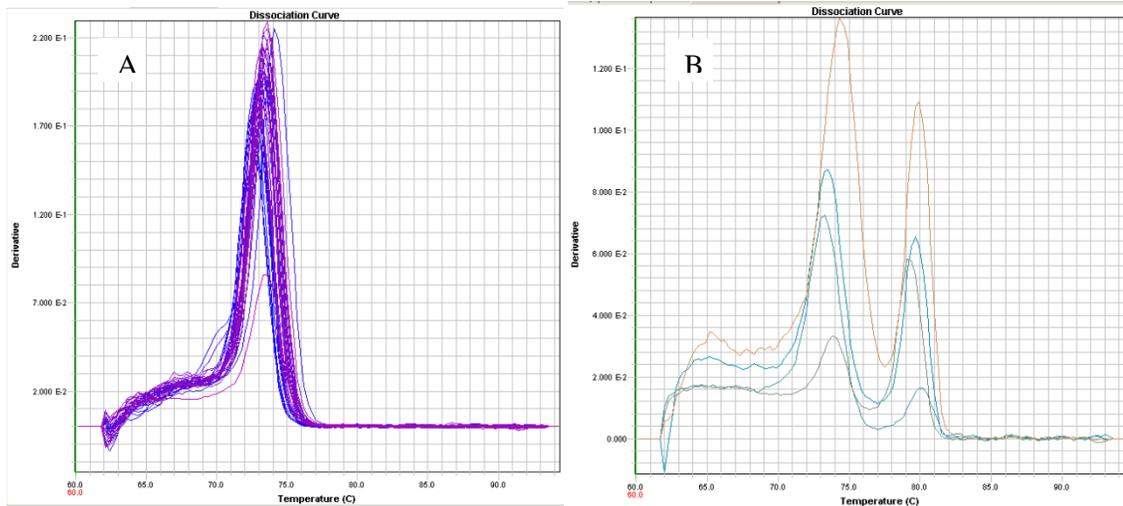
Figure 5-8: Dissociation curves showing primer specificity (A) and a primer that is not specific for a particular gene (B).
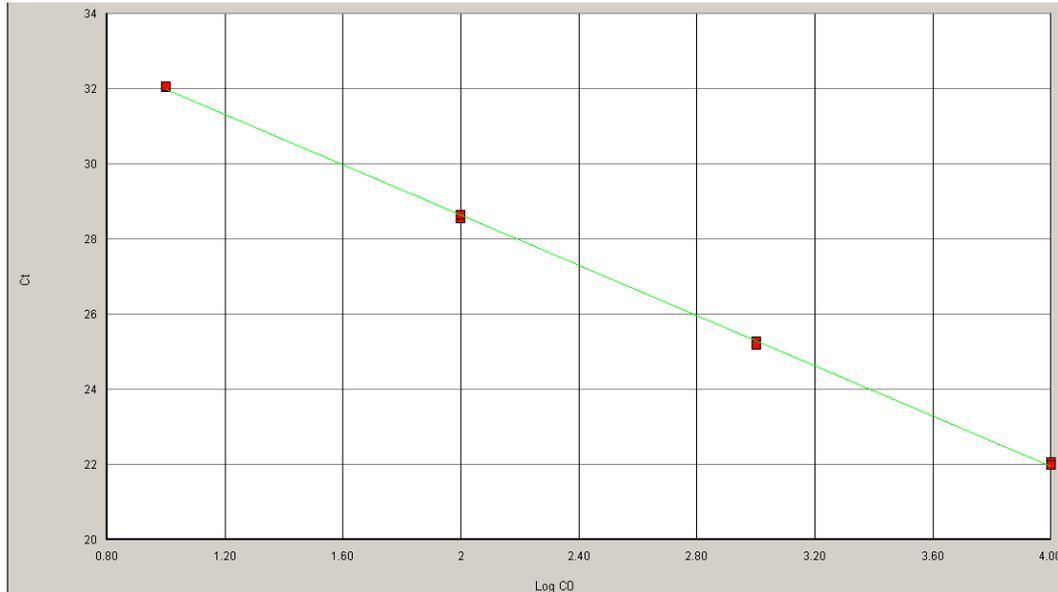
Figure 5-9: Ct values for Collagen XII primers plotted against the log of cDNA concentration. The gradient of the line is -3.34, which shows good amplification efficiency.

5.2.1.5. PCR

Gene expression levels were determined for each sample using an ABI 7900 HT fast real time PCR system in a 384 well plate format (Applied Biosystems). Plates were made up with 4.6 μl cDNA, 5 μl SYBR® master mix, 0.3 μl primers (9.1 mM) and 0.1 μl dH$_2$O per well. Primer sequences used are shown in Table 5-1. These genes were selected as they are either key tendon matrix proteins or their expression has been shown to be altered in tendons with evidence of pathology (Jones *et al.,* 2006). Scleraxis and tenascin expression was also measured as these genes have been proposed as markers of tendon phenotype

134

(Murchison *et al.,* 2007; Taylor *et al.,* 2009). Expression of COMP, tenascin and scleraxis were also assessed in a sample of equine skin and cartilage. All samples were measured in triplicate. The cycling conditions were 10 min. polymerase activation at 95 ºC followed by 40 cycles at 95 ºC for 15 sec. and 60 ºC for 60 sec.; this was followed by a dissociation step which determines if cDNA sequences for any genes other than the gene of interest are being amplified. If there was more than one peak in the dissociation curve the results for the well in question were discarded. The maximum acceptable Ct value was set at 40 cycles, and samples that had Ct values higher than this were re-measured; high Ct values will result in less precise results as the original mRNA concentration is very small.
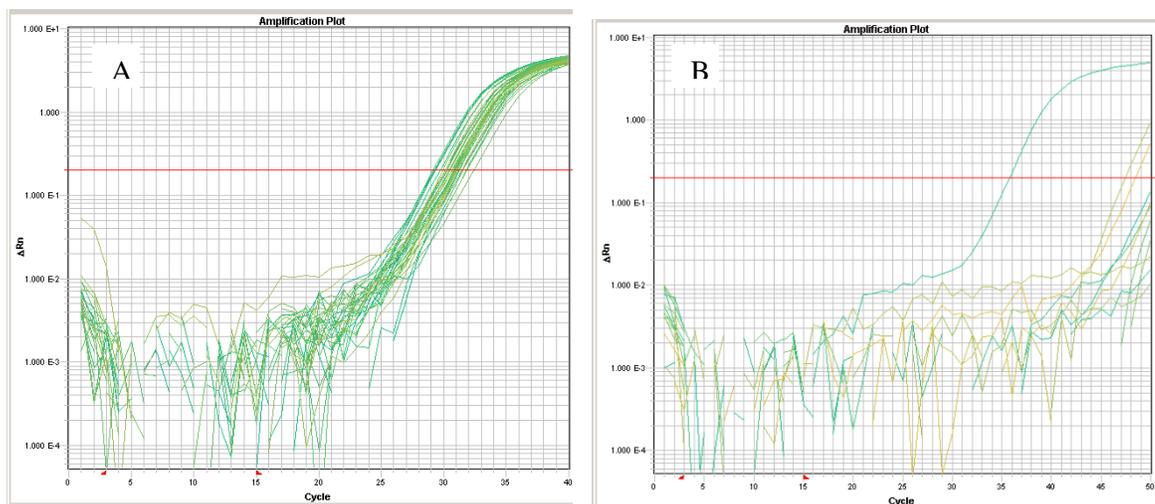
Figure 5-10: Amplification plot showing acceptable (A) and unacceptable (B) Ct values.

5.2.1.6. Calculation of mRNA Expression Ratios

Any wells that contained products with 2 peaks in the dissociation curve were omitted from the calculations. Ct values for each well were generated by the 7900 SDS software (version 2.2, Applied Biosystems). The geometric mean of the Ct values was calculated for the endogenous control genes and for each gene measured. The geometric mean was used rather than the arithmetic mean as it is less affected by outlying values (Vandesompele *et al.,* 2002). The relative differences in gene expression were calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). The Ct values for the endogenous control genes were subtracted from the Ct values for the genes of interest for each sample, and the data were converted to ratios using the equation $2^{-\Delta Ct}$. This expresses the data as a ratio of the control genes and indicates gene expression per cell. In addition the data were multiplied by the DNA concentration of each sample (measured in chapter 4) to show overall gene

expression per mg of tissue. As collagen type I and decorin are the most abundant collagenous and non-collagenous proteins present in tendon, the ratio of Col1A2 to decorin expression was calculated for all samples analysed.

5.2.2. MMP Protein Levels

5.2.2.1. Fluorogenic MMP Assay

A fluorogenic assay was used to assess protein levels of pro- and active forms of MMP-13 in the SDFT and CDET (Birch *et al.,* 2008b). Lyophilised tendon tissue (30 mg) was suspended in 1.2 ml extraction buffer (50 mM HEPES, 200 mM NaCl, 1 mM $CaCl_2$, 0.01% BRIJ-35, pH 7.3) and incubated at 22 °C for 60 min. in a water bath. The samples were vortex mixed several times during incubation to ensure maximal MMP extraction. Samples were centrifuged at 16 000 g for 5 min. and the supernatant was removed and stored at -80 °C. Aliquots (40 µl) of each sample including a blank (extraction buffer minus tissue) were pipetted into a 96 well fluorometer plate in duplicate. 50 µl of assay buffer (50 mM HEPES, 200 mM NaCl, 1 mM $CaCl_2$, 0.01% BRIJ-35, pH 7.3) containing 20 mM 4-aminophenylmercuric acetate (APMA) were added to each well. Plates were incubated at 37 °C for 1 h to allow the APMA to activate the latent enzyme. Fluorescence was also measured without the addition of APMA to determine the amount of active MMP-13 in the samples. A quenched fluorescence substrate for MMP-13 (Calbiochem) was added to each well to give a final concentration of 0.1 mM. This substrate is cleaved preferentially by MMP-13 and is less efficient as a substrate for MMP-1 or MMP-8. Plates were incubated at 37 °C and fluorescence measured after 2 h on a fluorescence reader (Bio-tek Instruments Inc. FLX800 microplate reader) with excitation and emission wavelengths of 325 nm and 393 nm respectively. Duplicate sample readings were averaged and the blank reading subtracted. Activity was expressed as relative fluorescence units per mg tissue.

5.2.2.2. Zymography

Zymography was used to assess MMP protein levels in the SDFT and CDET samples in a semi-quantitative manner. Proteins within each sample were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); the SDS denatures the proteins and gives them a negative charge. The samples are then loaded onto a polyacrylamide gel and a current is passed through the gel, separating the proteins out according to their molecular weight. Gels are co-polymerised with a protein that can be

digested by the MMPs. After electrophoresis, gels are incubated at 37 ºC to allow the MMPs to digest the protein; SDS also activates latent MMPs without the removal of the propeptide, allowing the concentration of pro- and active MMPs to be determined. After incubation the gels are stained in order to visualise the areas that have been digested.

Sample Preparation

Lyophilised tendon tissue from the SDFT and CDET was incubated in 2x non-reducing sample buffer (approximately 15 mg tissue in 600 µl; 125 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue at pH 6.8) at 22 ˚C for 30 min. The samples were centrifuged (16 000 g, 2 min.) and the supernatant was removed and stored at -80 °C.

Preparation of MMP-9 Standard

An MMP-9 standard was prepared using neutrophil cells from equine blood (collected from an equine abattoir). A density gradient was created using 70% and 85% Percoll™ (GE Healthcare, Amersham, UK) in 10x Hanks Balanced Salt Solution (HBSS, Gibco, Paisley, UK) and whole blood (12 ml) was added to the top. After centrifugation (1200 g, 20 min., 20 °C) the neutrophil containing lower middle and upper middle layers were removed to a clean tube and washed in HBSS (1X). The tube was centrifuged (800 g, 8 min, 20 °C) and the supernatant removed, leaving a pellet of neutrophil cells suspended in a small amount of supernatant, of which 3 µl was diluted in 3 ml sample buffer.

Gelatin Zymography

Gelatin zymography was used to assess the pro and active forms of the gelatinases (MMP-2 and -9) in the SDFT and CDET samples. For electrophoresis, 8% acrylamide separating gels containing bovine gelatin type B (1 mg/ml) were used with a 4% stacking gel. Initial experiments were carried out to determine the range in which the MMP-9 standard concentration was linear by measuring the area of digestion bands produced by serially diluting the MMP-9 standard in sample buffer (Figure 5-11). Area of digestion bands increased linearly with increasing concentration up to an area of 8000 units (Figure 5-12); all sample digestion bands had areas smaller than this.
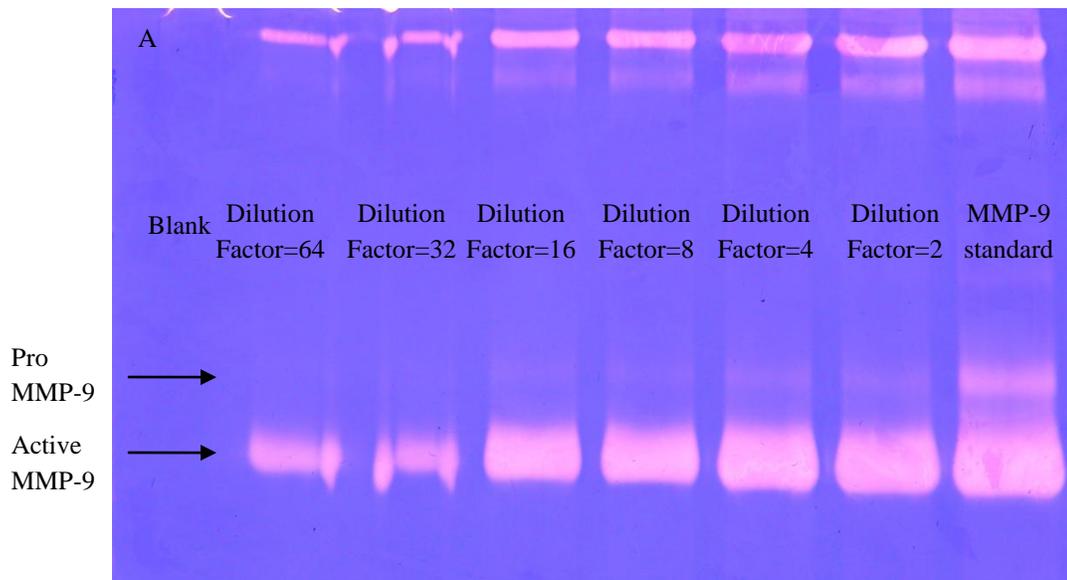
Figure 5-11: Gelatin zymogram showing digestion bands of serially diluted MMP-9 standards prepared from equine neutrophil cells.
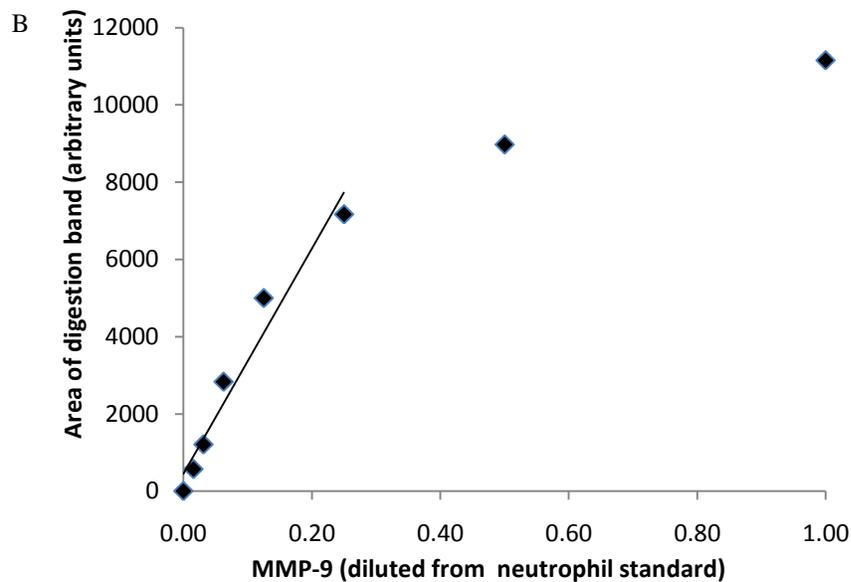
Figure 5-12: Area of digestion bands increased linearly with MMP-9 concentration up to a band area of 8000 (r=0.98).

Sample supernatant (15 µl) was loaded onto the gel, along with the prepared MMP-9 standard (10 µl) and a molecular weight marker (10 µl; Precision Plus Standards, Biorad Laboratories Ltd., Hemel Hempstead, UK). After loading, electrophoresis was performed at 20 mA constant current per gel for 1.5 hours. Gels were washed in 100 ml 2.5% Triton X-100 solution for 1 hour using a shaker. Gels were subsequently rinsed in incubation buffer (50 mM Tris, 0.2% Sodium Azide, 5 mM Calcium chloride, pH 7.6) and then incubated in 100 ml incubation buffer (as above) at 37 °C for 40 hours. Gels were rinsed in D.I. H_2O and

stained for 30 min in 100 ml stain solution (0.5% Coomassie Blue R250, 30% methanol, 10% acetic acid). After staining, the gels were de-stained in 200 ml de-stain solution (10% methanol, 5% acetic acid) for 20 hours in order to visualise the areas where the gelatin had been enzymatically digested. Gels were placed on a light box and photographed from a predefined distance using a Nikon Coolpix camera (5700) set at the highest resolution. Images were analysed using Scions β 4.0.2 image analysis software (Scion Corporation, Maryland, USA) (Figure 5-13). Comparison to the molecular weight marker indicated that pro-MMP-9 had a molecular weight of approximately 100 kDa and active MMP-9 had a molecular weight of approximately 90 kDa. Pro-MMP-2 had a molecular weight of approximately 70 kDa and the active form of the enzyme had a molecular weight of approximately 65 kDa. Digestion bands were expressed per mg of tissue and relative to the MMP-9 standard to allow comparison between gels.
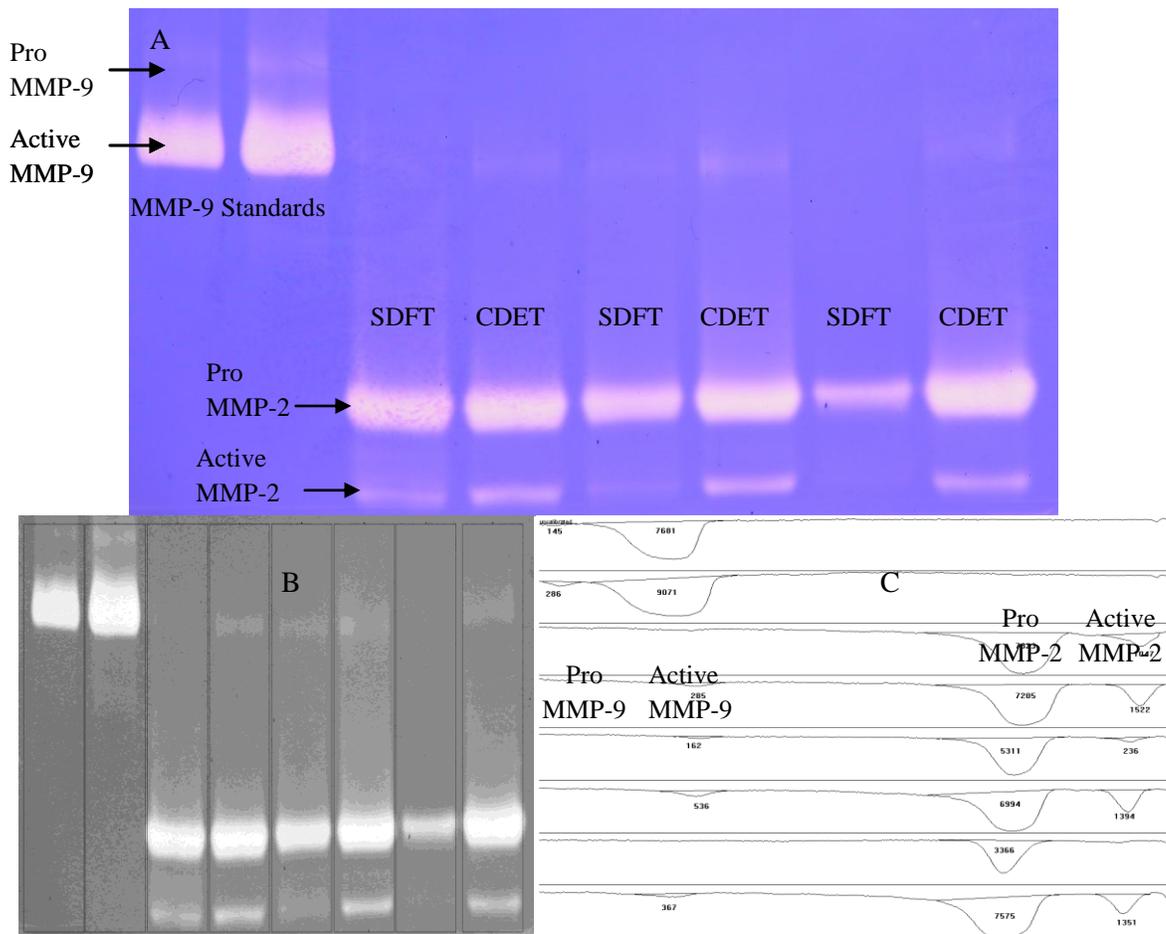
Figure 5-13: Semi-quantification of MMPs extracted from tendon tissue. A – Gelatin zymogram showing standards and pro- and active forms of MMP-2 and -9 in SDFT and CDET samples from three different horses. B – Rectangles mark standard and sample lanes. C – Resulting lane plot from B; the area of each digestion band is calculated and expressed relative to the standards for each gel.

Casein Zymography

Casein zymography was used to assess MMP-3 concentration at the protein level in the SDFT and CDET. Sample supernatant (20 µl) was loaded onto 10% separating gels containing β-casein (1 mg/ml) using a 4% stacking gel. A molecular weight marker (10 µl) was included on each gel. The MMP-9 standard prepared from neutrophils was also run alongside the samples on each gel to act as a negative control. Electrophoresis, gel staining and destaining, and image analysis was performed as for gelatin zymography. To allow comparison of samples separated on different gels, an SDFT sample with a relatively high concentration of MMP-3 was identified and run on every gel, and the activity of MMP-3 in all other samples were expressed relative to this sample (Figure 5-14). Comparison with the molecular weight marker gave an approximate molecular weight of 50 kDa for pro-MMP-3 and 45 kDa for the active form of the enzyme.
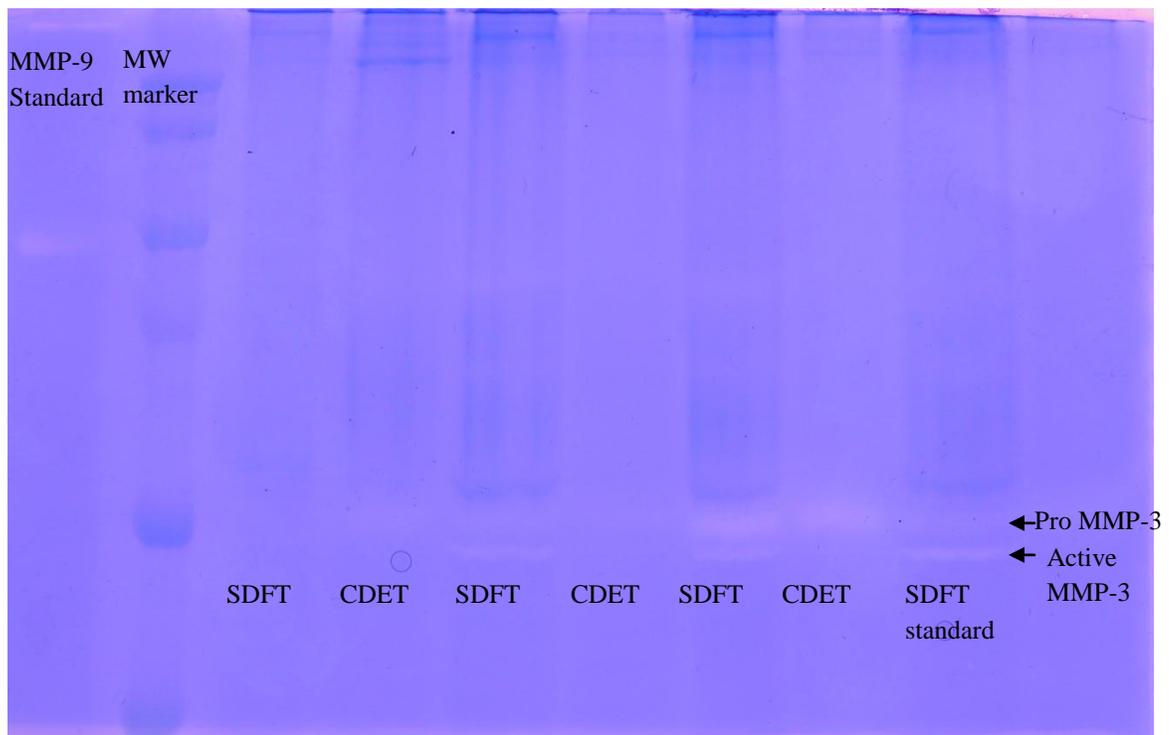
Figure 5-14: Casein zymogram showing pro and active forms of MMP-3 in SDFT and CDET samples from three horses. Areas of digestion bands were quantified as for gelatin zymography (see Figure 5-13).

5.2.3. Statistical Analysis

Data were analysed using linear mixed effects in SPlus (Version 6.1, Insightful). Results were grouped by horse number to account for individual variation and the effects of age and tendon type on mRNA and protein levels were determined. The distribution of the data were tested in Minitab (Version 15) using a Kolmongorov-Smirnoff test for normality. The

gene expression data did not follow the normal distribution and so these data were $\log_{10}$ transformed and re-tested for normality. However, the transformed data did not follow the normal distribution and so the un-transformed data were used for statistical analysis. Correlation analysis was performed in SPSS (Version 14) using parametric (Pearson's product moment correlation) or non-parametric (Spearman's rank correlation) tests depending on whether the data followed the normal distribution. Statistical significance was set at $p < 0.05$. All data are displayed as mean ± SEM.

5.3. Results

5.3.1. Primer Design and Validation

Equine specific primers had not been designed previously for several genes that have been implicated in tendon injury (Jones *et al.,* 2006) and so primers were designed for ADAM-8, -12 and -17; ADAMTS-2, -3, -8 and -12; Col-12A1 and -14A1; MMP-2, -7, -10, -19, -23 and -25; and TIMP-3 and -4. From these, primers for ADAM-12 and -17, ADAMTS-2; Col-12A1; MMP-10 and -23; and TIMP-3 and -4 met the criteria for specificity and efficient amplification and so the other primers were not used to quantify gene expression in the tendon samples. Average gene expression per cell and per tissue weight is shown in Table 5-3.

	SDFT		DDFT		SL		CDET	
	Per cell	**Per mg tissue**	**Per cell**	**Per mg tissue**	**Per cell**	**Per mg tissue**	**Per cell**	**Per mg tissue**
Col1A2	34.93±7.23	19.42±4.30	61.40±10.94	17.99±2.83	29.58±4.81	20.96±4.19	78.46±13.83**	30.88±6.12
Col3A1	18.56±3.93	10.72±2.75	29.57±10.72	8.28±2.59	19.06±4.41	14.65±4.24	14.95±3.16	6.04±1.46
Col5A1	1.49±0.40	0.84±0.22	1.95±0.72	0.58±0.18	0.69±0.15	0.51±0.13*	0.64±0.12	0.27±0.06**
Col12A1	9.51±1.38	5.20±0.79	15.08±3.50	6.26±2.09	15.52±4.30	9.61±2.27	15.64±2.59	6.00±1.07
Aggrecan	5.55±1.81	3.07±0.96	12.80±2.91**	3.57±0.70	3.95±0.80	2.70±0.47	0.21±0.03*	0.08±0.02***
Biglycan	63.27±13.76	35.84±8.44	78.36±16.11	23.49±4.77	75.65±30.15	55.44±23.19	25.00±4.60**	9.35±1.87***
Decorin	1343.42±302.85	780.99±198.98	793.59±67.81	245.38±20.64***	763.11±75.97	527.61±57.03	483.48±53.23***	190.83±25.68***
Fibromodulin	32.80±6.68	18.53±3.90	42.33±10.25	12.46±2.76*	13.28±2.19*	8.83±1.42***	19.89±3.58	7.99±1.58***
Lumican	48.50±7.45	27.62±5.30	73.09±10.48*	22.20±3.13	58.21±8.48	41.10±7.35*	13.06±1.57***	5.19±0.70***
MMP-1	0.03±0.01	0.01±0.01	0.08±0.05	0.04±0.02	0.01±<0.01	0.01±<0.01	0.14±0.03*	0.05±0.01*
MMP-3	3.52±0.83aa	1.89±0.45aa	4.03±1.20	1.14±0.29*	0.70±0.28**	0.57±0.27***	1.39±0.30*aa	0.52±0.12***aa
MMP-9	0.43±0.20	0.23±0.11	0.66±0.19	0.23±0.08	0.84±0.30	0.58±0.21	2.34±0.59***	0.99±0.28*
MMP-10	17.46±4.48	8.61±2.09	14.46±3.98	4.42±1.01*	2.23±0.77***	1.50±0.51***	7.39±1.71**	2.76±0.63***
MMP-13	0.16±0.12	0.07±0.05	0.13±0.05	0.05±0.03	0.03±0.01	0.02±0.01	0.72±0.13***	0.27±0.05***
MMP-23	0.03±0.01	0.02±0.01	0.05±0.03	0.02±0.01	0.03±0.01	0.02±0.01	0.08±0.03	0.04±0.02
TIMP-3	55.08±9.29	31.01±6.19	64.20±10.90	20.34±3.57*	48.69±9.46	33.79±8.52	36.36±5.78*	14.62±2.71**
TIMP-4	0.53±0.15	0.27±0.07	0.54±0.09	0.19±0.05	0.64±0.13	0.46±0.10	0.88±0.24	0.35±0.0
ADAM-12	0.05±0.01	0.03±0.01	0.10±.03	0.03±0.01	0.03±0.01	0.02±0.01	0.05±0.02	0.02±0.01
ADAM-17	1.21±0.22	0.65±0.13	1.13±0.21	0.35±0.07*	0.90±0.18	0.63±0.14	1.66±0.32	0.71±0.16
ADAMTS-2	1.22±0.27	0.64±0.12	1.45±0.37***	0.48±0.13	0.63±0.15	0.44±0.12	1.22±0.40	0.46±0.15
Tenascin	1.86±0.49	1.07±0.29	6.31±1.84***	1.83±0.53*	1.15±0.30	0.85±0.23	1.24±0.28**	0.53±0.13
Scleraxis	3.79±0.99	2.35±0.64	2.05±0.41*	0.61±0.10***	2.51±0.53	1.77±0.37	1.48±0.22	0.60±0.10***
COMP	2674.33±570.18	1516.06±320.36	2171.91±365.16	647.99±96.65**	2111.21±335.53	1463.28±251.1	1495.1±282.1**	577.50±109.10***

Table 5-3: Relative gene expression in equine forelimb tendons per cell and per tissue weight (corrected for DNA content) (mean ± SEM) n = 32. * Indicates significant difference relative to SDFT. *** p>0.001; ** p>0.005; * p>0.05. a indicates significant correlation with age. aaa p>0.001; aa p>0.005; a p>0.05.

5.3.2. Collagen Gene Expression

The genes coding for the various collagens found in tendon were expressed in all tendon samples. As expected, Col1A2 was the most highly expressed of all the collagens in all tendons; expression levels of Col1A2 were on average twofold greater than expression of Col3A1 and fourfold greater than Col12A1 (Figure 5-15). Col5A1 was expressed at the lowest levels in all tendons, expression levels were approximately 20 fold less than expression of Col1A2 (Figure 5-15). Expression of Col1A2 was significantly higher in the CDET than in the SDFT per cell (p=0.002) (Figure 5-16), but due to a lower cellularity in the CDET there was no significant difference per tissue weight (p=0.053). Expression of Col3A1 and Col12A1 did not differ between tendons, but expression of Col5A1 was significantly lower in the CDET (p=0.001) and in the SL (p=0.049) per tissue weight than in the SDFT (Figure 5-15). Collagen gene expression was not correlated with horse age in any tendon.
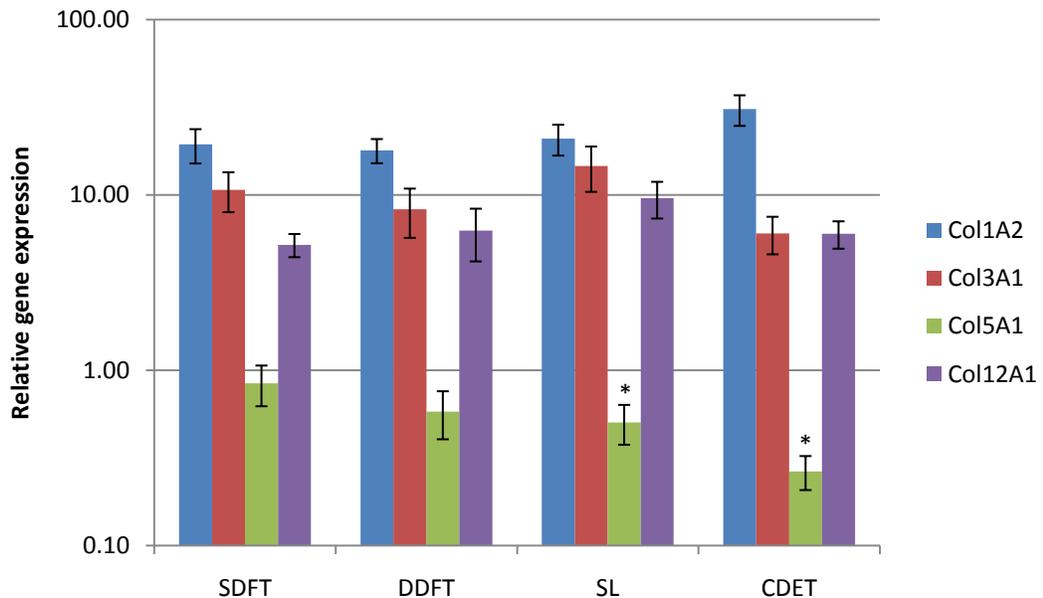
Figure 5-15: Relative expression of genes coding for collagen in equine tendon corrected for tendon DNA content (mean ± SEM) n = 32. *indicates significant difference to the SDFT. Data are displayed on a Log_{10} scale.
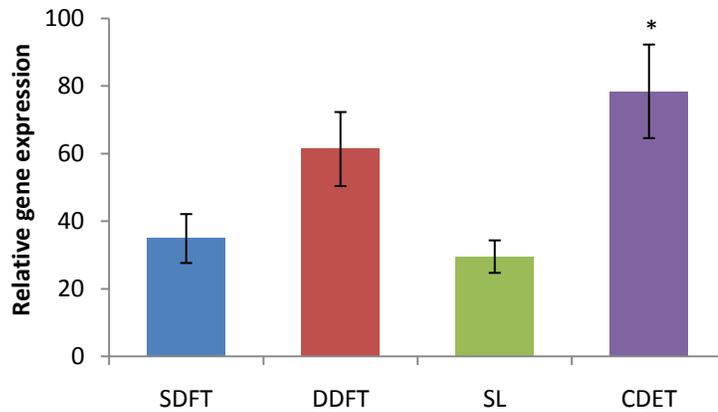
Figure 5-16: Relative expression of COL1A2 per cell (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

5.3.3. Proteoglycan Gene Expression

Expression of decorin was greater than expression of other proteoglycans in all tendons (Figure 5-17). Expression of the proteoglycans aggrecan and biglycan was significantly higher in the SDFT than in the CDET per tissue weight (p≤0.0004). Expression of decorin was greater in the SDFT than in the DDFT and CDET (p<0.0001), and fibromodulin expression was greater in the SDFT than in the other tendons (p≤0.03). Lumican expression was significantly greater in the SDFT than in the CDET (p=0.0002), and significantly greater in the SL when compared to the SDFT (p=0.02). Proteoglycan gene expression was not correlated with horse age in any tendon.
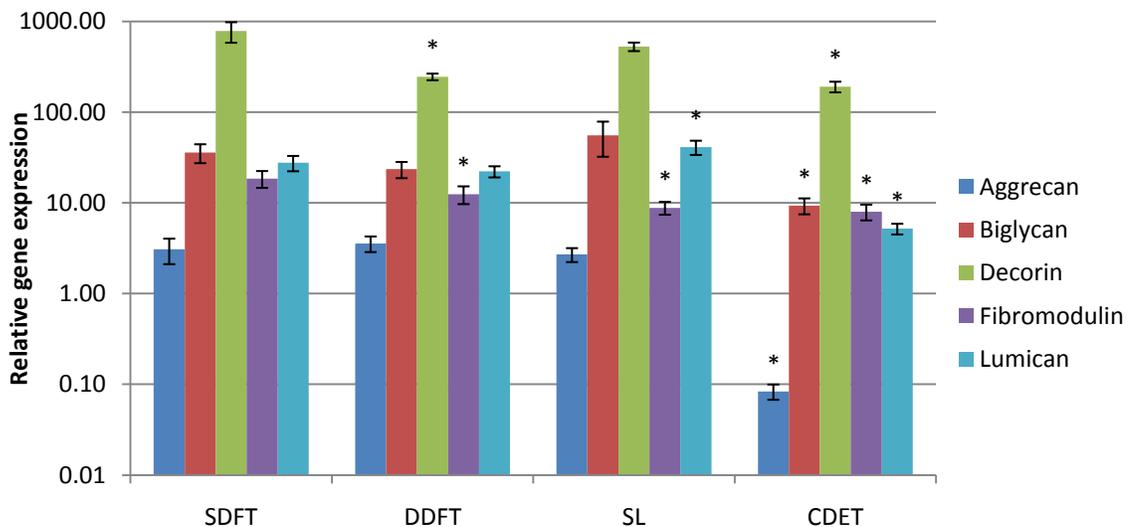
Figure 5-17: Relative expression of genes coding for proteoglycans in tendon corrected for DNA content (mean ± SEM) n = 32. * Indicates significant difference compared to SDFT. Data are plotted on a Log_{10} scale.

5.3.4. Ratio of Collagen Type I to Decorin Expression

The ratio of Col1A2 to decorin expression was calculated for each tendon; this ratio was significantly greater in the DDFT and CDET than in the SDFT ($p \leq 0.017$) (Figure 5-18). The ratio did not alter with increasing horse age in any tendon.
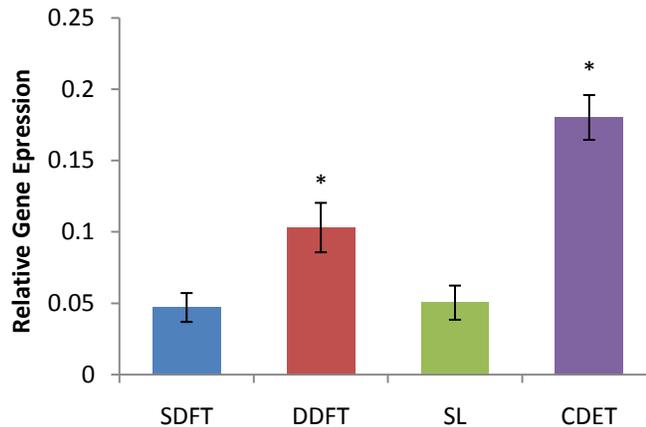
Figure 5-18: Ratio of Col1A2 to decorin expression in the forelimb tendons (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

5.3.5. Matrix Degrading Enzyme Gene Expression

Collagenase gene expression (MMP-1 and -13) was significantly higher in the CDET than in the SDFT both per cell ($p \leq 0.0049$) and per tissue weight ($p \leq 0.0281$) (Figure 5-19), and was not correlated with horse age in any tendon. Gelatinase expression (MMP-9) was also significantly higher in the CDET per cell ($p=0.0009$) and per tissue weight ($p=0.0124$) when compared to the SDFT (Figure 5-19) and showed no significant correlation with horse age in any tendon.
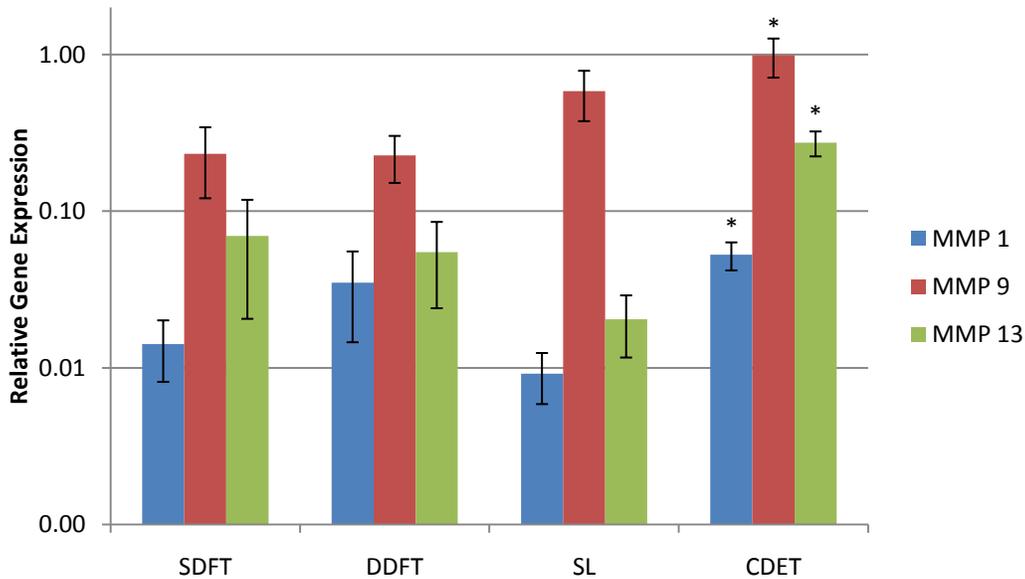
Figure 5-19: Relative gene expression of collagenases (MMP-1 & -13) and gelatinase (MMP-9) in equine tendon corrected for tendon DNA content (mean ± SEM) n = 32. * Indicates significant difference relative to SDFT. Data are plotted on a Log_{10} scale.

Stromelysin gene expression (MMP-3 and -10) was significantly higher in the SDFT than in the other tendons per tissue weight ($p \leq 0.03$) (Figure 5-20). There was no difference in MMP-23 expression between tendons.
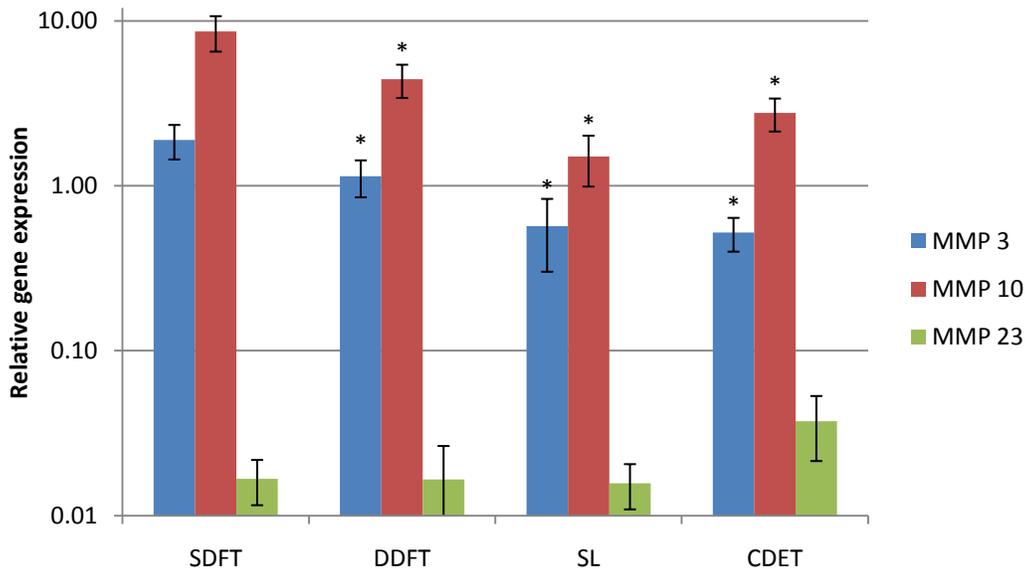
Figure 5-20: Relative gene expression of stromelysins (MMP-3 & -10) and MMP-23 corrected for tendon DNA content (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT. Data are plotted on a Log_{10} scale.

146

MMP-10 expression increased significantly with horse age in the SDFT and CDET (p≤0.005) (Figure 5-21), whereas MMP-3 showed a trend towards increased expression with horse age in the SDFT (r=0.3) but this was not significant (p=0.06).
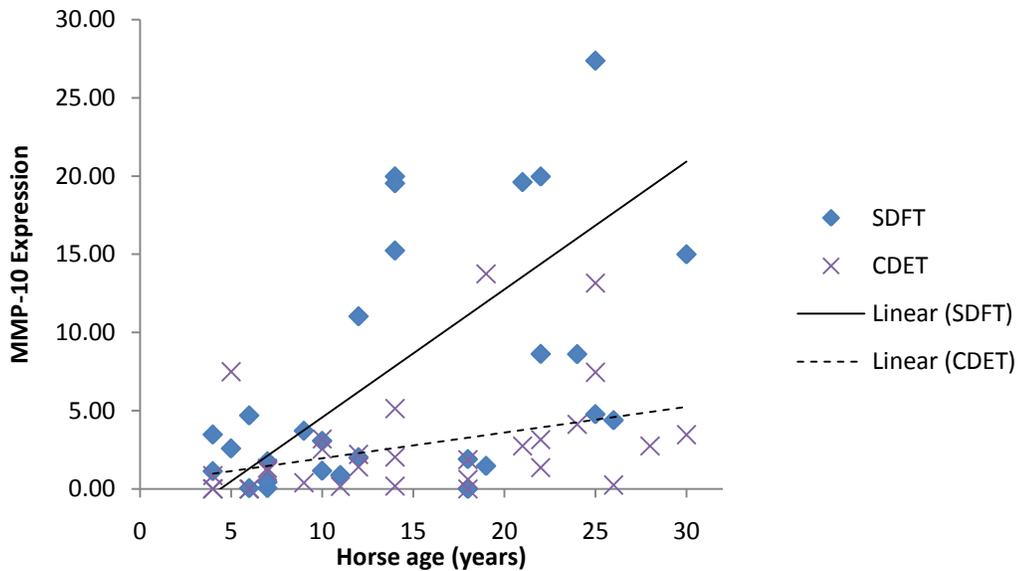
Figure 5-21: MMP-10 gene expression in the SDFT and CDET as a function of horse age. MMP-10 increased significantly with age in both the SDFT (n=32, r=0.531, p=0.003) and in the CDET (n=32, r=0.496, p=0.005).

ADAM-17 was expressed at significantly lower levels in the DDFT than in the SDFT (p=0.04); and expression of ADAM-12 and ADAMTS-2 did not differ between tendons (Figure 5-22). Expression of these enzymes did not show a correlation with age in any tendon.
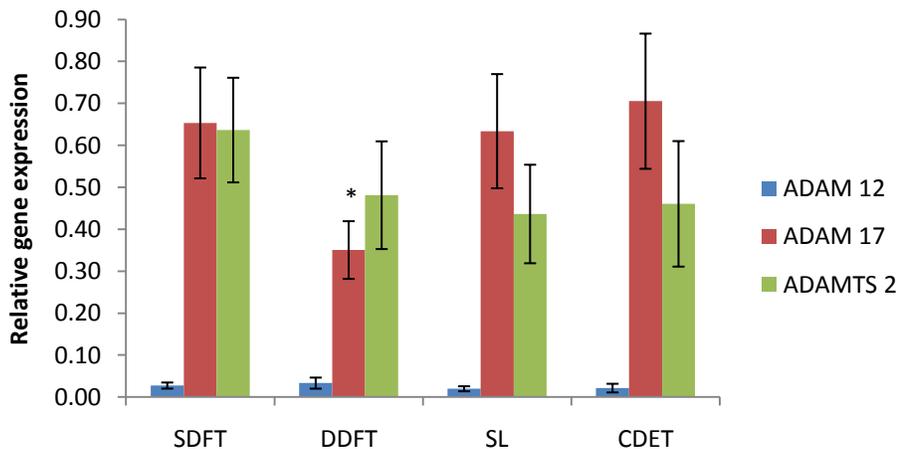
Figure 5-22: Gene expression of ADAM-12 & -17, and ADAMTS-2 in equine tendon corrected for DNA content (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

TIMP-3 expression was significantly higher in the SDFT than in the DDFT and CDET (p≤0.04) (Figure 5-23), and TIMP-4 expression did not differ between tendons. TIMP-3 & -4 expression was not correlated with horse age in any tendon.
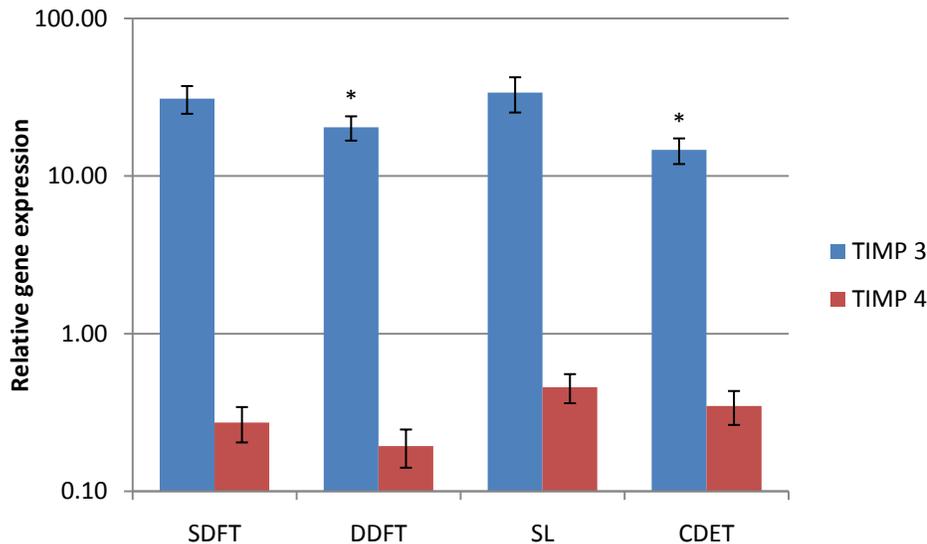
Figure 5-23: TIMP-3 and -4 expression in equine tendons (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT. Data are displayed on a Log_{10} scale.

5.3.6. Expression of Other Genes Associated with Tendon Matrix

COMP was the most highly expressed gene in all tendons, and was expressed at greater levels in the SDFT and SL than in the DDFT and CDET (p≤0.002) (Figure 5-24). COMP expression did not change with horse age in any tendon.
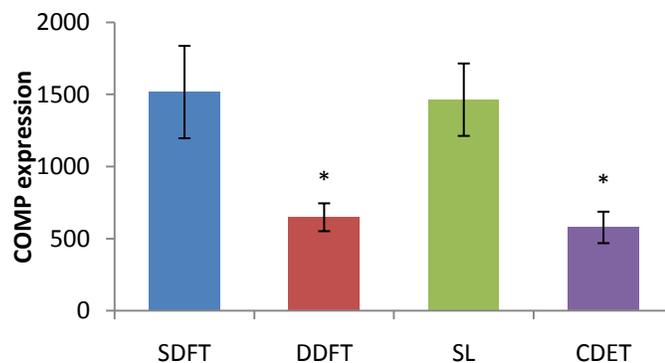
Figure 5-24: COMP expression in equine tendon corrected for tendon DNA content (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

Scleraxis also showed greater expression in the SDFT than in the DDFT and CDET (p=0.0009) and was significantly positively correlated with Col1A2 expression in all tendons (r≥0.55, p<0.0001). Tenascin expression did not differ between tendons (Figure 5-25). Expression of scleraxis and tenascin was not correlated with horse age in any of the tendons.
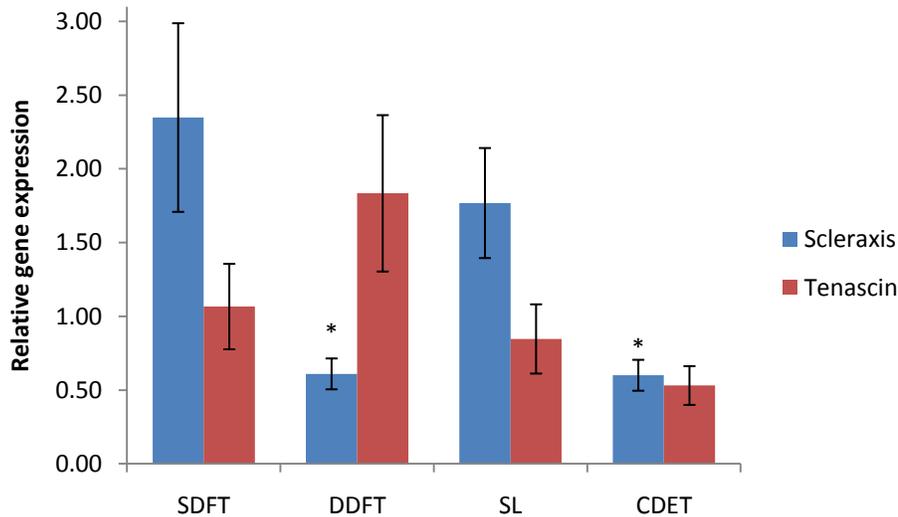
Figure 5-25: Expression of scleraxis and tenascin in equine tendon corrected for DNA content (mean ± SEM) n = 32. * Indicates difference relative to the SDFT.

Expression of COMP was approximately 20 000 times lower in equine skin than in tendon, and scleraxis expression was 20 times less. Expression of tenascin was also lower in skin than in tendon (approximately 3 times lower).

5.3.7. Comparison of Reference Genes

There was no significant difference in expression ratio between different tendons of any of the genes when calculated relative to GapDH or MRPS7 and HIRP5 (Figure 5-26), although absolute values did differ as GapDH is expressed at overall greater levels than MRPS7 and HIRP5 (approximately 30 fold greater expression of GapDH).
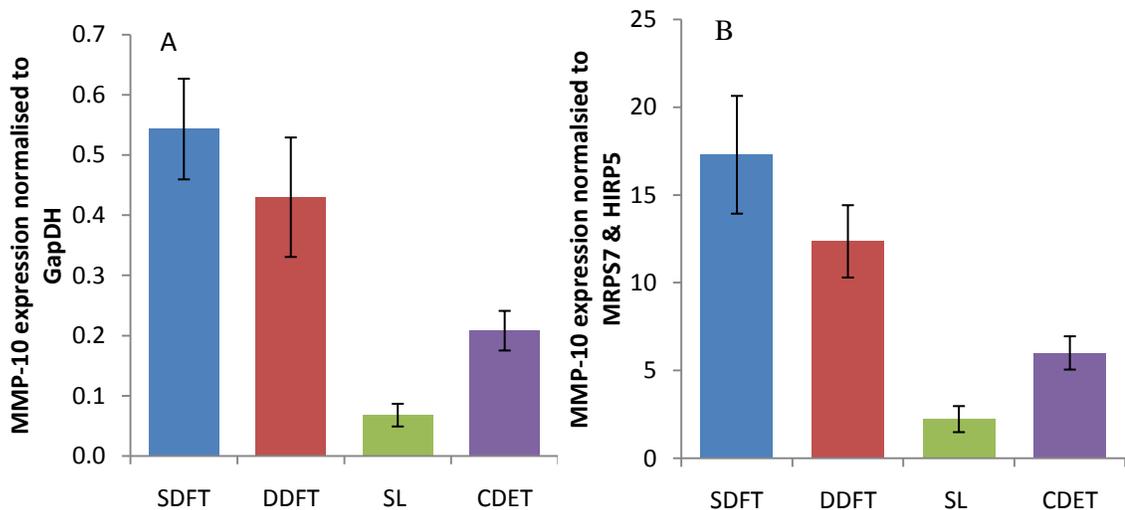
Figure 5-26: A - Expression of MMP-10 normalised to GapDH expression; B - MMP-10 expression normalised to expression levels of MRPS7 and HIRP5. Note the almost identical expression profiles when using different endogenous control genes.

5.3.8. MMP Protein Levels

Levels of the collagenase MMP-13 were assessed in the SDFT and CDET using a fluorogenic assay; MMP-13 protein levels are shown in Table 5-4. MMP-13 was detected only in the active form in both the SDFT and CDET, and was present at greater concentrations in the CDET relative to the SDFT (p=0.001) (Figure 5-27). MMP-13 concentration was not affected by increasing horse age in either tendon. There was no correlation between MMP-13 mRNA levels and MMP-13 protein concentration.

	SDFT	CDET
Pro-MMP-13 (RFU/mg tissue)	0	0
Active MMP-13 (RFU/mg tissue)	45.46±5.66	65.80±4.62[***]

Table 5-4: MMP-13 protein levels in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT. *** p<0.001. RFU; Relative fluorescence units.
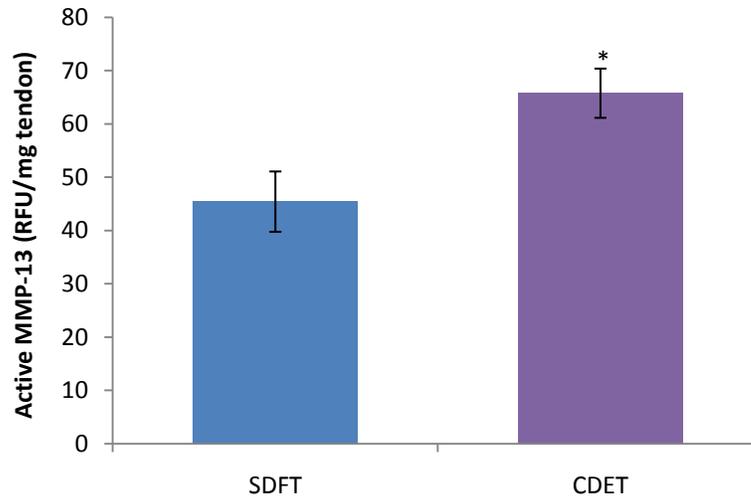
Figure 5-27: Concentration of active MMP-13 in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

The gelatinases MMP-2 and -9 were detected in all tendon samples; protein levels are shown in Table 5-5. MMP-9 was present entirely in the active form whereas MMP-2 was detected mainly in the latent form, although small amounts of the active form were also present in most tendon samples (Figure 5-28). MMP-2 (both the pro and active forms) and MMP-9 were detected in higher concentrations in the SDFT than in the CDET ($p \leq 0.006$). Neither MMP-2 nor -9 were correlated with age in either tendon. There was also no correlation between MMP-9 mRNA and protein levels.

	SDFT	CDET
Pro-MMP-2 (Units/mg)	6.53±0.32	5.46±0.38[*]
Active MMP-2 (Units/mg)	1.13±0.13	0.35±0.064[***]
Pro-MMP-9 (Units/mg)	0	0
Active MMP-9 (Units/mg)	0.43±0.066	0.13±0.033[***]

Table 5-5: Protein levels of MMP-2 and -9 in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT. * $p<0.05$; ** $p<0.005$; *** $p<0.001$.
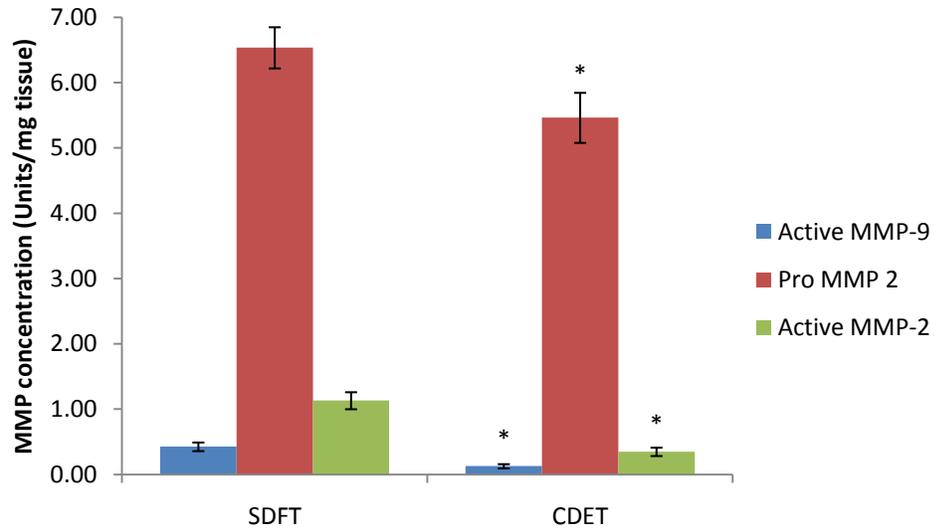
Figure 5-28: Concentration of the gelatinases MMP-2 and -9 in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.

Casein zymography showed that MMP-3 was present mainly in the latent form with small amounts of active enzyme present in some tendon samples. MMP-3 levels are shown in Table 5-6. Both the pro and active forms were present in higher concentrations in the SDFT than in the CDET (p≤0.04) (Figure 5-29).

	SDFT	CDET
Pro-MMP-3 (Units/mg)	5.17±0.57	3.34±0.54[*]
Active MMP-3 (Units/mg)	0.93±0.23	0.11±0.093[***]

Table 5-6: MMP-3 protein levels in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT. * p<0.05; **p<0.005; ***p<0.001.
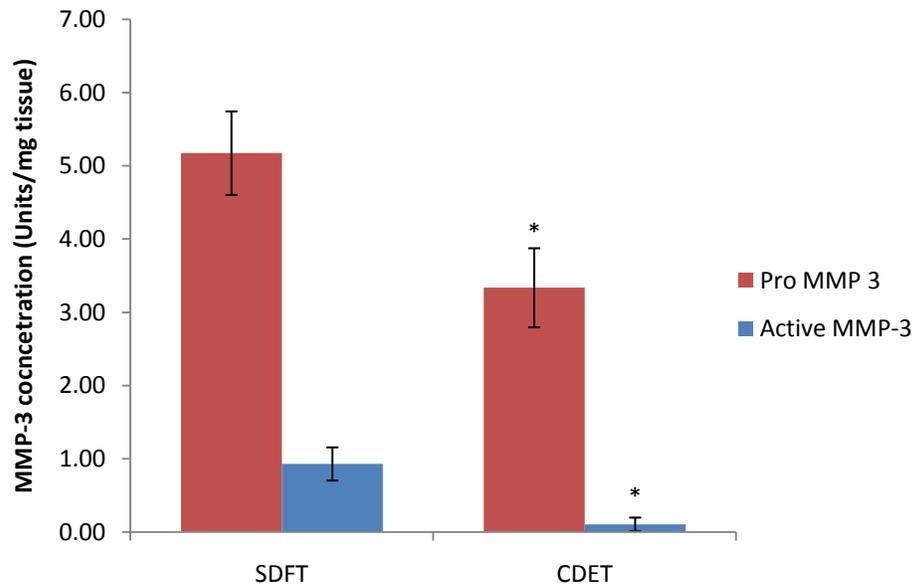
Figure 5-29: Concentration of MMP-3 in the SDFT and CDET (mean ± SEM) n = 32. * Indicates a significant difference relative to the SDFT.

Pro MMP-3 concentration increased significantly with age in the SDFT (p<0.0001) but was not correlated with age in the CDET. Active MMP-3 concentration was not significantly associated with age in the CDET but showed a significant increase with age in the SDFT (p=0.02) (Figure 5-30). Correspondingly, total MMP-3 concentration was significantly greater in the SDFT than in the CDET (p=0.003) and showed a significant positive correlation with age in the SDFT (p<0.0001) but not in the CDET (Figure 5-31).
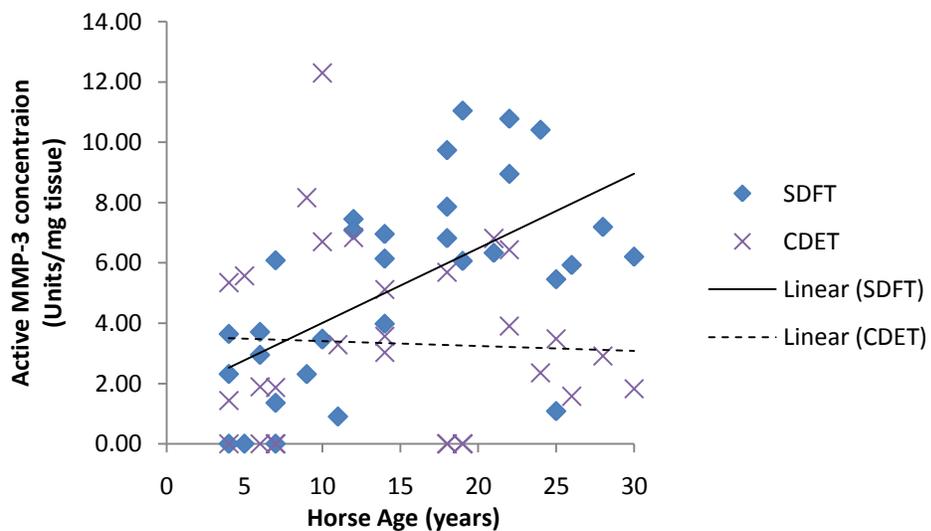
Figure 5-30: Concentration of active MMP-3 as a function of horse age in the SDFT and CDET. Active MMP-3 concentration increased significantly with age in the SDFT (p<0.0001, r=0.6) and was not correlated with age in the CDET.

153
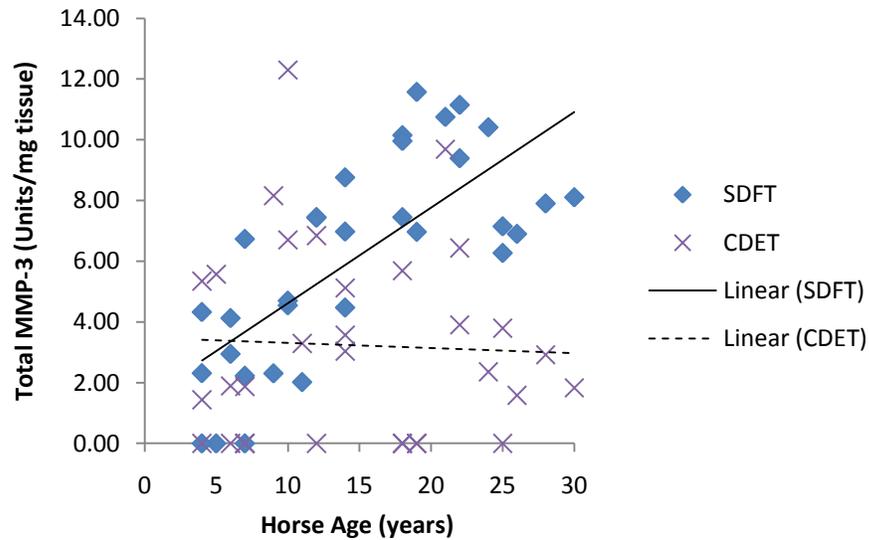
Figure 5-31: Total MMP-3 concentration in the SDFT and CDET as a function of horse age. Total MMP-3 concentration was significantly greater in the SDFT than in the CDET (p=0.003) and increased significantly with age in the SDFT (p<0.0001, r=0.8) but showed no relationship with age in the CDET.

Total and active MMP-3 protein levels showed a weak but significant positive correlation with MMP-3 mRNA levels in the SDFT (p≤0.04) but not in the CDET (Figure 5-32).
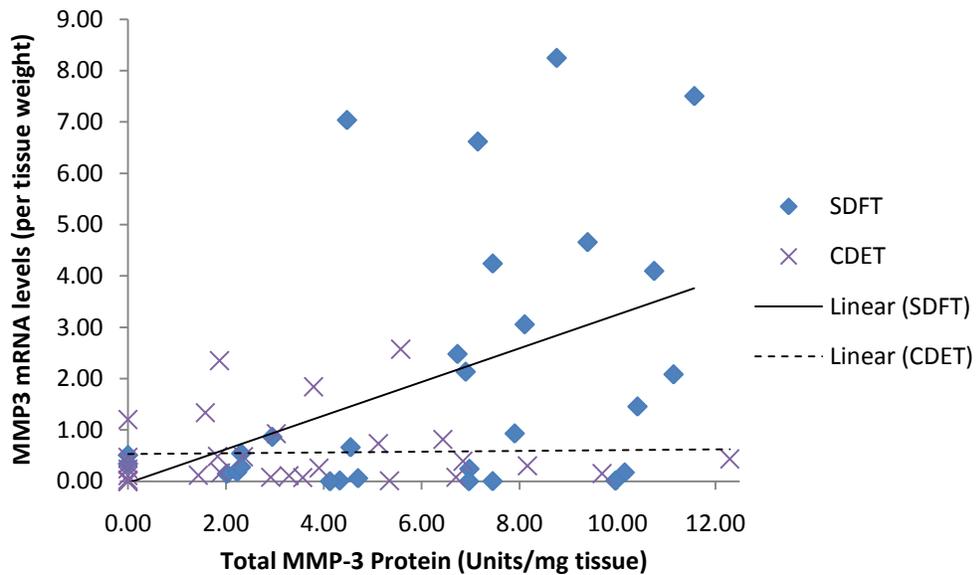
Figure 5-32: Total MMP-3 protein levels plotted against MMP-3 mRNA levels. Total protein increased concomitantly with MMP-3 mRNA levels in the SDFT (p=0.04, r=0.4) but there was no relationship between MMP-3 protein and mRNA levels in the CDET.

It was not possible to determine which of the MMPs assessed at the protein level were present in the highest concentration as the measured activities were relative not absolute

activities which allowed comparison between tendon samples but not between different MMPs.

5.4. Discussion

As hypothesised, the results of this work show a different pattern of gene expression by cells from the functionally distinct tendons in the equine forelimb. In addition, differences in the levels of matrix degrading enzymes at both the gene and protein level support the earlier work and indicate a difference in the rate of turnover between the different tendons. However, few alterations in the expression levels of any gene were identified with increasing horse age.

5.4.1. Normalising Gene Expression

Normalising gene expression has inherent problems, usually relating to the selection of appropriate control genes. Many studies select reference genes without determining if their expression is constant. This may generate misleading results as the expression of control genes may alter with disease, between tissues or with increasing age. Appropriate control genes can be selected by determining which genes are most stably expressed in the tissue or condition of interest (Maccoux *et al.,* 2007; Vandesompele *et al.,* 2002). GeNorm was used to identify the two most stably expressed control genes in mature equine tendon (MRPS7 and HIRP5); all data were expressed relative to these genes to account for differences in starting amount of mRNA. As previous work (Birch *et al.,* 2008b) has normalised data to GapDH, in some samples data were also normalised to this gene. This did not result in significant alterations in expression levels suggesting that GapDH is also suitable as a reference gene in equine tendon. To ensure reference genes did not show altered expression with age cDNA from horses aged 4, 11, 12 and 24 were used to assess the candidate reference genes. MRPS7 and HIRP5 have been found to be the most stably expressed genes in several tissues and disease states (Clements *et al.,* 2009; Maccoux *et al.,* 2007). HIRP5 interacts with HIRA proteins, which bind to histones in the nucleus and are involved in DNA packaging (Lorain *et al.,* 2001). MRPS7 is involved in protein synthesis within the mitochondria and it is thought to be a factor that initiates mitochondrial translation (Cavdar *et al.,* 1999). MRPS have been used previously as reference genes as their expression does not alter significantly with age or disease (Maccoux *et al.,* 2007; Szabo *et al.,* 2004).

5.4.2. Differences in Potential for Matrix Turnover between Tendons

The gene expression data show that cell phenotype differs between functionally distinct tendons; per cell more message for collagen type I production is expressed in the CDET tenocytes than by SDFT cells. This supports previous findings (Birch *et al.,* 2008b) and the data presented in chapter 4, which indicates an 'older' collagenous matrix in the SDFT than in the CDET. The capacity to synthesise collagen depends on tendon cell number in addition to the amount of collagen synthesised per cell. Collagen content is similar between the SDFT and CDET but cellularity is significantly greater in the SDFT (see chapter 4). When this is taken into account, the difference in the potential for collagen type I synthesis is no longer significant between the SDFT and the CDET, although there is a trend towards greater expression in the CDET (p=0.053). However, the significantly higher expression of collagenases (MMP-1 and -13) in the CDET relative to the SDFT suggests the cells in the CDET are more able to degrade collagen. This is supported by the higher concentration of MMP-13 at the protein level in the CDET than in the SDFT. Expression of Col1A2 was only two to fourfold greater than Col3A1 and Col12A1 expression in all tendons; however the heterotrimeric nature of type I collagen means that expression of Col1A2 accounts for one third of the total collagen type I gene expression. In contrast, both collagen type III and XII are homotrimers and therefore are only coded for by a single gene; when this is taken into account levels of collagen type I expression are approximately twelve times greater than collagen type III and XII expression. This reflects the percentage of these minor collagens present within tendon.

The pattern of gene expression in the DDFT and SL suggests that the cells from these tendons exhibit phenotypes that are intermediate between the SDFT and the CDET. This is likely to be related to the functions of these tendons and the strains they experience during galloping exercise. The strain experienced by the SL during high speed locomotion has not been assessed, but it can be assumed that it is not exposed to the extremely high strains experienced by the SDFT; although overstrain injury does occur to the SL the incidence of injury is approximately 13 fold greater in the SDFT in racehorses (Ely *et al.,* 2004). In a similar manner, although the DDFT and CDET do not act as energy stores, the DDFT is likely to experience higher strains than the CDET as it situated on the palmar aspect of the forelimb and will therefore undergo deformation when the metacarpophalangeal joint is hyper-extended during stance phase. This provides further evidence to suggest that gene

expression in the equine forelimb tendons is regulated by the amount of strain each tendon experiences *in vivo*. However, it is not known if this phenotype is pre-set or if the cells are able to alter their phenotype according to the strains they experience.

The matrix half-life data (chapter 4) show that in both energy storing and positional tendons the non-collagenous matrix proteins are turned over more rapidly than the collagenous fraction of the matrix. This is supported by the gene expression data which show greater expression of genes coding for the predominant proteoglycans in tendon than those that code for collagen. Furthermore, non-collagenous matrix half-life in the SDFT is lower than in the CDET, in support of this the gene expression data show the potential for proteoglycan synthesis and degradation is greatest in the SDFT. This would be expected as the levels of proteoglycan as determined from sulphated GAG content are higher in the SDFT than the CDET (see chapter 4). The greater potential for proteoglycan degradation is also apparent at the protein level, with higher levels of the stromelysin MMP-3 in the SDFT than in the CDET. This is likely to be of functional significance; proteoglycans are thought to be involved in strain transfer between collagen fibrils (Scott 2003), and so may be at higher risk of damage in the high strain SDFT than in the low strain CDET, especially as it has been shown that they play a greater role in strain transfer at higher strains (Puxkandl *et al.,* 2002).

5.4.3. Function of Proteoglycans in Functionally Distinct Tendons

It is well established that the small leucine rich proteoglycans (SLRPs) are involved in the regulation of collagen fibril formation (Banos *et al.,* 2008), but more recently it has been proposed they contribute significantly to the transfer of strain between collagen fibrils in mature tendon (Scott 2003). In agreement with previous studies (Ilic *et al.,* 2005; Rees *et al.,* 2000; Vogel and Meyers 1999), decorin was the most abundantly expressed SLRP in all tendons. Decorin is likely to have several functions within tendon; it inhibits the lateral fusion of fibrils, resulting in the formation of thinner fibrils (Birk *et al.,* 1995). Fibril mass average diameter is lower in the SDFT than in the CDET (Birch 2007); fibril size may impact on strain transfer between fibrils as fibrils with a smaller diameter have a greater relative surface area and therefore be able to form a greater number of intermolecular crosslinks. The decorin binding site on type I collagen molecules is close to the predominant intermolecular cross-linking site (Keene *et al.,* 2000), therefore the greater

levels of decorin in the SDFT may influence enzymatic crosslink formation. Decorin has also been implicated directly in the transfer of strain between collagen fibrils by interacting with other decorin molecules bound to adjacent collagen fibrils via its GAG side chain (Gupta *et al.,* 2010; Scott 2003). However, the precise mechanism of strain transfer is unclear and proteoglycan removal results in conflicting data; one study showed that proteoglycan removal did not have a significant effect on tendon mechanical properties (Fessel and Snedeker 2009), whereas others have found a decrease in tendon strain in certain tendon regions (Rigozzi *et al.,* 2009). This suggests that tendon response to strain is complex and heterogeneous throughout the tendon. Proteoglycans are proposed to provide the viscous properties to tendon, which is viscoelastic in nature. Fibre sliding, which is thought to be controlled by collagen-proteoglycan interactions, has been shown to be the major mechanism of tendon extension at high strains (Screen *et al.,* 2004). In support of this, tendons from decorin-null mice have decreased strain rate sensitivity (Robinson *et al.,* 2004b) and increased rate of strain relaxation (Elliott *et al.,* 2003). Therefore proteoglycans may play a more important role in the high strain SDFT, than in low strain tendons such as the CDET.

The second most abundant SLRP in tendon, biglycan also regulates fibrillogenesis; it is similar in structure and thought to have complementary functions to decorin (Banos *et al.,* 2008; Zhang *et al.,* 2005), suggesting that biglycan may also be involved in regulating lateral fusion of fibrils. Lumican and fibromodulin are able to regulate fibrillogenesis by inhibiting fibril fusion (Hedbom and Heinegard 1989) but it is not known if they have additional functions in mature tendon. Aggrecan is found at relatively low concentrations in the tensile region of tendon; in this study aggrecan expression was significantly greater in the SDFT than in the CDET. One of the main functions of aggrecan is to attract water into the matrix (Benjamin and Ralphs 1998); this may account for the higher water content in the SDFT (see chapter 4). It is well established that the SLRPs regulate collagen fibrillogenesis during embryogenesis and development. However, the half-life data presented in chapter 4 indicate that collagen turnover in tendon occurs relatively slowly, especially in the high strain SDFT, and therefore the rate of fibrillogenesis in mature tendon is likely to be low. The main function of proteoglycan in mature tendon may therefore be to transfer strain between fibrils. The data show that the potential for proteoglycan turnover is greater in the high strain energy storing SDFT than in the low strain positional CDET. This

supports the conclusions drawn in chapter 4 suggesting that proteoglycans in the SDFT are more likely to be damaged and require repair due to the levels and rates of strain the SDFT experiences.

5.4.4. Regulation of Matrix Turnover in Functionally Distinct Tendons

In order to be able to respond appropriately to mechanical and chemical signals the rate of tendon matrix turnover is tightly controlled by the tenocytes. Control of protein synthesis, including synthesis of degradative enzymes occurs at the transcriptional, translational and post-translational levels (see chapter 1); therefore it cannot be assumed that if mRNA is expressed for a protein that protein will necessarily be synthesised and correctly modified to have a functional role in the matrix. Collagen type I is synthesised from two mRNA sequences to form procollagen chains, which undergo hydroxylation and folding to form a triple helix (Bellamy and Bornstein 1971). The N- and C-terminal pro-peptides are then removed before it is incorporated into fibrils (Birk *et al.,* 1995; Kadler *et al.,* 1996). It is likely that in tendon some of the mRNA coding for collagen never undergoes translation, in addition some pro-collagen molecules may be degraded instead of being incorporated into the matrix. It is therefore important to assess synthesis of proteins within tendon at as many of these levels as possible in order to determine the rate of matrix turnover accurately.

As well as depending on the successful transcription, translation and activation of specific enzymes, matrix degradation is further limited by the hierarchical structure of tendon and the presence of enzyme inhibitors. The enzymes assessed in this chapter show there are differences between the mRNA and protein levels, and the pro- and active forms that are specific to each enzyme. There was no correlation between the mRNA and protein levels of the gelatinase MMP-9 and the collagenase MMP-13, while there was a weak correlation between mRNA and protein levels of the stromelysin MMP-3 in the SDFT but not in the CDET. Although MMP-9 mRNA levels were greater in the CDET than in the SDFT, protein levels of MMP-9 were greater in the SDFT. MMP-2 and -9 are able to degrade denatured collagen and so the higher levels of these enzymes in the SDFT suggest a greater potential for degradation of damaged collagen in this tendon. At the protein level MMP-13 and MMP-9 were only detected in the active form, suggesting they are activated shortly after synthesis. MMP-2 and -3 were present mainly in the latent form, with small amounts of active enzyme in the matrix. ProMMP-2 can only be activated by membrane bound

MMPs (Visse and Nagase 2003). Cell density is relatively low in the CDET, this may therefore explain the lower levels of active MMP-2 in this tendon. These data give an indication of the complexity of the regulation of matrix degrading enzyme activity within tendon and also suggest that control of enzyme activation is specific to each MMP, allowing the degradation of different fractions of the matrix to be tightly controlled. Although MMP-1 and -13 are the main collagen degrading enzymes, the stromelysin MMP-3 is likely to play an important role in tendon matrix degradation; as well as degrading a variety of matrix proteins, it is also involved in the activation of collagenases and gelatinases (Olson *et al.,* 2000; Suzuki *et al.,* 1990) and is thought to assist in collagen degradation (Sweeney *et al.,* 2008). Levels of collagenases were significantly higher in the CDET than in the SDFT, but mRNA and protein levels of MMP-3 were greater in the SDFT, which may result in comparable rates of collagen degradation between these tendons. TIMP-3, the predominant enzyme inhibitor in tendon, was expressed at higher levels in the energy storing SDFT and SL suggesting that there is an overall greater enzyme activity in the positional CDET and DDFT. Collagen may be further protected from collagenases by the SLRPs, which bind to the surface of the fibrils. Coating type I collagen fibrils with decorin, fibromodulin or lumican decreases collagenase degradation when compared to uncoated fibrils (Geng *et al.,* 2006). The higher proteoglycan content in the SDFT may therefore protect collagen within this tendon from degradation.

The role of other degradative enzymes within tendon matrix is less clear. The main function of ADAMTS-2 is cleavage of the amino pro-peptides of type I collagen during synthesis (Colige *et al.,* 1997) so levels of this enzyme may be more associated with synthesis than degradation. ADAMTS-2 expression is increased in tendinopathy (Jones *et al.,* 2006); this may therefore reflect increases in collagen synthesis in an attempt to repair matrix damage. There was no difference in ADAMTS-2 levels between functionally distinct tendons; however it has been shown that ADAMTS-14 functions as the main type I procollagen N-propeptidase in tendon (Colige *et al.,* 2002). ADAM-12 is able to cleave a variety of matrix components (Roy *et al.,* 2004) and is also involved in cell attachment and migration (Thodeti *et al.,* 2003). ADAM-12 expression is up-regulated in tendinopathy (Jones *et al.,* 2006), possibly reflecting an overall increase in matrix turnover in an attempt to repair the lesion. MMP-23 has also been implicated in tendinopathy; expression increases in painful Achilles tendons (Jones *et al.,* 2006). MMP-23 is a transmembrane protein (Pei *et al.,*

2000); its substrate specificity is yet to be determined but it is upregulated during endochondral bone formation (Clancy *et al.,* 2003). Up-regulation of this gene in tendinopathy may therefore be an indication of alterations in tenocyte cell phenotype. Expression of genes associated with tendinopathy in the human were not significantly different between tendons and did not alter with increasing age. This suggests that processes involved in ageing are different from those that result in tendinopathy.

5.4.5. Expression of Other Proteins in Tendon

COMP had the greatest levels of expression of all genes in all tendons. This glycoprotein has been proposed to act as a catalyst in collagen fibrillogenesis (Halasz *et al.,* 2007; Sodersten *et al.,* 2005). The concentration of COMP is correlated with tendon mechanical properties (Smith *et al.,* 2002b) and so this protein may also play a role in strain transfer between fibrils (Smith *et al.,* 1997). However, COMP-null mice do not exhibit a tendon phenotype (Svensson *et al.,* 2002) and so this glycoprotein may not be as critical to tendon function as previously thought. COMP expression was greater in the high strain SDFT and SL than in the low strain DDFT and CDET, suggesting expression may be modulated by mechanical load. Tenascin-C and scleraxis were expressed at lower levels than COMP in all tendons; these genes are often used as markers of tendon phenotype, but their functions in mature tendon are yet to be determined.

5.4.5.1.   Markers of Tendon Phenotype in Functionally Distinct Tendon

Several genes have been proposed as markers of tenocyte phenotype, including tenascin-C and scleraxis (Murchison *et al.,* 2007; Taylor *et al.,* 2009), but no individual gene has been identified that is only expressed by tenocytes. The results in this chapter are similar to those reported previously (Taylor *et al.,* 2009), with greater expression of scleraxis than tenascin-C. Comparison of expression in equine tendon to expression of tenascin-C and scleraxis in skin and cartilage samples confirm the cells in the tendons studied exhibit a tendon phenotype. Transfection experiments have shown that over-expression of scleraxis results in a 200% increase in Col1A1 expression (Lejard *et al.,* 2007). Correspondingly, the data presented in this chapter show a correlation between expression of scleraxis and Col1A2 in all tendons. Scleraxis expression was significantly lower in the low strain CDET and DDFT than in the high strain SDFT; it is well established that expression of this gene is modulated by mechanical load (Eliasson *et al.,* 2009; Farng *et al.,* 2008; Kuo and Tuan 2008).

The data presented in this chapter has also identified a difference in cell phenotype between functionally distinct tendons which is most obvious between the SDFT and CDET. Cells in the SDFT can be characterised by greater expression of proteoglycans and enzymes that degrade them, whereas cells in the CDET express greater levels of genes coding for collagen type I (per cell) and collagenases. Therefore the ratio of collagen type I to decorin expression could be used to differentiate between cells from functionally distinct tendons. This ratio is on average fourfold greater in the low strain positional CDET than in the high strain energy storing SDFT (Figure 5-18), suggesting that turnover of proteoglycans is more critical to maintain tendon function than turnover of the collagenous matrix in energy storing tendons and supporting the data presented in chapter 4. This ratio would be of use to determine if cell phenotype alters with the progression of tendinopathy, and also in *in vitro* systems to compare the phenotype of cultured cells to that of cells in native tendon tissue.

5.4.6. Effect of Age on Cell Phenotype

In contrast to the hypothesis tested, gene expression is not altered with increasing horse age; the only gene that showed a significant relationship with horse age was MMP-10, expression of which increased with ageing in both the SDFT and CDET. Changes in gene expression that occur during tendon development and maturation and with tendinopathy have been determined previously, but there is little information available regarding alterations in gene expression that occur with normal ageing. There was also no alteration in the concentrations of the assayed MMPs at the protein level with increasing age, with the exception of MMP-3, which increased with ageing in the SDFT. These data indicate that there is no decrease in the ability of aged cells to turnover the matrix components at least with respect to transcription of matrix macromolecules and synthesis of matrix degrading enzymes. This suggests that the increased incidence of tendinopathy with ageing may not be a cell mediated problem. Although the data presented in this chapter do not identify a decrease in the synthesis of degradative enzymes with increasing age, it is possible that the synthesis of structural matrix proteins decreases with ageing; studies have identified a decrease in overall protein synthesis rates in aged individuals (Tavernarakis 2008). Alternatively, age related modifications to the matrix may increase the resistance of both the collagenous and non-collagenous matrix to degradation; it has been shown that glycated type VI collagen is significantly more resistant to degradation by MMPs *in vitro* than non-glycated collagen (Mott *et al.,* 1997). Furthermore, pentosidine levels have been associated

162

with decreased proteoglycan synthesis, suggesting that AGEs may inhibit protein synthesis (DeGroot *et al.,* 1999). This chapter assessed the potential for matrix synthesis and degradation; the rate of collagen protein synthesis and matrix collagen degradation can be further assessed by measuring molecular markers of collagen synthesis and degradation.

5.5. Conclusions

- Cell phenotype differs between the functionally distinct SDFT and CDET and this corresponds to the differences in matrix protein half-life identified in chapter 4.

- Cells in the CDET have a greater potential to synthesise and degrade collagenous matrix components than those in the SDFT.

- Cells in the SDFT show a greater potential for non-collagenous matrix turnover than their counterparts in the CDET.

- These differences are also evident at the protein level, with greater concentrations of collagen degrading enzymes in the CDET and higher levels of proteoglycan degrading enzymes in the SDFT.

- The ability of cells to synthesise matrix components and degradative enzymes does not alter with increasing age.

CHAPTER SIX

6. Markers of Collagen Turnover Indicate an Accumulation of Partially Degraded Collagen within Energy Storing Tendons

6.1. Introduction

Although gene expression data, as measured in the previous chapter, gives an indication of protein synthesis it does not provide conclusive evidence that the protein is present within the matrix. Likewise, the presence of matrix degrading enzymes does not show that these enzymes are actively degrading the matrix. In fact, the data presented in the previous chapters show disparity; the half-life of the collagenous matrix increases with age in the superficial digital flexor tendon (SDFT), indicating a reduced rate of collagen turnover in aged energy storing tendons. However, the data presented in chapter 5 show that there is no overall decrease in the genes expressed by tenocytes with increasing horse age in either the SDFT or the common digital extensor tendon (CDET) and there also does not appear to be any alteration in the ability of cells to degrade the matrix with increasing age in equine tendon, based on measurement of MMP protein levels. The incidence of tendon injury increases with age in both horses and humans (Clayton and Court-Brown 2008; Kannus *et al.,* 1989; Kasashima *et al.,* 2004) and this has previously been attributed to a decrease in cell activity in aged tendons (Smith *et al.,* 2002a) although the data presented in this thesis do not support this.

This may be because the rate of degradation of damaged collagen within the matrix and subsequent repair that occurs within tendon is not only dependant on the ability of tenocytes to synthesise pro-collagen molecules and synthesise and activate degradative enzymes; it also depends on how resistant the matrix is to degradation. Intact triple helical collagen is much more resistant to degradation than collagen monomers in solution (Slatter *et al.,* 2008). In tendon, neighbouring collagen molecules are cross-linked to one another and packed tightly together to form fibrils, and only the molecules on the surface of the fibrils are available for degradation as the collagenases are unable to access the collagen molecules located in the centre of the fibrils. Furthermore, in order for collagenases to be able to degrade intact triple helical collagen the C-terminal telopeptide must be unwound (Chung *et al.,* 2004; Perumal *et al.,* 2008). This action cannot be performed by the collagenases; the stromelysin MMP-3 is thought to play a key role in this process (Sweeney

et al., 2008), and so collagen degradation depends on the presence of several MMPs with different substrate specificity.

The rate of degradation of matrix collagen can be assessed further by using the concentration of protein fragments generated when triple helical collagen is degraded as markers of degradation. In a similar manner, the concentration of pro-collagen with tendon matrix can be used as a marker of collagen synthesis.

6.1.1. Collagen Synthesis

Collagen is synthesised as procollagen molecules (see chapter 1), with non-triple helical amino- and carboxy- pro-peptide regions (Figure 6-1). During synthesis, the pro-peptide regions are removed by proteolytic enzymes (Colige *et al.,* 2002). The cleaved collagen molecules are then able to assemble into fibrils, which are incorporated into the tendon matrix. As such, collagen molecules within the tendon matrix that have not had the N- and C- terminal pro-peptides removed are newly synthesised and so measurement of the concentration of pro-collagen peptides within the matrix can be used as markers of the rate of collagen synthesis at the level of translation.

Collagen synthesis was first quantified in this manner by Taubman *et al.* (1974) by measuring the concentration of the C-terminal peptide (PICP) using a competitive radio immunoassay (RIA). PICP is now a well established marker of collagen synthesis and has been used to identify changes in collagen synthesis in a variety of disease states, including investigating the pathogenesis of osteoporosis (Parfitt *et al.,* 1987) and the identification of bone metastases (Klepzig *et al.,* 2009). More recently changes in levels of collagen pro-peptides have been used to assess the effect of exercise on the rate of collagen synthesis, both in humans (Karlsson *et al.,* 2003a; Karlsson *et al.,* 2003b) and horses (Billinghurst *et al.,* 2003; Price *et al.,* 1995b). While most of these studies have focused on changes in bone or cartilage metabolism, concentration of collagen pro-peptides has also been used to assess alterations in tendon turnover with exercise or disease. Levels of serum PICP have been shown to be elevated in horses with tendinopathy, and have also been shown to decrease with increasing horse age, representing an overall decrease in collagen synthesis (Jackson *et al.,* 2003). However, this study measured changes in serum levels of PICP and so may not reflect the specific changes that occur in tendon tissue with increasing age. Localisation of pro-collagen molecules within tendon matrix has also been achieved using

166

immunohistochemistry (Halper *et al.,* 2005; Young *et al.,* 2009b). However, no previous studies have measured the total concentration of pro-collagen molecules extracted from equine tendon tissue *ex vivo*.
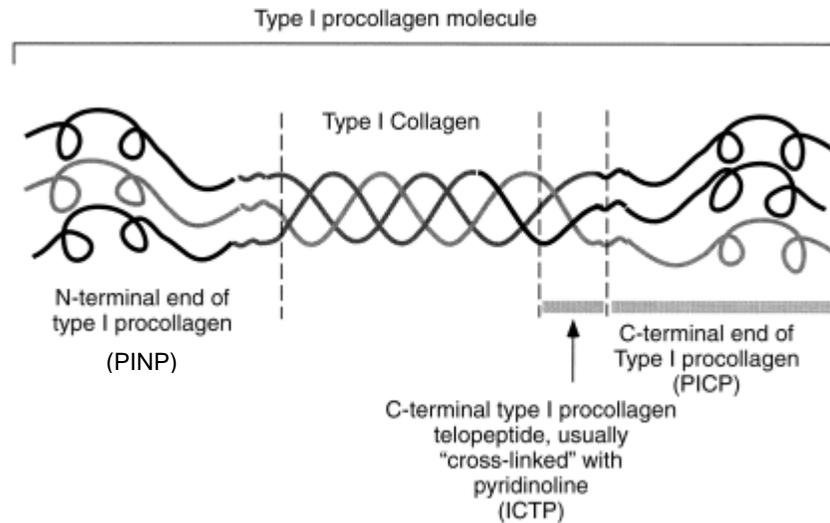
Figure 6-1: Representation of the type I pro-collagen molecule showing the N- and C- terminal propeptides, which are removed during processing and the C-terminal region where pyridinolines crosslink the molecule to adjacent molecules. Adapted from Urena and De Vernejoul (1999).

6.1.2. Collagen Degradation

Intact triple helical collagen can only be degraded by the collagenases (MMP-1, -8 and -13) (Lauer-Fields *et al.,* 2000; Visse and Nagase 2003), which cleave the triple helix into ¼ and ¾ fragments (Figure 6-2). This exposes new ends of the collagen molecule (neoepitopes) which can be measured using antibodies specific to the end sequences. Therefore the concentration of the ¼ and ¾ fragments within the matrix can be measured and used as indicators of the rate of collagen degradation. The concentration of the ¾ fragment (C1,2C neoepitope) has been measured to assess collagen degradation rates in lung disease (Armstrong *et al.,* 1999) and both equine and human osteoarthritic cartilage (Billinghurst *et al.,* 1997; Billinghurst *et al.,* 2004; Frisbie *et al.,* 2008), but has not been used previously to assess collagen degradation in tendon tissue.
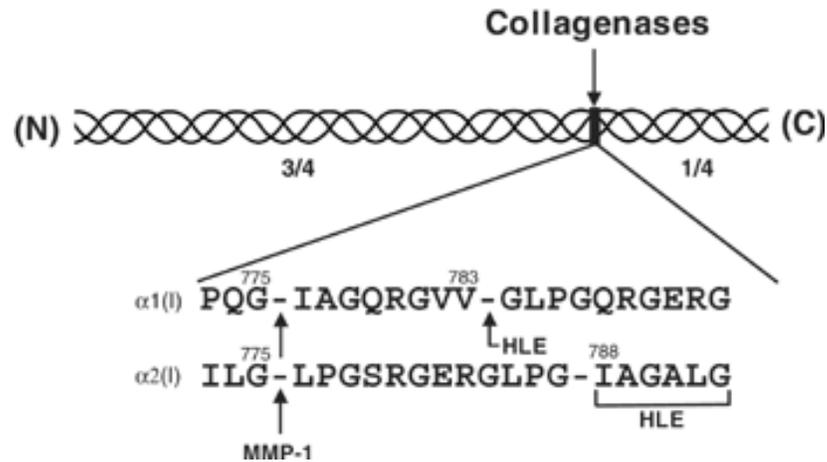
Figure 6-2: Schematic showing the collagenase cleavage site on type I collagen molecules, which results in the generation of ¼ and ¾ fragments. Adapted from Chung *et al.* (2004).

Another marker of collagen degradation is the cross-linked C-terminal telopeptide of type I collagen (ICTP). ICTP consists of a trivalent collagen cross-link which connects three polypeptide chains (Figure 6-1); two are α1 chains from one collagen molecule while the third is derived from either an α1 or an α2 chain from the helical region of an adjacent molecule (Risteli *et al.,* 1993). Therefore any ICTP present in the matrix must have been generated when collagen molecules that had been incorporated into fibrils were degraded; mature inter-molecular crosslinks would not be present between newly synthesised collagen molecules and so ICTP would not be produced when they were degraded. The concentration of ICTP has been used widely as a marker of collagen degradation; serum levels of ICTP are often used to assess bone degradation in a variety of disease states, including rheumatoid arthritis (Hakala *et al.,* 1993) and myelomas (Elomaa *et al.,* 1992). Collagen degradation in tendon has also been assessed by measuring the concentration of ICTP in peritendinous tissue of the Achilles tendon (Christensen *et al.,* 2008; Langberg *et al.,* 1999; Langberg *et al.,* 2001). ICTP has also been used to study bone and tendon turnover in horses; several studies have used serum levels of ICTP to assess changes in collagen degradation with increasing age or injury (Jackson *et al.,* 2003; Lepage *et al.,* 1998; Price *et al.,* 1995a; Price *et al.,* 2001). However, measurement of serum levels of ICTP will only give an indication of general collagen turnover and is not specific to a particular tissue. ICTP has also been used previously as a marker of the rate of collagen degradation in equine tendon (Birch *et al.,* 2008b); this study reported that levels of extracted ICTP were lower in the SDFT than in the CDET.

6.1.3. Aims and Hypothesis

The aims of this chapter were to assess collagen synthesis at the protein level and actual degradation of the matrix in the functionally distinct SDFT and CDET from horses with a wide age range. Collagen synthesis was assessed by measuring pro-collagen levels in a semi-quantitative manner using Western blotting. Matrix collagen degradation was assessed by measuring the concentration of protein fragments that are generated when type I collagen is cleaved by collagenases; the concentrations of ICTP and the C1,2C neoepitope were measured using commercially available enzyme linked immunosorbent assay (ELISA) and RIA. It was hypothesised that the rate of collagen synthesis and degradation in the high strain energy storing SDFT would be lower than that in the low strain positional CDET and that collagen turnover would decrease with increasing horse age in the SDFT.

6.2.   Materials and methods

Lyophilised tendon tissue from the SDFT and CDET of 32 horses collected and processed as described in chapter 3 was used for the following studies.

6.2.1. Markers of Collagen Synthesis

6.2.1.1. PINP

Collagen synthesis was assessed at the protein level in the SDFT and CDET from 14 horses selected from the sample group based on age range (6 – 30 years) by measuring the concentration of pro-collagen that could be extracted from the matrix. Pro-collagen was detected using an antibody for the N-terminal propeptide of type-I collagen (PINP) using Western blotting. Initial work found that the more commonly used commercially available EIAs for PICP (Takara procollagen Type I C-peptide EIA, Takara Bio Inc., Japan; MicroVue CICP EIA kit, Quidel) did not show cross reactivity with equine collagen. Previous work has shown that an antibody for PINP raised against ovine protein (SP1.D8, Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa, UK) shows good cross reactivity with the horse (Young *et al.,* 2009b) and so this antibody was used to assess collagen synthesis in equine tendon. Protein was precipitated from Guanidine-HCl (GuHCl) extracts (see chapter 4) (Birch *et al.,* 2008b); 900 μl of 95% ethanol in 50 mM sodium acetate at pH 7.4 was added to a 100 μl aliquot of the supernatant from the GuHCl extraction. Protein was precipitated overnight at -20 °C, and the supernatant was removed following centrifugation (16 000 g for 25 min).

The pellet was air dried and re-dissolved in 100 µl of 50 mM sodium acetate, pH 7.4 and precipitated again. The remaining samples were reconstituted in 100 µl 2x reducing buffer (10% mecaptoethanol in 125 mM Tris, 2% SDS, 10% glycerol, pH 6.8) and heated at 60 ºC for 5 min. before loading 20 µl of each sample onto the gels. Proteins were separated by SDS-PAGE on a 5% acrylamide gel with a 4% stacking gel; a molecular weight standard (10 µl; Precision Plus Standards, Biorad Laboratories Ltd., Hemel Hempstead, UK) was run alongside the samples on each gel. Proteins were separated by applying a constant current of 20 mA per gel to the gels for 55 min.

Initial work was preformed to confirm the position of the collagen α1 and α2 chains when proteins were separated by electrophoresis, pepsin digested type I collagen purified from equine and rat tail tendon was heated at 90 ºC for 5 minutes and separated by SDS-PAGE. Gels were stained with Coomassie Blue R-250 stain solution for 1 hour, and destained in de-stain solution for 2 hours. Collagen α1(I) and α2(I) chains had a molecular weight of approximately 130 kDa and 120 kDa respectively (Figure 6-3). These values are similar to those reported in the literature for pepsin extracted equine collagen (Falini *et al.,* 2004).
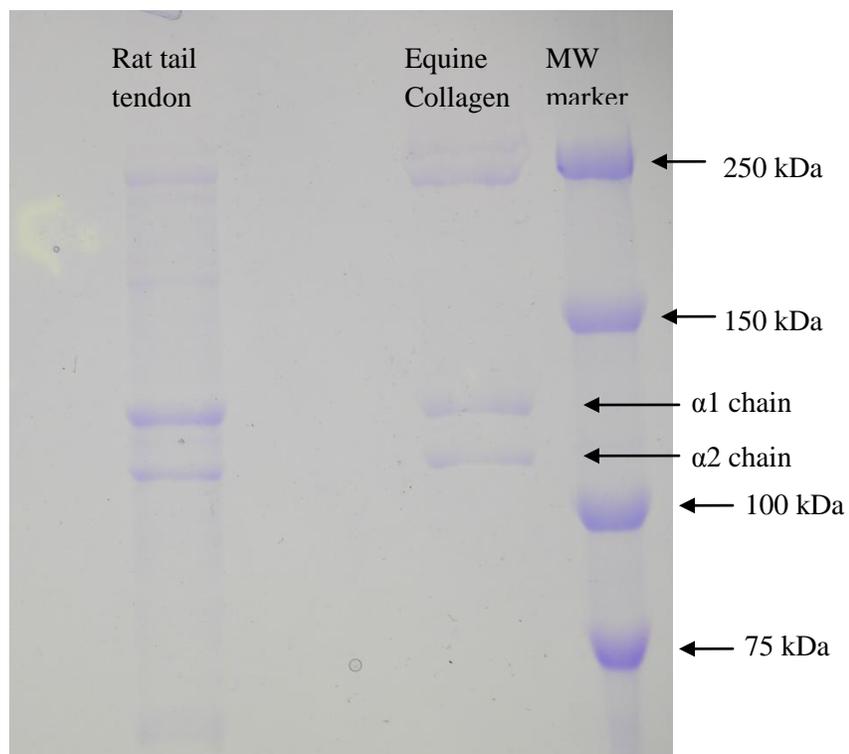
Figure 6-3: Gel stained with Coomassie Blue R-250 showing molecular weight marker and position of collagen α1 and α2 chains.

After electrophoresis, gels were incubated in transfer buffer (10% 10 x running buffer, 20% methanol, 70% deionised water) and proteins were transferred to PVDF membranes (Amersham Hybond-P, GE Healthcare, Amersham, UK) by blotting for 75 min at 100 V. Membranes were stored overnight in transfer buffer. Membranes were then washed in Tris buffered saline (0.05 M, pH 8) with 0.1% Tween 20 (TBS-T) containing 4% skimmed milk powder for 1 hour at room temperature on an orbital shaker. Membranes were rinsed in 2 changes TBS-T and then incubated in ovine PINP antibody raised in mouse (SP1.D8, diluted 1 in 1000 in TBS-T) for 2 hours at room temperature with shaking. Membranes were rinsed in 2 changes of TBS-T, then washed for 15 min. in TBS-T, followed by three 5 min. washes in TBS-T. Membranes were incubated in an enhanced chemi-luminescent (ECL) peroxidase labelled anti-mouse secondary antibody (GE Healthcare, diluted 1 in 10 000 in TBS-T) for 2 hours at room temperature with shaking. After washing as described above blots were developed with Amersham ECL Plus™ Western blotting detection reagents according to the manufacturer's instructions (GE Healthcare). Membranes were then placed in an x-ray film cassette with autoradiography film (Hyperfilm, Amersham Biosciences). Exposure time was varied from 10 minutes to 18 hours to determine the best exposure time; the time that resulted in the best contrast was 10 minutes. Resulting x-ray films were developed and the x-rays were photographed on a lightbox using a Nikon Coolpix camera (5700, high resolution) set at a defined distance from the lightbox. Band areas were quantified using Scion Image (Version 4.0.3.2, Scion Corporation, Maryland, USA). To allow comparison between blots, a CDET sample was selected and included on each gel, and levels of pro-collagen were expressed relative to this CDET sample and per tissue weight. Comparison to the molecular weight marker showed four bands with molecular weights ranging from approximately 155 kDa to 200 kDa (Figure 6-4). These bands are likely to represent pro-collagen α1(I) and α2(I) chains either with both the N- and C-terminal propeptides still attached, or with just the N-terminal propeptide attached. The molecular weight of the collagen α1(I) chain is 139 kDa (Urtasun *et al.,* 2009), the C-terminal pro-peptide has a molecular weight of 35 kDa (Pedersen and Bonde 1994) and the N-terminal pro-peptide has a molecular weight of 27 kDa (Jensen *et al.,* 1998). The collagen α2(I) chain is slightly smaller, with a molecular weight of 130 kDa (Urtasun *et al.,* 2009), PICP on the α2(I) chain has a molecular weight of 33 kDa (Pedersen and Bonde 1994) and PINP has a molecular weight of 20 kDa. Therefore the band at 200 kDa

represents the procollagen α1(I) chain with PICP and PINP attached and the band at 190 kDa represents the procollagen α2(I) chain with PICP and PINP attached. The bands at 165 kDa and 155 kDa correspond to the α1(I) and α2(I) chains with PINP, but not PICP attached. The band corresponding to the pro-collagen α2 chain with both PICP and PINP attached (190 kDa) was the clearest band in the majority of samples and so the area of this band was measured to assess the rate of collagen synthesis in the SDFT and CDET.

Figure 6-4: X-ray showing bands of protein corresponding to PINP concentration in the SDFT and CDET from 3 horses.

To ensure the PINP antibody did not recognise mature collagen molecules that no longer contained an N-terminal pro-peptide region, rat tail tendon and equine collagen were separated by SDS-PAGE and probed using the SP1.D8 antibody. Development of the subsequent blot did not result in the visualisation of any bands (Figure 6-5).
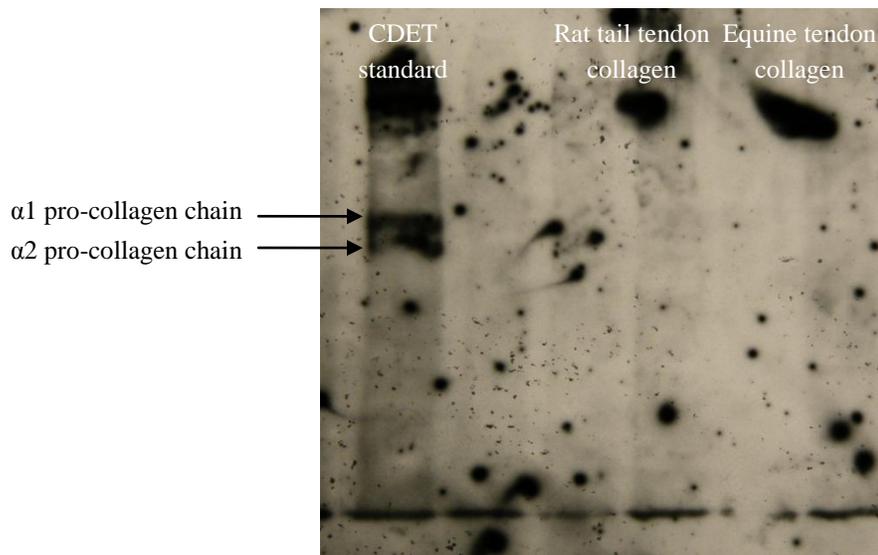
Figure 6-5: X-ray confirming that the SP1.D8 antibody does not recognise mature collagen molecules.

6.2.2. Markers of Collagen Degradation

6.2.2.1. ICTP

The concentration of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) was assessed in the SDFT and CDET using a commercially available competitive RIA (Orion Diagnostica, distributed in the UK by Oxford Biosystems, Oxford, UK). A known amount of ^{125}I-labelled ICTP competes with an unknown amount of unlabelled ICTP for antibody binding sites. Although designed for use with human serum, it has been demonstrated previously that this RIA shows good cross-reactivity with the horse (Price *et al.,* 1995a). Protein was precipitated from GuHCl extracts as described above. The remaining pellet was re-dissolved in 1 ml of dilution buffer (0.8% SDS, 140 mM sodium chloride, 8 mM disodium hydrogen phosphate, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate, 3.1 mM sodium azide, pH 7.4). Previous work has validated this RIA for use with samples that have undergone GuHCl extraction (Birch *et al.,* 2008b); there was a very strong correlation between diluted GuHCl extracted samples and ICTP reference standards (Thorpe *et al.,* 2010). Preliminary work was carried out to determine the dilution factors required, to measure the concentration in trypsin digested samples and to determine the effect of different dilution buffers. The RIA was performed in duplicate according to the manufacturer's instructions and a blank containing dilution buffer with no tissue was included to account for any difference in absorbance due to the dilution buffer used. Radioactivity was measured using a gamma counter and the standards

provided were used to construct a calibration curve, from which sample ICTP concentration was determined. Concentration of ICTP was expressed as ng per mg dry weight tissue.

As expected, the concentration of ICTP was approximately 400 times greater in the trypsin digested samples, and was similar between the SDFT and CDET, reflecting a similar overall collagen content. Reconstituting the GuHCl extracted pellets in PBS was found to result in incomplete dissolution of the pellets and low readings, and so pellets were redissolved in the buffer used in the previous study (Birch *et al.,* 2008b). It was determined that dissolving samples from the SDFT in 1 ml dilution buffer resulted in readings that were within the range of the standard curve whereas samples from the CDET had to be further diluted 10 fold to bring them within range of the standard curve.

6.2.2.2. Neoepitopes of Type I Collagen

The ¾ fragment generated when intact type I collagen is cleaved by collagenases was extracted from the matrix by collagen denaturation using heat followed by trypsin digestion, and concentration was measured in the SDFT and CDET samples using a commercially available ELISA kit (IBEX Technologies Inc., Montreal, Canada), using an antibody which recognises an 8 amino acid sequence at the C-terminal cleavage site (Billinghurst *et al.,* 1997). Approximately 30 mg lyophilised powdered tissue from the SDFT and CDET was accurately weighed out and suspended in 0.75 ml 3-(N-Tris (hydroxymethyl) methylamino)-2-hydroxypropanesulfonic acid (TAPSO) buffer (100 mM TAPSO, 10 mM CaCl, pH 8.2). The samples were heated at 90 °C for 35 minutes and then cooled to 37 °C before 0.4 ml trypsin solution (10 000 units/ml TAPSO buffer) was added. The samples were incubated for 20 hours at 37 °C with shaking. After 20 hours, trypsin activity was inhibited with 20 µl N-p-tosyl-L-lysin chloromethyl ketone (18.2 mg in 1 ml TAPSO buffer). A blank containing no tissue was included with the samples and was used to dilute the standards to account for any difference in absorbance caused by differences in buffer composition. The ELISA was performed in duplicate according to the manufacturer's instructions and the resulting absorbance was measured at 450 nm using a plate reader (BioRad 3550 microplate reader). Previous studies (Billinghurst *et al.,* 1997) have used chymotrypsin to extract the peptide from the matrix so preliminary work was carried out to determine the effects of using trypsin instead of chymotrypsin, and denaturing the collagen prior to enzymatic digestion. The C1,2C antibody recognises the

following sequence at the carboxy terminus of the ¾ fragment: Pro-Gly-Thr-Pro-Gly-Pro-Glu-Gly (Billinghurst *et al.,* 1997); this sequence will not be digested by trypsin as this protease is only able to cleave at the carboxyl side of lysine and arginine (Olsen *et al.,* 2004). To ensure trypsin digestion did not alter the antibody recognition site, reference standards with concentrations of 4.16 nmoles/ml and 1.37 nmoles/ml were heated and digested with trypsin before being assayed for C1,2C. Absorbance readings for trypsin digested standards were very similar (on average 1.06 times greater) to those for undigested standards; indicating that trypsin digestion does not affect the antibody binding site and therefore is suitable for extraction of the neoepitope from tendon tissue.

The absorbance of the reference standards supplied with the kit was used to calculate a standard curve. This was not linear so curve fitting software (GraphPad Prism, Version 5, GraphPad Software Inc., California, USA) was used. Mean absorbance was plotted on y-axis versus Log_{10} concentration on x-axis. Non-linear regression was performed to fit an appropriate curve, using the sigmoidal dose-response (variable slope) function (Figure 6-6) and the concentration in the samples was calculated. The assay is a competitive immunoassay, therefore sample concentration is inversely proportional to the absorbance measured. The collagen content of the trypsin digested samples was measured using an assay for hydroxyproline as described in chapter 4 and the concentration of the ¾ fragment was expressed per mole of collagen to correct for any difference in collagen content and digestion efficiency between the tendon samples.
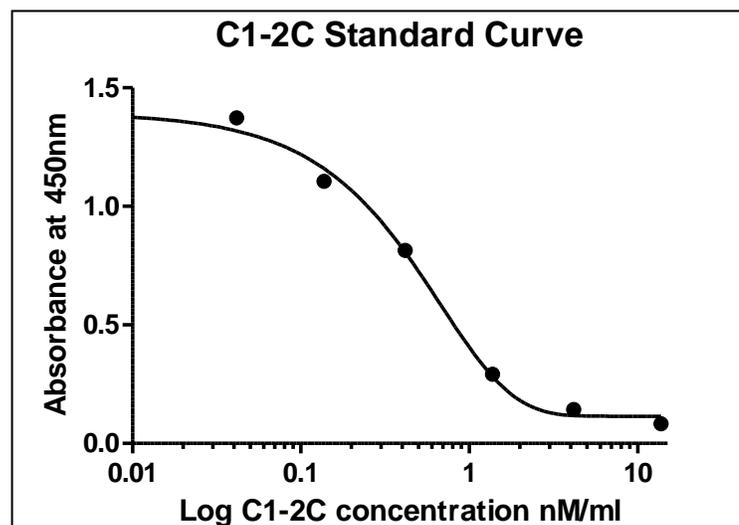
Figure 6-6: Non-linear curve fitting was performed and the concentration of C1,2C present in the samples was calculated from the equation for the curve using GraphPad Prism (Version 5).

6.2.3. Statistical Analysis

Statistical significance was assessed using a linear fixed effects model in SPlus (Version 6.1, Insightful) with horse as a grouping factor. Data were tested for normality using Kolmongorov-Smirnoff test (Minitab, Version 15) and correlation with age was assessed for each tendon using Pearson Product Moment Correlation and Spearman Rank correlation (SPSS, Version 14). All data are displayed as mean ± SEM.

6.3.   Results

Concentration of the markers of collagen synthesis and degradation in the SDFT and CDET, and correlation of these markers with horse age are shown in Table 6-1.

	Mean ± SEM		Correlation with age (r)	
	SDFT	**CDET**	**SDFT**	**CDET**
Pro-collagen (Units/mg tissue) (n = 14)	43.09±14.85	198.68±37.47***	0.189	-0.429
ICTP (ng/mg tissue) (n = 32)	86.09±7.31	448.21±104.89***	-0.651§§§	-0.499§§
C1,2C (mM/M collagen) (n = 32)	12.65±0.71	15.49±0.86*	0.458§	0.056

Table 6-1: Concentration of marker of collagen synthesis and degradation in the SDFT and CDET (mean ± SEM). *Indicates significant difference from SDFT: *p<0.05; **p<0.005, ***p<0.001; §Indicates significant correlation with age: §p<0.05; §§p<0.005, §§§p<0.001.

6.3.1. Collagen Synthesis

Pro-collagen was detected in all CDET samples analysed, and was not detected in some SDFT samples. Correspondingly, levels of pro-collagen extracted from the matrix were significantly higher in the CDET than in the SDFT (p=0.0008) (Figure 6-7). Pro-collagen levels did not change significantly with age in either tendon; although there was a trend towards decreased pro-collagen levels in the CDET, this was not significant (p=0.1, r=-0.4) (Figure 6-8). Furthermore, there was no significant correlation between pro-collagen at the protein level and expression of Col1A2 at the mRNA level (data presented in chapter 5).
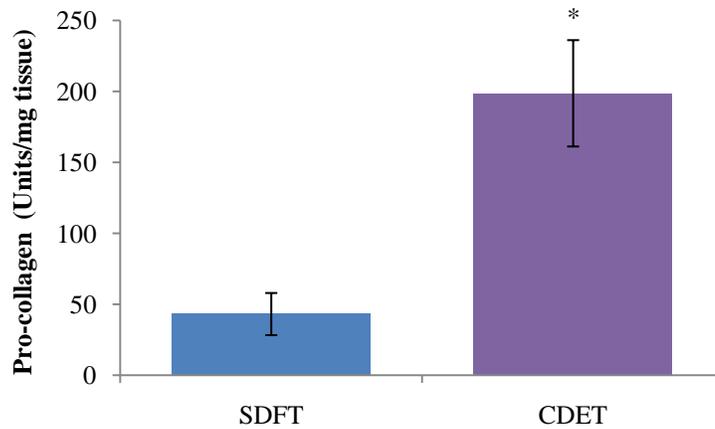
Figure 6-7: Concentration of pro-collagen in the SDFT and CDET (mean ± SEM) n = 14. * Indicates significant difference relative to the SDFT.
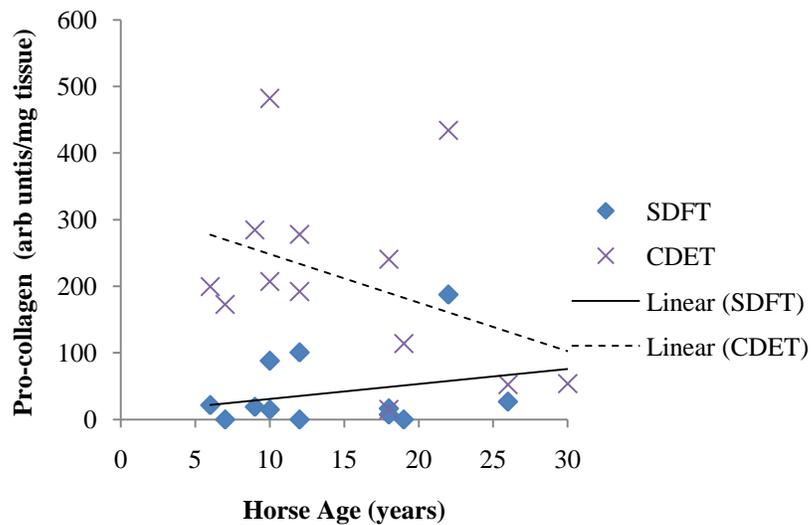
Figure 6-8: Pro-collagen concentration as a function of horse age in the SDFT and CDET from 14 horses. Pro-collagen concentration was not significantly correlated with horse age in either tendon type.

6.3.2. Collagen Degradation

ICTP was detected in the majority of tendon samples extracted with GuHCl. The RIA used showed good repeatability, with an average variation of 4.84% between duplicate samples. Levels of ICTP extracted from the matrix were significantly greater in the CDET when compared to the SDFT (p<0.0001) (Figure 6-9); and decreased significantly with age in both tendons (p<0.0001) (Figure 6-10).
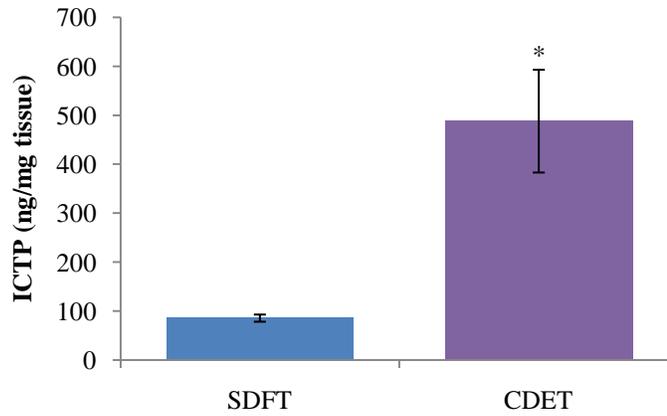
Figure 6-9: Concentration of ICTP in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.
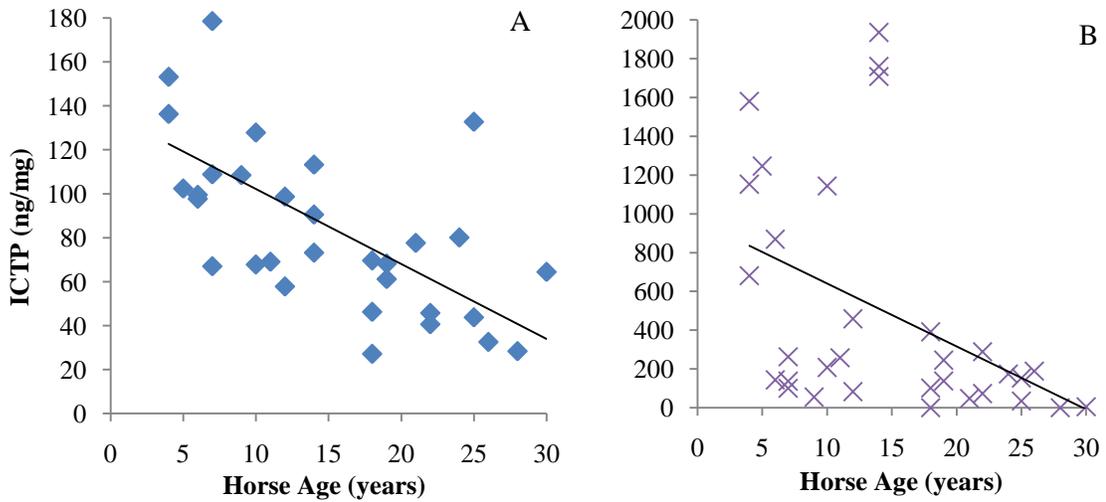
Figure 6-10: Concentration of ICTP extracted from the SDFT (♦) (A) and CDET (×) (B) as a function of horse age. A linear equation was found to give the best fit (p<0.0001, r=-0.64 and p<0.000, r=-0.50 for the SDFT and CDET respectively). The amount of ICTP extracted from the matrix was significantly greater in the CDET than in the SDFT (p<0.001).

Levels of ICTP extracted from the SDFT showed a significant negative correlation with levels of the AGE pentosidine (p<0.001) (Figure 6-11) but there was no correlation between ICTP and pentosidine concentration in the CDET.

178
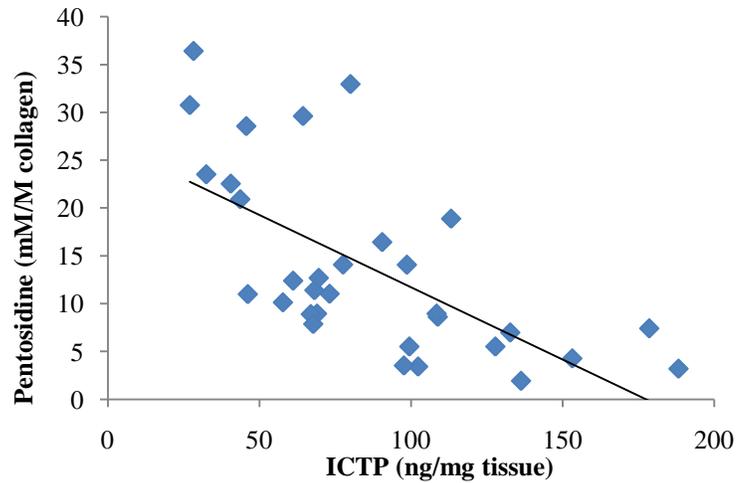
Figure 6-11: Correlation of ICTP levels with pentosidine concentration in the SDFT, r=-0.65, p<0.001.

The C1,2C neoepitope was detected in all tendon samples. The ELISA used showed good repeatability between duplicate samples, with a mean variation of 6.77%. Levels of the C1,2C neoepitope in the matrix were found to be significantly higher in the CDET than in the SDFT (p=0.007) (Figure 6-12); and accumulated with age in the SDFT (p=0.008) but not in the CDET (Figure 6-13).
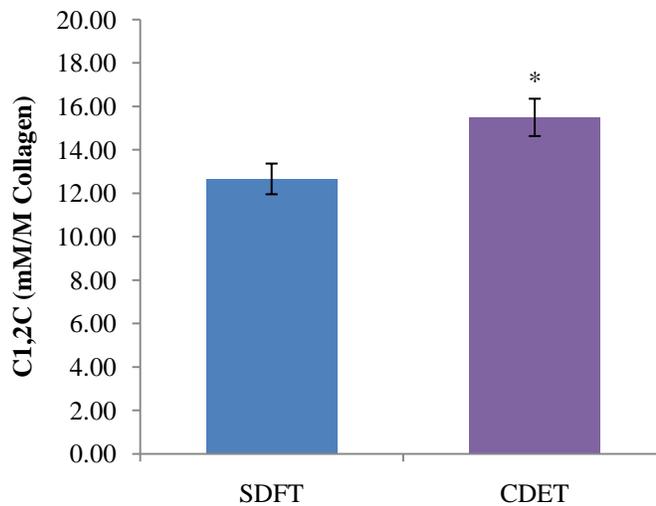
Figure 6-12: Concentration of C1,2C neoepitope (mM/M collagen) in the SDFT and CDET (mean ± SEM) n = 32. * Indicates significant difference relative to the SDFT.
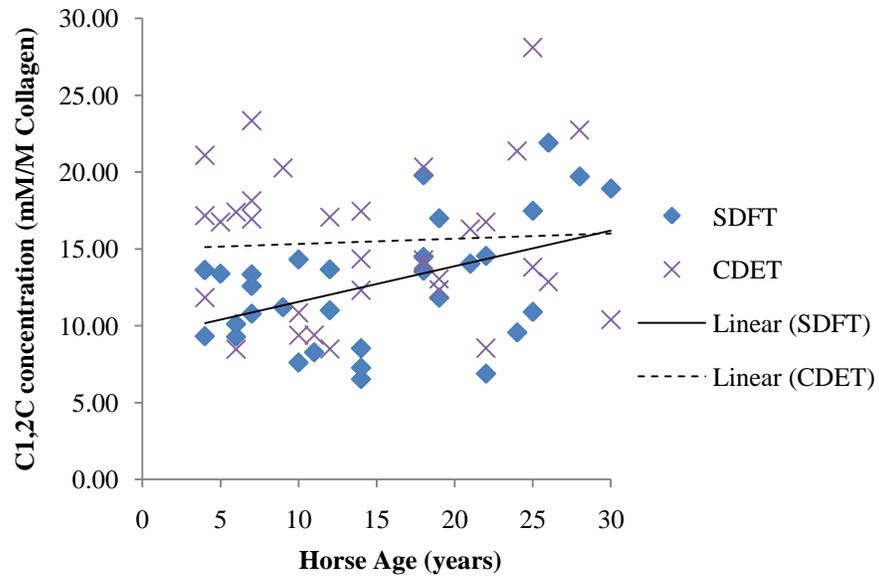
Figure 6-13: Concentration of C1,2C as a function of horse age in the SDFT and CDET. C1,2C concentration increased significantly with age in the SDFT (p=0.007, r=0.458) and showed no correlation with age in the CDET.

C1,2C levels were significantly correlated with pentosidine levels in the SDFT (p=0.02) (Figure 6-14), but showed no relationship with pentosidine concentration in the CDET. There was also no correlation between C1,2C concentration and mRNA or protein levels of the collagenase MMP-13 in either tendon.
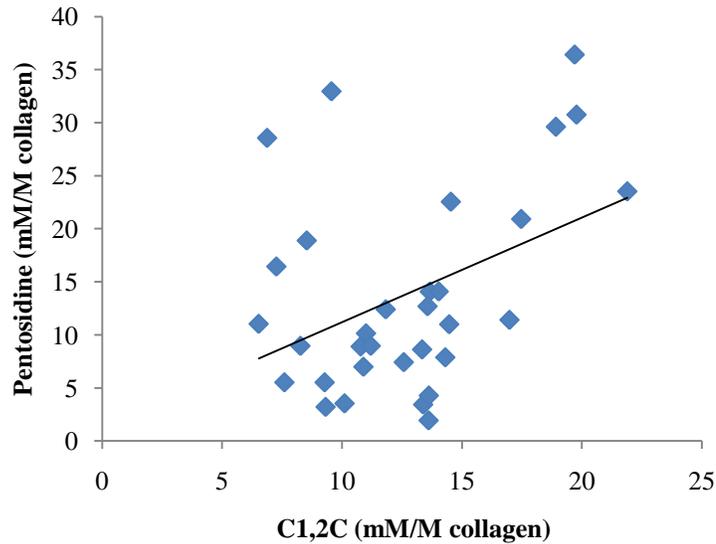
Figure 6-14: Correlation of pentosidine concentration with C1,2C concentration in the SDFT (r=0.41, p=0.02).

180

6.4. Discussion

The results of this chapter show that transcription of the type I collagen gene (measured in the previous chapter) results in translation into pro-collagen protein and that MMP-13 (and other MMPs) are functional within the tendon matrix. The levels of pro-collagen show that collagen synthesis at the translational level is greater in the CDET than in the SDFT and does not decrease with age in either tendon, reflecting differences identified at the transcriptional level. The amount of matrix collagen degradation is also greater in the CDET than in the SDFT and corresponds with the higher levels of message for, and protein levels of matrix degrading enzymes in this tendon. These results support the hypothesis tested in this chapter, which stated that the rate of collagen turnover at the protein level would be greater in the CDET than in the SDFT.

6.4.1. Methods Used to Assess Collagen Synthesis and Degradation

Previous studies have assessed the rate of collagen synthesis in equine tissues using a commercially available competitive RIA that recognises PICP (Orion Diagnostica, Finland) (Price *et al.,* 1995a; Price *et al.,* 2001). However, this kit is no longer manufactured and so two alternative enzyme immunoassay kits were tested for cross-reactivity with the horse (Takara procollagen Type I C-peptide EIA, Takara Bio Inc., Japan; MicroVue CICP EIA kit, Quidel). These commercially available kits are designed to assess PICP levels in human serum. The EIA manufactured by Takara Biosciences appeared to recognise the PICP sequence in equine samples in this study but showed extremely high variability between duplicate samples (up to 50%), suggesting it is not specific for PICP in the horse. The EIA manufactured by Quidel was tested in this study using horse and human serum samples; levels were successfully detected in human serum but the kit failed to recognise equine PICP. Collagen production in equine tendon tissue was therefore assessed in a semi-quantitative manner using Western blotting. A primary antibody that recognises the amino terminal pro-peptide of type I collagen has been validated previously for use in the horse (Young *et al.,* 2009b). Western blotting requires a high level of optimisation to determine the appropriate amount of sample that should be loaded on the gels, dilution and incubation time for primary and secondary antibodies, washing procedure and x-ray exposure time. The resultant blots produced four bands with molecular weights ranging from 150 to 200 kDa; these are likely to correspond to α1(I) and α2(I) pro-collagen chains with both the N- and C-terminal pro-peptides still attached and pro-collagen chains where the C-terminal

pro-peptide has been removed. The area of the band corresponding to the α2(I) chain with both pro-peptides attached was used as a marker of collagen synthesis as this band was the clearest in most samples and collagen synthesis was assessed at the transcriptional level by measuring Col1A2 mRNA levels. In a few cases excess sample was loaded onto the gel such that bands on resulting blots were too large to quantify accurately (see Figure 6-4). Due to time constraints it was not possible to re-assay all samples and so band size was measured as the minimum amount present in several CDET samples. As this results in underestimation rather than overestimation, the difference in collagen synthesis between the SDFT and CDET may actually be greater than reported in this chapter. In future experiments it would be prudent to assess total protein content in the samples (Smith *et al.,* 1985) and load a known amount of protein onto the gel for electrophoresis, which would allow more accurate quantification of pro-collagen levels. Alternatively, after separation of the protein by electrophoresis mass spectrometry could be used to accurately measure the concentration of the pro-collagen molecules within tendon.

Assessment of collagen degradation in the SDFT and CDET was possible with commercially available assays; the ICTP assay has been validated for use with horse serum (Price *et al.,* 1995a) and has been used previously to measure ICTP levels in equine tendon extracts (Birch *et al.,* 2008b). Denaturation and extraction of tendon with GuHCl results in the separation of the soluble and insoluble fractions of the matrix; intact collagen will remain in the insoluble pellet whereas collagen that is not cross-linked into the matrix will be separated from the insoluble collagen, along with non-collagenous proteins. Any difference in extraction efficiency between tendon types may therefore affect the final ICTP concentration; it is possible that a proportion of the higher levels of ICTP in CDET extracts are due to greater levels of soluble collagen in this tendon as a result of different crosslinks present in the matrix. No previous studies have used the concentration of C1,2C to assess collagen degradation within tendon; the concentration of this fragment is normally used to assess the degradation of type II collagen. However, the sequence recognised by the primary antibody at the cleavage site is identical in collagen types I and II, therefore this EIA can also be used to assess degradation of type I collagen (Sukhova *et al.,* 1999). This EIA has been validated previously for use with equine tissue (Billinghurst *et al.,* 2004).

6.4.2. Collagen Synthesis

As hypothesised, levels of pro-collagen were greater in the CDET than in the SDFT, indicating that the rate of collagen synthesis is greater in the low strain positional CDET. This supports the data presented in chapters 4 and 5, which show the cells in the CDET synthesise more mRNA for collagen type I than those in the SDFT, resulting in a lower half-life of the collagenous matrix in the CDET. However, the large difference in pro-collagen content is somewhat surprising as there was not a significant difference in expression of Col1A2 once the difference in DNA content between the tendons had been corrected for. This and the lack of any correlation between expression of the Col1A2 gene and pro-collagen protein levels give an indication of the complexity of the processes involved in collagen synthesis. These data suggest that the level of translation of Col1A2 mRNA into protein is greater in the CDET than in the SDFT. In contrast to the hypothesis, the rate of collagen synthesis did not decrease significantly with age in either tendon. This supports the gene expression data and suggests that the ability of the cells to synthesise matrix components does not decrease with increasing horse age.

These data are supported by several previous studies which have assessed collagen synthesis in human and equine tendon. An immunohistochemical study reported that collagen synthesis decreased in mature SDFTs compared to foetal tendons, whereas collagen synthesis increased in mature CDETs compared to immature tendon, resulting in overall greater levels of pro-collagen in the mature CDET than in the SDFT (Young *et al.,* 2009b). The pro-collagen levels reported in this previous study were approximately four times greater in the CDET than in the SDFT, which is similar to the values reported in this chapter. Collagen pro-peptide concentration in human Achilles peritendinous tissue has been assessed in response to exercise; it has been reported that levels of PICP decrease immediately after a bout of exercise but are increased 72 hours after exercise (Langberg *et al.,* 1999). Training for a period of 4 weeks resulted in a sustained increase in peritendinous PICP levels (Langberg *et al.,* 2001). Changes in pro-collagen peptides have also been reported in injured tendons; PINP levels decreased in the soluble fraction of injured Achilles tendons when compared to non-injured controls (Eriksen *et al.,* 2002). Interestingly, results from another study indicate that normal and injured tendons respond differently to the same exercise programme; levels of PICP were elevated in the tissue surrounding previously injured tendons in response to eccentric exercise, whereas levels did

not change in uninjured tendons (Langberg *et al.,* 2007), suggesting that the cell response to loading may be altered in injured tendons.

These studies indicate that, contrary to previous studies which have proposed that cells within mature energy storing tendons are inactive (Smith *et al.,* 2002a), cells within tendon show a basal level of turnover and are able to respond to exercise induced alterations in loading by increasing collagen production. Furthermore, studies have not identified a decrease in collagen synthesis, as assessed by measuring the concentration of PICP and PINP in Achilles tendon extracts, with increasing subject age (Eriksen *et al.,* 2002), and immunohistochemical labelling of PINP and PICP was relatively high in both normal and ruptured mature Achilles tendons (Pajala *et al.,* 2009), suggesting that collagen synthesis is maintained in aged tendons. However, unlike in other tissues such as bone and muscle, exercise does not result in alterations in tendon mechanical properties in the energy storing tendons in mature animals (Birch *et al.,* 1999b; Birch *et al.,* 2008a; Legerlotz *et al.,* 2007; Smith *et al.,* 1999). Whilst fibrillogenesis that occurs during tendon development has been studied extensively, the mechanisms of collagen turnover and repair in mature tendon are poorly understood. Although collagen is synthesised in response to exercise, this does not mean that it will be incorporated into the functional tendon matrix; rather it is possible that unless repair is required, the majority of the newly synthesised collagen is degraded as increasing the collagen content of energy storing tendons such as the equine SDFT and human Achilles would increase the stiffness of these tendons, decreasing their energy storing capacity. Extensive remodelling of the tendon would also result in a transient decrease in mechanical properties, which would actually further increase the risk of injury. In a similar manner, although pro-collagen molecules were present at low levels in most of the SDFT samples assessed, they may be degraded rather than be incorporated into the matrix. It is possible the increase in collagen production in tendon in response to exercise is to enable the repair of micro-damaged collagen molecules but does not result in an overall increase in tendon strength or stiffness. The data presented in this and previous studies has not identified a decrease in collagen protein production with increasing age, suggesting that the increased collagenous matrix half-life in aged tendons is not due to a decreased ability of the cells to synthesise structural matrix proteins.

6.4.3. Collagen Degradation

The higher levels of markers of collagen degradation in the CDET support the conclusions from the previous chapters that collagen degradation occurs more rapidly in the low strain positional CDET than in the high strain energy storing SDFT. Levels of ICTP were approximately 6 fold higher in the CDET than in the SDFT, and decreased with increasing age in both tendons. These data are supported by previous findings (Birch *et al.,* 2008b), which reported that levels of ICTP were 5 fold greater in the CDET than in the SDFT. Several studies have used ICTP levels to assess alterations in collagen degradation within tendon that occur with exercise or injury. Levels of ICTP have been found to increase in synovial fluid from patients with torn rotator cuff tendons (Tajana *et al.,* 2009), however analysis of the insoluble fraction of ruptured Achilles tendons found no difference in ICTP levels when compared to controls (Eriksen *et al.,* 2002). ICTP has also been measured as a marker of collagen turnover in the Achilles tendon in response to training; ICTP concentration in peritendinous tissue increased transiently at the beginning of a training program, and then decreased to pre-training levels (Langberg *et al.,* 2001), suggesting an initial increase in collagen degradation at the start of a training program, possibly acting to repair micro-damage caused in the initial training period. ICTP levels have also been reported to decrease with increasing age in the insoluble fraction of the matrix of Achilles tendons, both in ruptured and normal tendons (Eriksen *et al.,* 2002). Assessment of ICTP in the insoluble fraction of the matrix is a measure of mature crosslink concentration rather than collagen degradation and this study may therefore indicate a disruption in crosslink formation with increasing age. The alterations in ICTP levels with exercise and injury indicate that tenocytes in mature tendon are able to degrade and remodel the matrix.

C1,2C levels were higher in the CDET than in the SDFT, again suggesting an overall greater level of collagen turnover in the low strain positional tendon. The C1,2C data indicate that approximately 1.5% of the collagen within the matrix of the CDET is cleaved, this figure is irrespective of horse age. In contrast, C1,2C concentration increased significantly with age in the SDFT; in the youngest horse assessed 0.9% of the collagen within the SDFT had been cleaved whereas in the oldest horse this figure increased to 1.9%. These data contradict the results presented in chapter 4 that show that collagen half-life increases with increasing horse age. Taken together these results could be explained by an accumulation of partially degraded collagen within the matrix. GuHCl extraction is not

able to remove the partially cleaved collagen resulting in a decline in ICTP levels whereas trypsin digestion releases the partially cleaved collagen which has accumulated with increasing age in the SDFT. The C1,2C levels measured in this chapter may be an underestimation as other enzymes present in the matrix would tend to digest the newly exposed neoepitope. Partially degraded collagen that remains in the matrix would still be subjected to age related changes such as glycation and aspartate racemization hence an increased collagen half-life is apparent.

6.4.4. Effects of Accumulation of Partially Degraded Collagen within Tendon Matrix

This accumulation of collagen fragments within tendon matrix is likely to be due to increased resistance to degradation as a result of modifications that occur to the extracellular matrix with age. Glycation results in the formation of AGEs between the collagen molecules, which causes an increase in the stiffness and strength of the matrix in artificially glycated tendons *in vitro* (Reddy *et al.,* 2002; Reddy 2004) and also as a result of ageing or diabetes *in vivo* (Avery and Bailey 2005; Monnier *et al.,* 2008). Enzymatically derived crosslinks are only able to form at the telopeptide regions of the collagen molecules, whereas glycation can occur throughout the triple helical region (Avery and Bailey 2005). This has important implications for collagen degradation as collagen molecules may be partially degraded by collagenases but the ¾ and ¼ fragments may remain cross-linked into the matrix (Figure 6-15) and so may be more resistant to degradation by other degradative enzymes present within tendon.
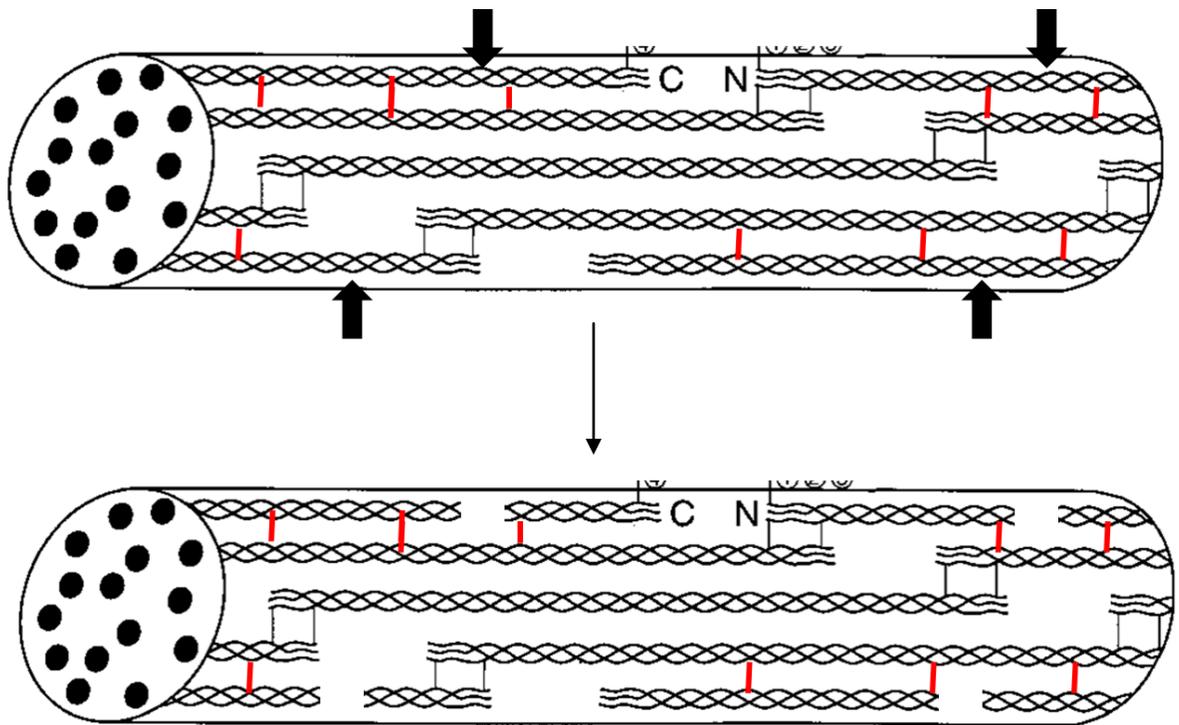
Figure 6-15: Accumulation of partially degraded collagen within a collagen fibril as a result of non-enzymatic glycation. Lysyl oxidase mediated crosslinks are represented by black lines; glycated crosslinks are represented by red lines; black arrows represent the site of collagenase cleavage. Adapted from Diab *et al.* (1996).

Increased resistance to degradation with increasing age is not unexpected. There is considerable experimental evidence to support decreased digestibility of collagen with increasing age, the fraction of collagen than could be extracted from skin samples by pepsin digestion decreased as a function of age (Schnider and Kohn 1981), indicating an increased resistance to degradation with increasing subject age. Furthermore, glycated type II collagen shows greater resistance to degradation by collagenases *in vitro* than non-glycated collagen (DeGroot *et al.,* 2001; Verzijl *et al.,* 2000a). Formation of AGEs within tendon has also been shown to affect the molecular stability of collagen in response to tensile overload; immature bovine tail tendon showed a significant decrease in collagen thermal stability when overloaded, whereas the decrease in thermal stability in older bovine tail tendon, which contained a greater concentration of crosslinks, was reduced (Willett *et al.,* 2010). It has been proposed that the decrease in thermal stability in response to overloading occurs as a result of disruption of the structure of the collagen fibrils, such that there is an increase in the conformational freedom of the collagen molecules, increasing the potential for proteolysis (Willett *et al.,* 2008). This would suggest that collagen within the high strain SDFT would be more susceptible to degradation than the low strain CDET. However, the

data presented here indicates the collagenous matrix in the SDFT is more resistant to degradation than that in the CDET. The longer half-life of collagen molecules within the SDFT identified in chapter 4 means that there is a greater potential for AGE formation in this tendon. Increased levels of crosslinking in glycated collagen would mean that overloading would result in maintenance of collagen structure, such that there would be minimal increases in the potential for proteolysis. The data presented in this chapter show that increased pentosidine levels are correlated with decreased extractability of ICTP and increased C1,2C levels within the matrix of the SDFT, suggesting levels of this AGE contribute to the accumulation of partially degraded collagen in high strain energy storing tendons. Interestingly, pentosidine showed no correlation with the markers of collagen degradation in the CDET, suggesting glycation does not affect the resistance of the matrix to degradation in this tendon.

6.5. Conclusions

- Collagen synthesis at the level of translation is lower in the high strain energy storing SDFT than in the low strain positional CDET.

- Matrix collagen degrading enzymes are functional within both tendons but the higher levels in the CDET result in an overall greater rate of collagen degradation in this tendon.

- Accumulation of markers of collagen degradation within the SDFT matrix suggests that the collagenous matrix becomes more resistant to degradation with increasing age, leading to a reduced rate of repair.

- This increased resistance to complete degradation is likely to be related to the increase in age related matrix modifications including the formation of AGEs in aged tendon, which results in the accumulation of partially degraded collagen within the matrix.

- Accumulation of partially degraded collagen within the SDFT matrix could have a deleterious effect on tendon mechanical properties, decreasing the ability of the tendon to resist force and therefore resulting in an increased risk of tendon injury in aged individuals.

CHAPTER SEVEN

7. Tendon Cell Phenotype is Altered by Cell Environment

7.1 Introduction

The data presented in the previous chapters of this thesis show that there is a difference in cell phenotype between the functionally distinct superficial digital flexor tendon (SDFT) and common digital extensor tendon (CDET). This difference is most apparent in the expression of genes that code for collagen type I and decorin; cells in the low strain positional CDET express higher levels of the Col1A2 gene whereas those in the high strain energy storing SDFT express more mRNA for decorin. Correspondingly, the ratio of collagen type I to decorin expression is approximately four times greater in cells from the CDET than those from the SDFT and therefore this ratio is a potential marker that could be used to differentiate between cells from functionally distinct tendons. There is also a difference in the potential of the cells to degrade tendon matrix; SDFT tenocytes produce more message for enzymes able to degrade the non-collagenous matrix (MMP-3) whereas cells in the CDET express higher levels of collagenases (MMP-13). This difference in matrix synthesis and degradation is maintained at the protein level; collagenous proteins have a greater half life in the SDFT (see chapter 4). In contrast, turnover of the non-collagenous matrix occurs at a more rapid rate in the SDFT than in the CDET, based on estimates of non-collagenous protein half life. Due to their distinct functions, the SDFT and CDET experience different amounts and rates of deformation; strains of up to 16% have been recorded in the energy storing SDFT (Stephens *et al.,* 1989), whereas maximum strain in the positional CDET has been estimated as 2.5% (Birch *et al.,* 2008b). Therefore, cells in the SDFT are likely to experience greater strain than those in the CDET.

Previous work has identified differences in matrix composition and turnover both between different tendons and within regions of tendon that undergo different loading conditions. It has been reported that levels of matrix degrading enzymes are greater in the injury prone supraspinatus tendon than in the biceps brachii tendon, which is injured rarely (Riley *et al.,* 2002). Studies have also shown that there are significant differences in matrix composition between regions of tendon that experience tensile loading and areas that are placed under compressive load (Lin *et al.,* 2005b; Rees *et al.,* 2000). These studies indicate that the mechanical environment the cells experience may be an important determinant of cell phenotype.

However, it has not been established if the difference in phenotype between cells from functionally distinct tendons is due to the different levels and rates of strain that the cells experience *in vivo* or if it is pre-set during tendon development. Studies have shown that mature tenocytes are able to respond to application of mechanical load, both *in vivo* and *in vitro* (Archambault *et al.,* 2002; Banes *et al.,* 1999a; Langberg *et al.,* 2001; Miller *et al.,* 2005). It is therefore more likely that the distinct phenotype exhibited by cells from functionally distinct tendons is due to the different strains the resident tenocytes experience during normal function *in vivo* rather than being in-built. Cell phenotype in functionally distinct tendons can be investigated first by exposing tenocytes from the SDFT and CDET to the same conditions and second by exposing tenocytes to strains they would not experience *in vivo* and measuring alterations in gene expression.

Although the SDFT experiences strains approximately six times greater than those experienced by the CDET (16% compared to 2.5%), it cannot be assumed that the tenocytes in the SDFT are deformed six times more than the cells in the CDET. Tendon response to strain is dependent on cell matrix interactions (Ingber 1997) and the material properties of the tendon (Cheng and Screen 2007). Strain distribution within tendon is heterogeneous and complex; at high strains the majority of cells within a tendon will experience strains that are much lower than the strain experienced by the tendon as a whole. Studies have shown that when a strain of 8% is applied to tendon fascicles the strain experienced by the resident tenocytes rarely exceeds 2% (Screen *et al.,* 2003; Screen *et al.,* 2004). However, finite element analysis suggests that there are areas of stress concentration within tendon; tenocytes residing within these areas may experience higher strains, approaching, and even possibly exceeding, those experienced by the whole tendon (Screen and Evans 2009). Furthermore, there is also evidence to suggest that during loading tenocytes experience high compressive strains due to the exudation of water from within the fascicles (Cheng and Screen 2007).

The majority of studies that have investigated the effect of applied mechanical strain on tenocyte phenotype *in vitro* have placed cells in an environment very different to that which they experience *in vivo*, both in terms of the mechanical and physiochemical environments. This may have a significant effect on cell activity; it has been shown that tendon cell phenotype in culture is not stable but changes with increasing passage number (Yao *et al.,*

2006). Therefore results should be interpreted with caution when attempting to predict the effect of strain on tenocyte phenotype *in vivo*. Loading tenocytes within their normal matrix allows maintenance of cell matrix interactions and so is more representative of the conditions the cells will experience when exposed to load *in vivo*. However, whole tendons are large structures which cannot be maintained in culture conditions without loss of cell viability therefore most studies investigate the effect of load on tendon cell phenotype by dissecting fascicles from the tendon and exposing them to load *in vitro* (Maeda *et al.,* 2009; Maeda *et al.,* 2007). This involves cutting the tendon, a process which will affect the hierarchical structure of the tendon and therefore alter the manner in which strain is transferred throughout the fascicle. Further, making an incision into healthy tendon may induce a damage response. The medial accessory extensor tendon (MAET) is situated on the medial border of the CDET and has a much smaller cross sectional area compared to the SDFT and CDET. Furthermore, it is possible to dissect this tendon free from the border of the CDET without damaging its structure, making it ideal for use as scaffold for the application of mechanical load to tenocytes. No previous studies have used the MAET to expose tenocytes to strain and no studies have investigated the properties of this tendon and compared these to the SDFT or CDET.

7.1.1. Aims and Hypothesis

The aims of this chapter were firstly to determine if removing SDFT and CDET tenocytes from their *in vivo* environment and culturing these cells in the same environment would result in a loss of their distinct phenotypes. Tendon cell phenotype was assessed by measuring the expression of key matrix proteins and degradative enzymes in 2D monolayer culture and compared to the *in vivo* data presented in chapter 5. The second aim was to determine if tendon specific phenotype could be restored by culturing cells in an environment more similar to native tendon tissue. This was achieved by seeding cells in 3D collagen gels and assessing levels of gene expression. The final aim was to subject cells to an unfamiliar strain environment and determine if their phenotype is altered. The MAET was subjected to high and low levels of strain and differences in gene expression were assessed. It was hypothesised that tendon specific phenotype is due to the different *in vivo* cell environment and therefore culturing cells in monolayer in the absence of mechanical load would result in significant alterations in cell phenotype and differences between SDFT and CDET tenocytes would be lost. Culture of cells in collagen gels was hypothesised to

result in a phenotype more similar to that seen in native tendon tissue. In contrast, explant culture of the MAET would allow maintenance of tendon phenotype and application of high mechanical load would result in up-regulation of genes associated with SDFT tenocyte phenotype. Specifically, high strain levels were hypothesised to result in a significant decrease in the ratio of type I collagen to decorin expression.

7.2 Materials and methods

7.2.1. Cell culture

The SDFT and CDET were collected from 5 horses aged six to ten years euthanased for reasons other than tendon injury at a commercial equine abattoir. Cells were extracted from the tendon tissue on the same day as euthanasia as described by Birch *et al.* (1997a). A section of tendon (approximately 1 cm length) was removed from the mid-metacarpal region of the tendon and washed in sterile PBS (x3). The tissue was then rinsed in sterile PBS plus antibiotics (100 µg/ml streptomycin and 200 U/ml penicillin) and fungizone (5 µg/ml amphotericin). The outer tissue was trimmed away and the remaining tissue transferred to a clean tube. The tissue was washed 3 times in sterile PBS plus antibiotics and fungizone. The tissue was then transferred to a clean tube in sterile PBS plus antibiotics and fungizone and into the laminar flow cabinet. Samples of tendon tissue (approximately 1 mm^3) were placed in Petri dishes and covered with a sterile glass coverslip. Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen Corporation, Paisley, UK) supplemented with 10% foetal calf serum (FCS; Invitrogen Corporation) plus antibiotics (streptomycin (100 µg/ml) and penicillin (200 U/ml)) and fungizone (amphotericin (5 µg/ml)) was added to each Petri dish, which were then incubated in a humidified incubator at 37 ºC with 5% CO_2. Once the cells had emerged from the tissue and reached confluence, the medium and tissue were removed and cells were released with 0.05% trypsin in sterile phosphate buffered saline (PBS) with 0.02% EDTA and 0.01% Phenol Red. Cells were transferred to 25 cm^2 tissue culture flasks and cultured in DMEM (5 ml) with 10% FCS plus antibiotics (as above). Cells at passage 2 were frozen in vials of 0.5 x 10^6 cells in 0.5 ml 90% FCS plus 10% dimethyl sulphoxide (DMSO) and stored at -196 ºC in liquid nitrogen.

When required, vials of cells were thawed and cultured in DMEM supplemented with 10% FCS and antibiotics (as above) in 25 cm^2 tissue culture flasks. Media was changed after 24

hours to remove DMSO. Cells were grown to confluence before passaging; flasks were washed with sterile PBS and cells were released with 0.05% trypsin in sterile PBS with 0.02% EDTA and 0.01% Phenol Red and transferred to 75 cm^2 tissue culture flasks where they were re-suspended in DMEM supplemented with 10% FCS and antibiotics (15 ml). When the cells reached confluence at passage 4 they were released from the flasks with trypsin solution and an equal amount of media was added. The cell suspension was centrifuged (1000 g, 5 min.) and the resulting pellet was re-suspended in 1 ml media. An aliquot (20 µl) was removed and mixed with an equal amount of trypan blue for a live/dead stain. Cell number was determined using a cytometer. Cell viability was in excess of 90%. Flasks contained an average of 1.01×10^6 ($\pm 1.41 \times 10^4$) cells. Cells were re-pelleted by centrifugation and re-suspended in media to give a concentration of 5×10^6 cells per ml DMEM.

7.2.2. 2D and 3D Cell Culture

Collagen gels were set containing 5×10^5 cells in a 1 ml gel (Eastwood *et al.,* 1994). A solution was made up containing 0.8 ml rat tail tendon collagen in 0.1 M acetic acid (First Link, West Midlands, UK) and 0.1 ml 10x minimum essential medium (Gibco). This was neutralised by the addition of sterile filtered 5 M NaOH, using the colour change from yellow to cirrus pink as an indication the correct pH had been reached (Cheema *et al.,* 2003). Cell suspension (0.1 ml containing 5×10^5 cells) was added to the neutralised collagen solution and this was allowed to gel in a 12 well plate for 30 minutes at 37 ℃. 4 ml DMEM supplemented with 10% FCS was then added to cover each gel. Cells from the same passage were seeded in 25 cm^2 tissue culture flasks at a density of 5×10^5 cells per flask (monolayer) in 5 ml DMEM plus 10 % FCS. Cells in monolayer and in collagen gels were allowed to stabilise for 48 hours before the media was changed to DMEM supplemented with 1% FCS, antibiotics and ascorbic acid (50 µg/ml). Monolayers and gels were incubated for 24 hours before Tri Reagent solution was added to release the cells from the tissue culture flasks and lyse the gels. The media was removed from the gels and monolayers and Tri reagent was added directly to the tissue culture flasks (1 ml Tri reagent per 10 cm^2 surface area); the cell lysate was passed through a pipette several times to homogenise the sample. Gels were blotted on tissue paper and placed in Tri Reagent (2.5 ml per gel); the gels were passed through a pipette several times to homogenise the

collagen gel. Samples were left to stand at room temperature for at least 5 minutes before being frozen at -80 °C.

7.2.3. Loading Experiments

7.2.3.1   Harvesting of the MAET

The CDET and surrounding tissue were harvested from 12 horses euthanased for reasons other than tendon injury at a commercial equine abattoir. Forelimbs were collected on three separate occasions in order that the loading experiments could be carried out on the same day as harvesting. The MAET was dissected free from the medial border of the CDET (Figure 7-1). MAETs were excluded if they were either too thick or thin, or were fused to the CDET at the medial edge. The MAETs were washed (see below) and placed into culture or RNAlater on the same day as euthanasia.
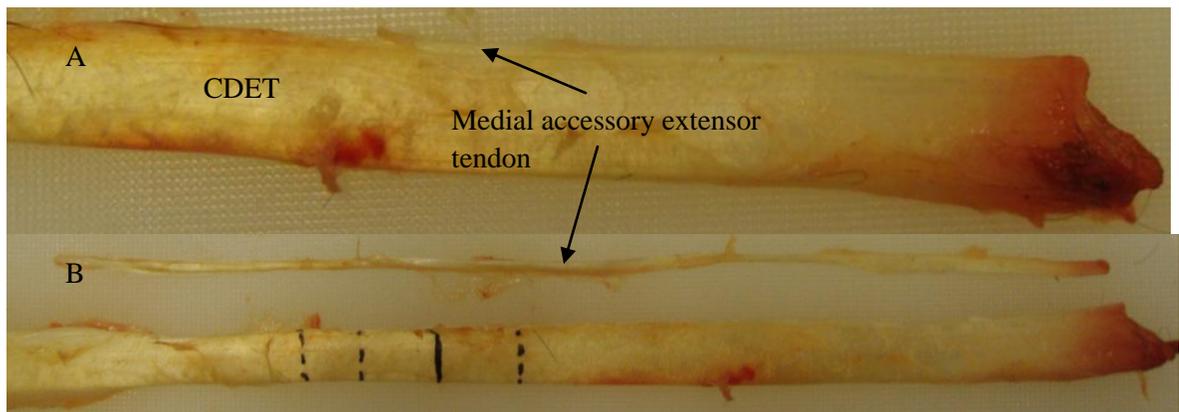
Figure 7-1: Location of the medial accessory extensor tendon (MAET). A: Proximal end of the CDET showing location of MAET on the medial border of the tendon. B: The MAET has been dissected free of the CDET proper.

7.2.3.2   Molecular Composition and Histological Parameters of the MAET

Preliminary work was undertaken to assess the molecular composition and histological parameters of the MAET along the length of the tendon and these data were compared with the CDET proper. The MAET appeared similar to the CDET in structure, consisting of longitudinal bundles of collagen fibres with elongated cells situated between the fibres (Figure 7-2). The collagen, DNA and GAG content of the MAET did not differ along the length of the tendon and was not significantly different from the CDET (Birch H.L., unpublished data).
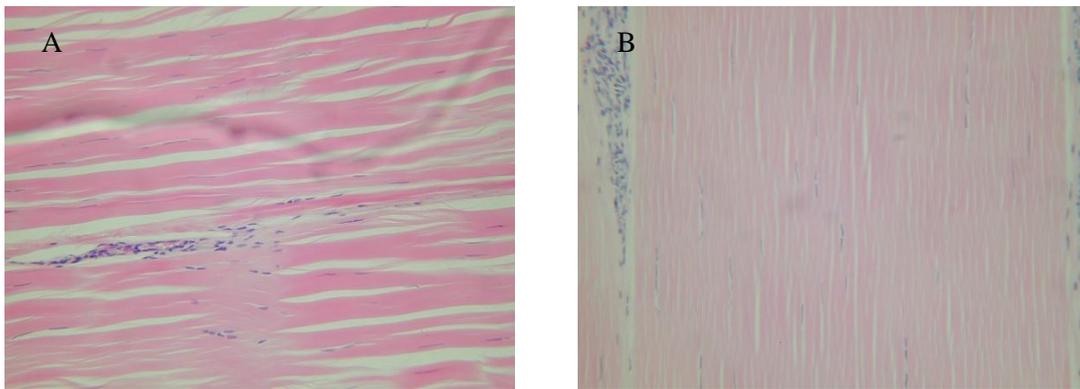
Figure 7-2: Longitudinal haemotoxylin and eosin stained section of CDET (A) and MAET (B).

7.2.3.3 Loading Protocol

The MAETs were divided into three sections approximately 65 mm in length. One tendon section from four horses was immediately placed in RNAlater in order to compare gene expression with the CDET and SDFT. The remaining strips were washed in three times in sterile PBS with antibiotics (streptomycin (100 µg/ml) and penicillin (200 U/ml)) and fungizone (amphotericin (5 µg/ml)). Strips of tendon were clamped into the custom made loading cassette (Figure 7-3) (Dudhia *et al.,* 2007) and each strip was bathed in 2 ml DMEM supplemented with 1% FCS, antibiotics, fungizone and ascorbic acid as above.
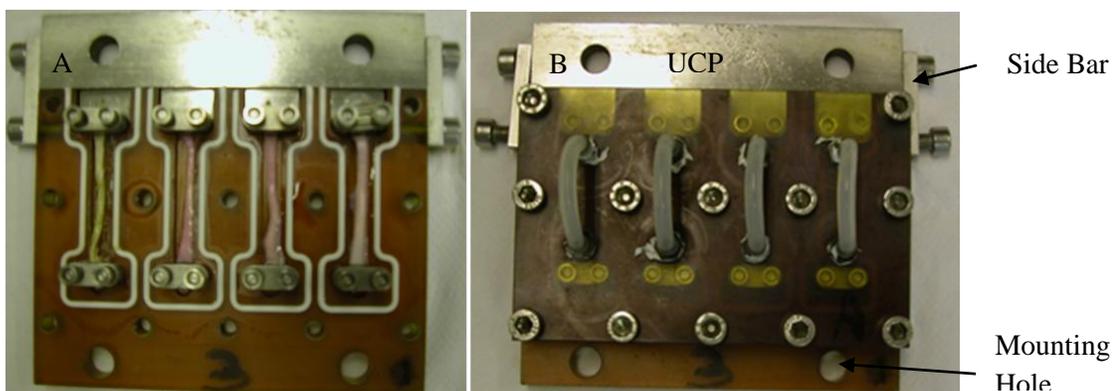
Figure 7-3: Cassette with four separate chambers for strain application to tendon strips. Each tendon strip was held in place by a clamp at each end (A). The top block of the cassette was bolted securely onto the base block. Medium was applied via the tubing on access ports over each chamber (B). UCP; upper clamp plate.

The strips of MAET were divided into three experimental groups; unloaded, low strain (2%) and high strain (12%). Tendon strips in the unloaded group were clamped into the chambers in the loading cassette but were not loaded during the culture period. The strips in the low strain group were clamped into the cassette which was placed in the modified servohydraulic materials testing device (Figure 7-4) (Zwick-Roell GmbH & Co, Ulm,

Germany) and were conditioned under cyclical strain of 2% at a frequency of 1 Hz for 20 hours. Tendons in the high strain group were exposed to cyclical strain of 12% at a frequency of 1 Hz for 20 hours. For each strip in the experimental groups, a section from the same MAET was maintained in normal tissue culture conditions for the same time period to act as a paired control.
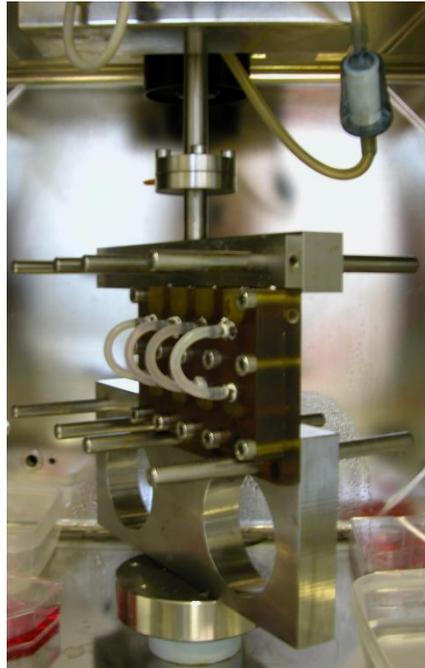
Figure 7-4: The loading cassette was placed onto the hydraulic ram via the mounting holes. Once the cassette was in place the side bars were removed, which allowed the upper clamp plate to slide relative to the lower body when strain was applied. The top end of each chamber was open to the humidified atmosphere of the incubator.

After the loading protocol was completed strips were removed from the loading cassette and the middle 2 cm section of each strip was placed in RNAlater. Samples were stored at 4 ºC for 24 hours before being stored at -20 ºC. MAET strips were then flash frozen in liquid nitrogen, reduced to a fine powder by the use of a dismembrator (2500 rpm, 2 min., Sartorius, Germany) and placed in Tri reagent solution (approx 100 mg/ml), which was stored at -80 ºC until required.

7.2.4. Assessment of Cell Phenotype

Messenger RNA was extracted from the samples stored in Tri reagent solution; mRNA from 2D and 3D culture experiments was extracted by the use of Qiagen RNeasy kits with an additional on-column DNA digestion step as described in chapter 5. An isopropanol precipitation was used to extract mRNA from the mechanically loaded MAET strips;

sample supernatants were mixed with choloroform (200 µl), vortex mixed and allowed to stand at room temperature for 10 minutes before being centrifuged (16 000 g, 4 ℃, 15 min.). The upper aqueous layer containing the mRNA was removed to a clean tube and an equal volume of isopropanol was added. Samples were incubated at room temperature for 5 min. and centrifuged for 10 min. (16 000 g, 4 ℃). The isopropanol was poured off and the tubes were inverted to allow the RNA-containing pellet to dry. The pellet was washed in 70% ethanol (1 ml, made up in 0.1% DEPC water) and centrifuged for 5 min. (16 000 g, 4 ℃). Tubes were inverted and allowed to dry completely. The mRNA was reconstituted in 25 µl 0.1% DEPC water. RNA concentration was determined using a NanoDrop spectrophotometer and up to 1 ng RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase and random primers according to the manufacturer's instructions (Promega). Samples were stored at -20 ℃ prior to quantification of gene expression. The expression of Col1A2, decorin, MMP-3, MMP-13, scleraxis, tenascin-C and tenomodulin was assessed using real time PCR assays in a 96 well plate format, with 5 µl cDNA (diluted 1 in 9), 12.5 µl SYBR green master mix (Applied Biosystems), 2.5 µl forward and reverse primers (3 µM) and 5 µl d H_2O per well. The cycling conditions were 10 min. polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 sec. and 60 ℃ for 60 sec. A dissociation step was then performed to check primer specificity. Primer sequences are the same as used in chapter 5, with the addition of tenomodulin: forward primer-5'ACGTGACCATGTATTGGATCAATC3'; reverse primer-3'CACCATCCTCCTCAAAGTCTTGT5' (Taylor *et al.,* 2009). Tenomodulin expression was expected to be low in the cultured cells, so more concentrated cDNA (diluted 1 in 2 rather than 1 in 9) was used to measure tenomodulin expression by cultured cells. Gene expression data were expressed relative to MRPS7 and HIRP5 expression levels, which were identified as the most stably expressed genes in equine tendon (see Chapter 5). Data were analysed using Sequence Detection Systems Software v2.1 (Applied Biosystems, Warrington, UK.).

7.2.5. Statistical Analysis

None of the gene expression data were normally distributed when analysed using the Kolmongorov-Smirnoff test for normality (Minitab, Version 15) and therefore data were analysed using non-parametric tests in SPlus. The effect of cell culture on gene expression was determined using Kruskal-Wallace rank sum tests; if the results of this were significant

post-hoc analysis was carried out using an exact Wilcoxon rank-sum test. To determine the effect of culture on gene expression in the MAET linear mixed effects analysis was used, with horse as a grouping factor. All data are displayed as mean ± SEM.

7.3 Results

7.3.1. Gene Expression in 2D and 3D Cell Culture

Average gene expression normalised to MRPS7 and HIRP5 is shown in Table 7-1. For comparison, the *in vivo* data reported in chapter 5 are displayed on the expression graphs for each gene. Expression of Col1A2 did not differ between tenocytes from the SDFT and CDET in 2D or 3D culture (Figure 7-5). Compared to *in vivo* data, Col1A2 expression was increased by tenocytes cultured in monolayer (p≤0.003). Culturing cells in 3D gels did not result in a significant change in Col1A2 expression when compared to 2D culture, although there was a trend towards decreased expression in both cell types; SDFT tenocytes cultured in collagen gels showed significantly higher levels of Col1A2 compared to *in vivo* data (p<0.0001), whereas CDET tenocytes cultured in 3D expressed similar levels of Col1A2 when compared to *in vivo* results (p=0.3) (Figure 7-5).
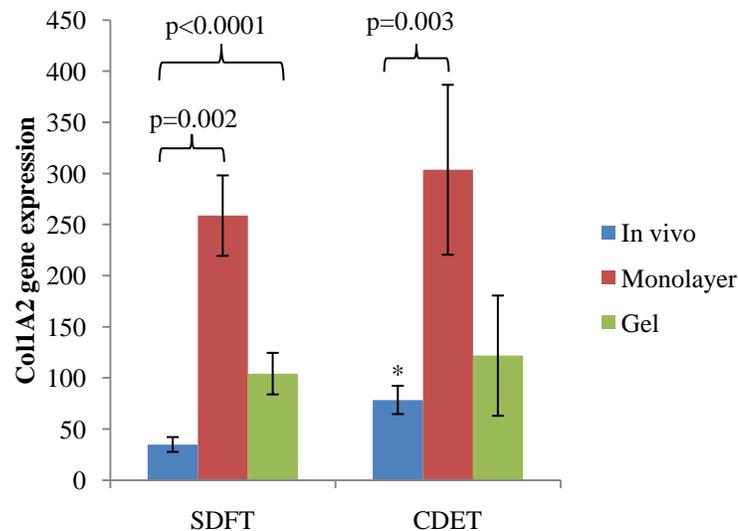
Figure 7-5: Col1A2 expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in tenocytes from the SDFT and CDET. * indicates significant difference relative to the SDFT.

Gene	Gene Expression (mean ± SEM)					
	SDFT			CDET		
	In vivo	Monolayer	3D Gel	*In vivo*	Monolayer	3D Gel
Col1A2	34.93±7.23	258.76±39.32*	104.19±20.34$^\lambda$	78.46±13.83$^\beta$	303.57±83.12*	121.77±58.81
Decorin	1343.42±302.85	21.16±5.23*	38.92±10.47$^\lambda$	483.48±53.23$^\beta$	24.83±9.66*	31.65±11.42$^\lambda$
MMP-13	0.16±0.12	0.33±0.12	10.36±5.15$^{\lambda\alpha}$	0.72±0.13$^\beta$	4.42±1.69*	91.55±55.70$^{\lambda\alpha}$
MMP-3	3.52±0.83	4.52×10^{-3}±$1.73 \times 10^{-3*}$	12.23×10^{-3}±$4.39 \times 10^{-3\lambda}$	1.39±0.30$^\beta$	24.36×10^{-3}±13.86×10^{-3}	36.78×10^{-3}±$15.8 \times 10^{-3\lambda}$
Scleraxis	3.79±0.99	0.40±0.15	0.34±0.14	1.48±0.22$^\beta$	0.33±0.09	0.47±0.16
Tenascin	1.86±0.49	6.60±2.21*	2.10±0.46$^\alpha$	1.24±0.28	19.94±7.94*	4.44±1.09
Tenomodulin	-	9.72×10^{-5}±2.06×10^{-5}	24.9×10^{-5}±9.74×10^{-5}	-	1.79×10^{-3}±1.51×10^{-3}	29.08×10^{-5}±9.37×10^{-5}
Col1A2:DCN	0.047±0.01	14.21±2.4*	3.61±1.29$^{\lambda\alpha}$	0.18±0.016$^\beta$	32.30±23.23*	4.19±0.72$^{\lambda\alpha}$

Table 7-1: Gene expression levels by tenocytes from SDFT and CDET *in vivo* (n = 32), and in 2D (monolayer) (n = 5) and 3D (collagen gel) (n = 5) culture systems. * indicates significant difference between *in vivo* expression levels and monolayer; $^\lambda$ indicates significant difference between *in vivo* data and 3D gels; $^\alpha$ indicates significant difference between gene expression in monolayer and gels; $^\beta$ indicates significant difference between SDFT and CDET gene expression *in vivo*.

There was no difference in expression of decorin between cell types, either when cultured in 2D or 3D (Figure 7-6). Decorin synthesis was significantly down-regulated by both cell types when cultured in monolayer (p<0.0001). There was a trend towards increased decorin expression when cells were cultured in 3D gels compared to monolayer culture but this was not significant; expression of decorin was significantly lower in SDFT and CDET tenocytes cultured in 3D than expression levels measured *in vivo* (p≤0.0003) (Figure 7-6).
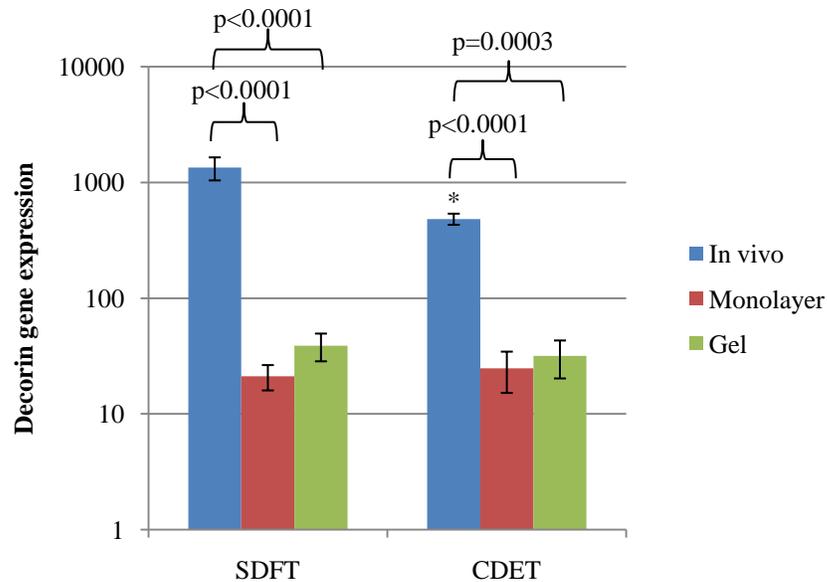
Figure 7-6: Decorin expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in SDFT and CDET tenocytes. * indicates significant difference between the SDFT and CDET. Data are plotted on a Log_{10} scale.

Expression of MMP-13 did not differ between SDFT and CDET tenocytes, either in 2D or 3D culture, although there was a trend towards increased MMP-13 expression in cells from the CDET in 3D culture (p=0.06). Expression of MMP-13 was increased in cells from the SDFT and CDET cultured in monolayer compared to *in vivo* data (p=0.005) (Figure 7-7). Culture of cells in 3D gels caused an upregulation of MMP-13 production in both tendons compared to *in vivo* data and when compared to 2D culture (p≤0.016) (Figure 7-7).
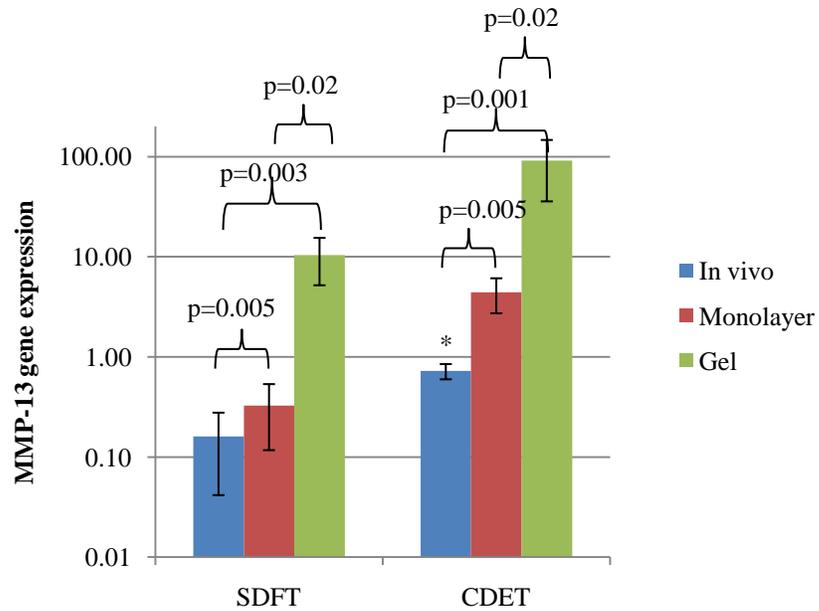
Figure 7-7: MMP-13 gene expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in tenocytes from the SDFT and CDET. * indicates significant difference between the SDFT and CDET. Data are displayed on a Log_{10} scale.

There was no difference in MMP-3 expression between cell types when cultured in either monolayer or gel (Figure 7-8). Expression of MMP-3 decreased significantly in SDFT tenocytes cultured in monolayer compared to *in vivo* data (p=0.001) but was not altered in CDET tenocytes. Culturing cells in 3D resulted in a trend towards increased MMP-3 expression compared to expression levels in monolayer, but this was not significant (Figure 7-8); cells expressed lower levels of MMP-3 in 3D culture than *in vivo* (p=0.02).
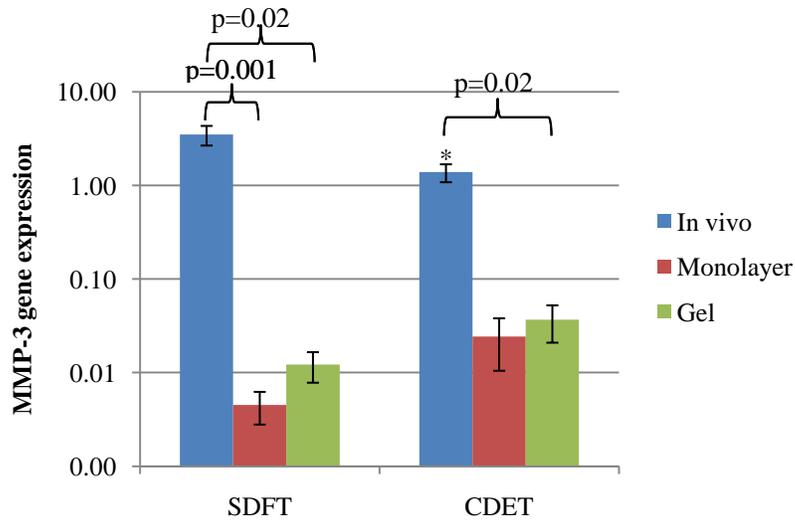
Figure 7-8: Expression of MMP-3 (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in tenocytes from the SDFT and CDET. * indicates significant difference between the SDFT and CDET. Data are plotted on a Log_{10} scale.

There was no difference in the amount of scleraxis expressed by cells from the SDFT or CDET in 2D or 3D culture. Scleraxis expression was significantly down-regulated when cells were cultured in 2D (p≤0.04) or 3D (p≤0.04) when compared to *in vivo* data (Figure 7-9), and values were not significantly different between the different culture conditions (Figure 7-9).
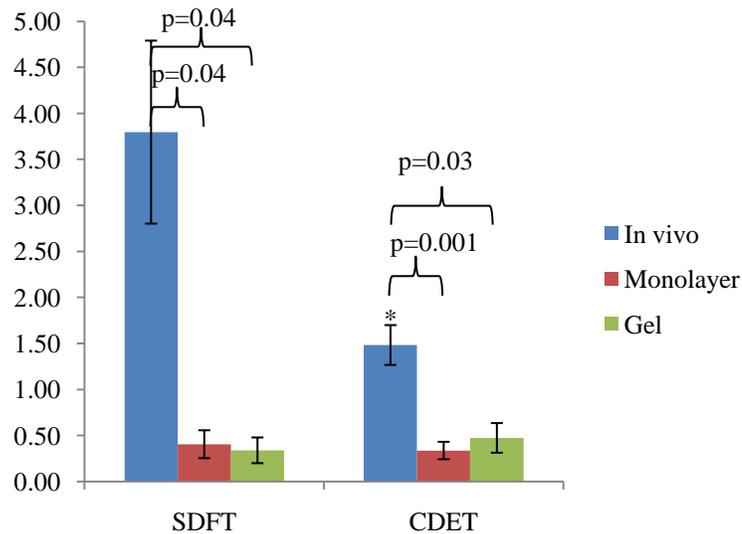
Figure 7-9: Scleraxis expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in tenocytes from the SDFT and CDET. * indicates significant difference between the SDFT and CDET.

Tenascin-C expression was not significantly different between cell types in either culture system (Figure 7-10). Tenascin-C expression was increased when cells were cultured in monolayer compared to the *in vivo* data (p≤0.02) (Figure 7-10). 3D culture resulted in a decrease in expression compared to 2D culture, although this was only significant in cells from the SDFT (p=0.008).
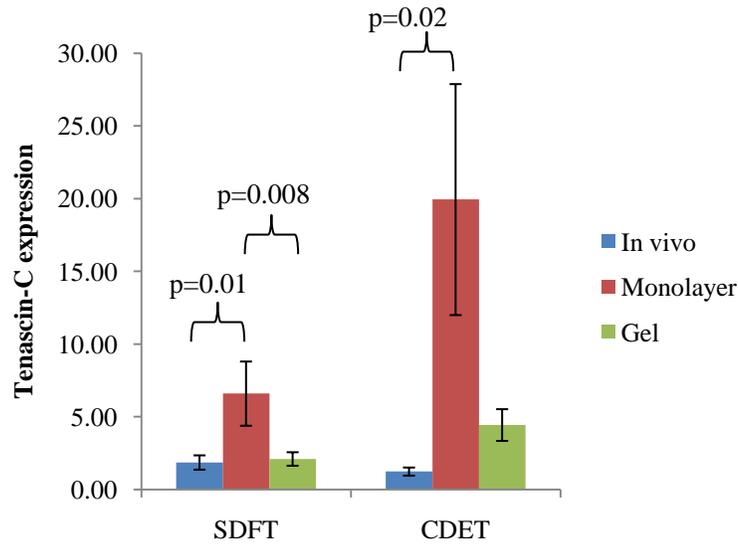
Figure 7-10: Tenascin-C expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in tenocytes from the SDFT and CDET.

Tenomodulin expression was very low in cultured cells, and was undetectable in some samples. There was no difference in tenomodulin expression in cells from the SDFT or CDET, and no difference in expression levels between 2D and 3D culture systems (Figure 7-11).
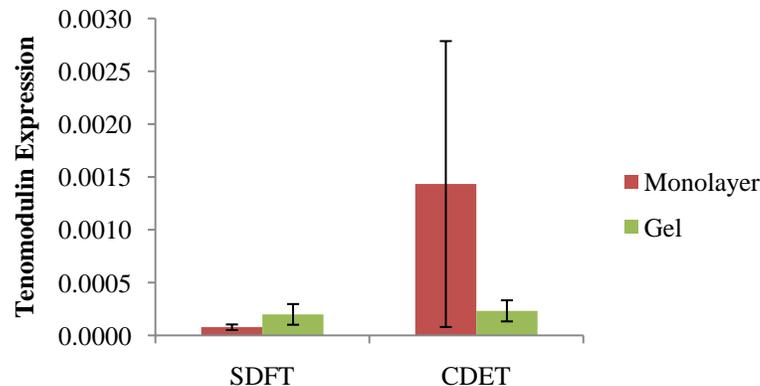
Figure 7-11: Tenomodulin expression (mean ± SEM) in tenocytes from the SDFT and CDET (n = 5) cultured in 2D and 3D culture systems.

There was no significant difference in the ratio of Col1A2 to decorin expressed by cells from the SDFT and CDET in either culture system (Figure 7-12). The ratio of Col1A2 to decorin expression was significantly greater in 2D culture than *in vivo* (p<0.0001) (Figure 7-12). Culturing cells in 3D resulted in a decrease in the ratio when compared to 2D culture (p≤0.02) but levels were still significantly increased when compared to *in vivo* data (p≤0.0001).
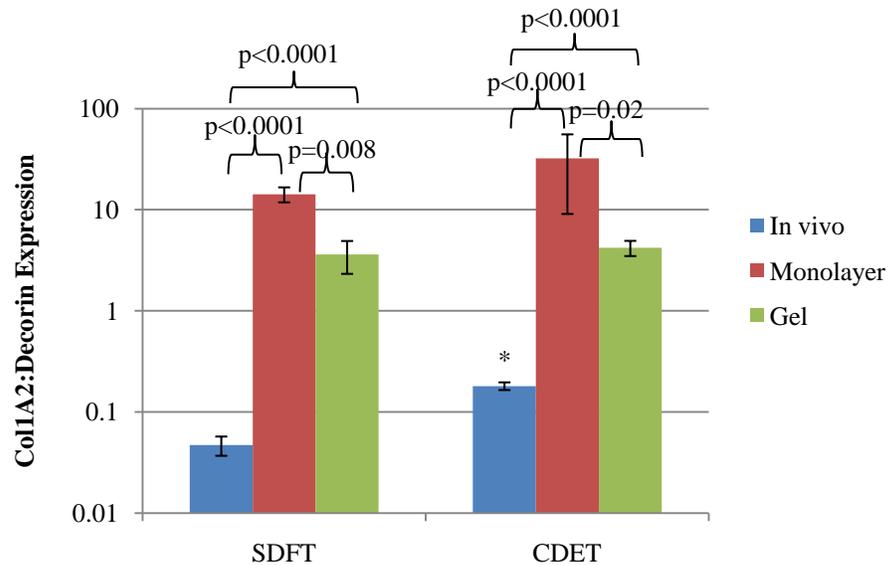
Figure 7-12: Ratio of Col1A2 to decorin expression (mean ± SEM) *in vivo* (n = 32), in monolayer culture and in 3D collagen gels (n = 5) in cells from the SDFT and CDET. * Indicates significant difference relative to the SDFT. Data are displayed on a Log_{10} scale.

7.3.2. Comparison of MAET, SDFT and CDET Gene Expression

As expected, gene expression was comparable between the MAET and CDET, with similar expression of Col1A2, decorin, MMP-13, resulting in a similar Col1A2 to decorin ratio in these tendons (Figure 7-13). However, the expression of MMP-3 was significantly greater in the MAET than in the CDET (p=0.01) as was the expression of tenascin-C (p=0.004), whereas expression of scleraxis was significantly lower in the MAET than in the CDET (p=0.007) (Figure 7-14). When gene expression data from the MAET were compared to the gene expression data from the SDFT (presented in chapter 5), it was found that there was no significant difference in the expression of Col1A2, decorin or MMP-3 between these tendons, although there was a trend towards increased Col1A2 expression (p=0.07) and decreased decorin expression (p=0.05) in the MAET compared to the SDFT (Figure 7-13).

Correspondingly, the ratio of Col1A2 to decorin expression was significantly greater in the MAET compared to the SDFT (p=0.002). Expression of MMP-13 (p=0.001) and tenascin (p=0.009) was significantly greater in the MAET than in the SDFT, whereas scleraxis expression was significantly lower (p=0.0001) (Figure 7-14).
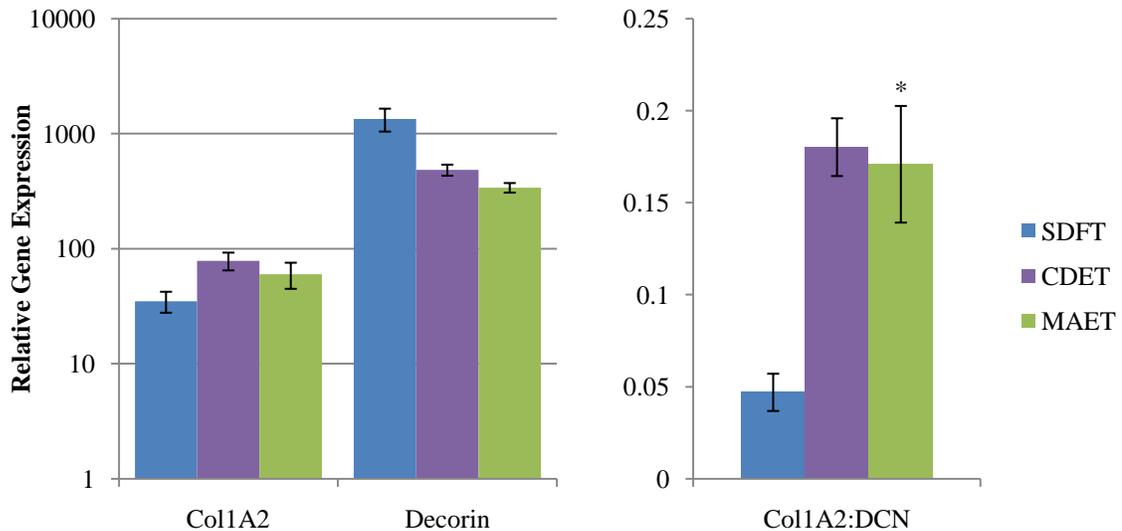
Figure 7-13: Expression (mean ± SEM) of Col1A2 and decorin (A – data displayed on Log_{10} scale) and ratio of Col1A2 to decorin expression (B) in the SDFT (n = 32), CDET (n = 32) and MAET (n = 4). * Indicates significant difference between SDFT and MAET.
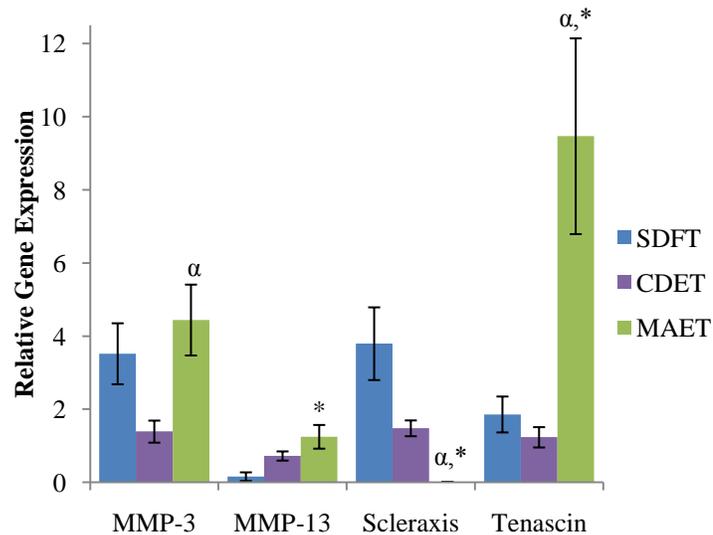
Figure 7-14: Expression (mean ± SEM) of MMP-3, MMP-13, scleraxis and tenascin in the SDFT (n = 32), CDET (n = 32) and MAET (n = 4). * Indicates significant difference between SDFT and MAET; $^{\alpha}$ Indicates significant difference between CDET and MAET.

7.3.3. Effect of Culture on Gene Expression in the MAET

Maintaining MAETs in organ culture for 24 hours did not result in significant alterations in gene expression, with statistically similar expression of Col1A2, decorin, MMP-3 and -13, scleraxis, tenascin and tenomodulin between cultured tendons and *in vivo* data. There was, however, a trend towards decreased decorin gene expression in cultured tendons (p=0.06) (Figure 7-15).
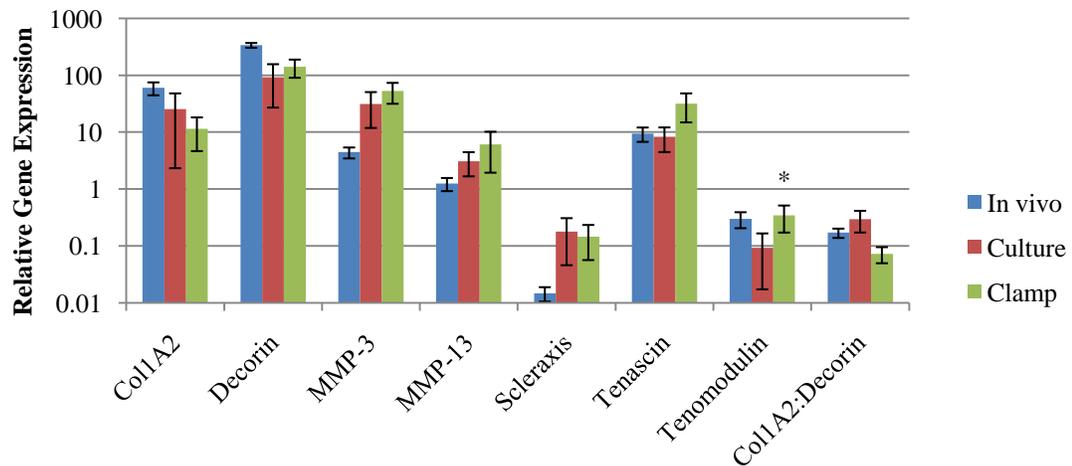
Figure 7-15: Expression (mean ± SEM) of genes coding for matrix proteins and degradative enzymes in native MAET tissue and after a 24 hour culture period under normal tissue culture conditions and when clamped into the loading cassette (n = 4). Data are displayed on a Log_{10} scale. * Indicates significant difference between normal culture conditions and clamping.

7.3.4. Effect of Clamping the MAET into the Loading Cassette

Clamping the MAET into the chambers of the loading cassette and culturing for a period of 24 hours in the absence of load did not result in significant alterations in cell phenotype (Figure 7-15). There was no significant difference in expression of Col1A2, decorin, MMP-3, MMP-13, scleraxis and tenascin-C between MAETs cultured for 24 hours in a 6 well plate and those clamped into the chambers and incubated within the loading cassette in the absence of load. Correspondingly, there was no difference in the ratio of Col1A2 to decorin expression between groups. The only gene that was affected by clamping was tenomodulin, which was significantly up-regulated in MAETs clamped into the loading cassette (p=0.04).

7.3.5. Effect of Cyclical Strain on Gene Expression

There was no difference between the gene expression in the low strain and high strain groups and so the data from these groups were combined to determine if there was a general loading effect. Cyclical loading did not result in significant alterations in gene

expression compared to unloaded controls; there was no difference in the expression of genes coding for matrix structural proteins Col1A2 and decorin, or corresponding degradative enzymes MMP-3 and -13 between groups (Figure 7-16). Cyclic loading also did not affect the ratio of Col1A2 to decorin expression. Scleraxis expression was significantly up-regulated in loaded samples compared to controls (p=0.002), whereas expression of tenascin-C and tenomodulin was not significantly affected by the loading protocol (Figure 7-17).
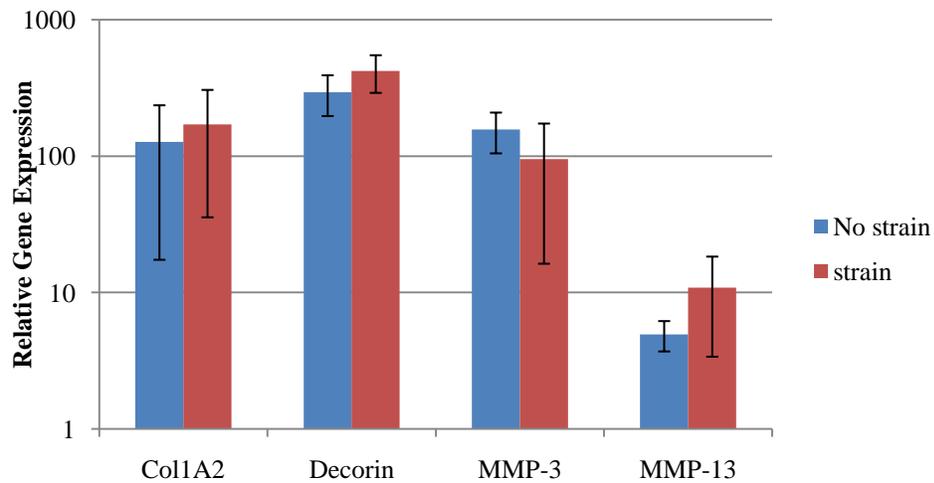
Figure 7-16: Expression of key matrix proteins and degradative enzymes did not differ between strained and unstrained groups (mean ± SEM) n = 4. Data are plotted on a Log_{10} scale.
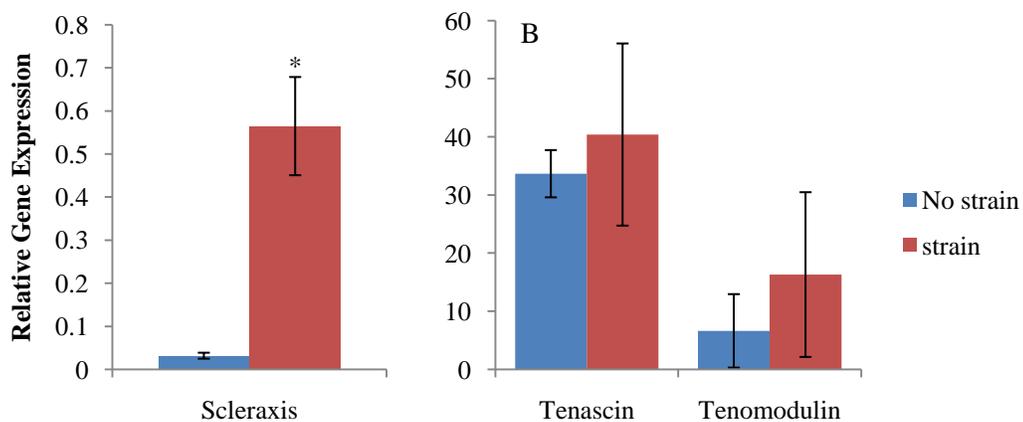
Figure 7-17: A – Scleraxis expression in loaded and unloaded MAETs. B – Tenascin-C and tenomodulin expression in loaded and unloaded MAETs (mean ± SEM) n = 4. * Indicates significant difference relative to the unstrained control.

7.3.6. Effect of Acidosis on Gene Expression

Due to problems with the CO_2 supply to the incubator during the low strain loading cycle, both the unloaded and loaded samples were exposed to high levels of CO_2 for the duration of this experiment. Comparing the unloaded controls in the low strain group with those in the high strain group therefore allows the effect of acidosis on gene expression to be determined. High CO_2 levels were found to significantly up-regulate decorin expression ($p=0.04$) and there was also a trend towards increased MMP-3 expression in tendons exposed to a high concentration of CO_2 ($p=0.06$) (Figure 7-18). Acidosis did not have a significant effect on the expression of Col1A2, MMP-13, scleraxis, tenascin or tenomodulin. There was also no difference in the ratio of type I collagen to decorin expression.
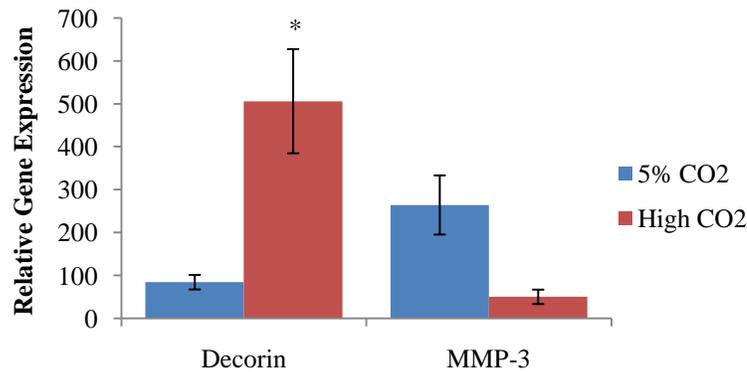
Figure 7-18: Expression levels of decorin and MMP-3 in MAETs exposed to normal carbon dioxide levels and high carbon dioxide levels (mean ± SEM) n = 4. * Indicates significant difference relative to normal CO_2 levels.

7.4 Discussion

The results of this chapter show that culture of tenocytes from the SDFT and CDET in monolayer caused the cells from these functionally distinct tendons to lose the phenotypic differences identified in chapter 5, and these differences were not restored when cells were cultured in 3D collagen gels. In contrast, organ culture of the MAET resulted in maintenance of cell phenotype and application of high strains to the MAET did not result in significant alterations in cell phenotype as assessed by measuring gene expression.

7.4.1. Effect of 2D culture on Tenocyte Phenotype

In support of the hypothesis, differences in gene expression exhibited by tenocytes from the functionally distinct SDFT and CDET in native tendon tissue were lost when cells were

removed from their *in vivo* environment. It is well established that culture of cells in monolayer results in significant alterations in cell phenotype when compared to native tissue (Almarza *et al.,* 2008; Darling and Athanasiou 2005; Schwarz *et al.,* 1976; Stoll *et al.,* 2010). Tenocytes maintained in 2D culture have been shown to de-differentiate rapidly; it has been found that human Achilles tenocytes cultured to passage 8 synthesised significantly lower amounts of decorin compared to those at passage 1 and increased the ratio of type III to type I collagen synthesis (Yao *et al.,* 2006). This supports the data presented in this chapter which identify a greater than 100 fold decrease in decorin expression at passage 5 in cells from both the SDFT and CDET. In addition, it has been previously shown that culture of immature rabbit Achilles tenocytes resulted in decreased decorin expression with increasing passage number (Bernard-Beaubois *et al.,* 1997) and cultured human tenocytes up-regulated collagen type I synthesis and down-regulated decorin synthesis when compared to native tendon tissue (Almarza *et al.,* 2008; Stoll *et al.,* 2010). The alterations in expression of matrix proteins identified in this and other studies show that monolayer culture causes cells to increase collagen synthesis and decrease proteoglycan production, resulting in an increase in the ratio of type I collagen to decorin expression and suggesting a loss of tenocyte specific phenotype. However in other studies fibroblasts that exhibit a different phenotype *in vivo* have been shown to maintain these differences in monolayer culture. Dermal fibroblasts proliferated more rapidly and synthesised lower levels of collagen and GAG than tendon fibroblasts cultured under the same conditions (Evans and Trail 1998).

7.4.2. Effect of 3D Culture on Tenocyte Phenotype

As hypothesised, culture of SDFT and CDET tenocytes in 3D collagen gels resulted in a phenotype more similar to the phenotype seen in native tendon tissue. Previous studies have attempted to maintain cell phenotype *in vitro* either by culturing cells in 3D high density cultures or embedding cells within constructs. Culture of tenocytes at high densities maintains cell phenotype over a 14 day culture period, with stable expression of type I collagen and scleraxis (Schulze-Tanzil *et al.,* 2004). However, this study did not compare *in vitro* cell phenotype with that in native tendon. Comparison of the 3D high density culture system and monolayer culture with native tendon tissue found that the high density culture system resulted in a phenotype more similar to that expressed in native tendon, although there were still significantly lower levels of scleraxis and higher levels of type III

collagen compared to native tendon (Stoll *et al.,* 2010). Embedding tenocytes within 3D constructs has also been explored as a method of maintaining tenocyte phenotype *in vitro*; it has been reported that tenocytes seeded in poly[lactic-co-glycolic-acid] matrices exhibit a phenotype more similar to native tendon when compared to monolayer and high density culture (Stoll *et al.,* 2010). In addition, seeding tenocytes in collagen gels resulted in decreased cell proliferation and type I collagen production compared to monolayer cultures (Lamberti and Wezeman 2002). Culture of corneal, dermal and tendon fibroblasts in 3D collagen gels also resulted in the maintenance of the distinct phenotypes these cell types exhibit *in vivo* (Doane and Birk 1991). These results support those reported in this chapter, which suggest that culture of tenocytes in 3D collagen gels results in a phenotype more similar to that seen in tendon tissue. However, significant differences were still present in the expression levels of decorin, MMP-3, MMP-13 and scleraxis between native tendon tissue and in 3D culture, showing that phenotype is not regained in 3D conditions.

7.4.3. Effect of Culture on Cells from Functionally Distinct Tendons

In contrast to cell phenotype in native tendon tissue, tenocytes from the SDFT and CDET cultured in 2D and 3D systems showed similar levels of expression for all the genes assessed. These results are supported by a study that reported that *in vitro*, human flexor and extensor tenocytes had similar morphology and produced similar amounts of protein (Evans and Trail 2001). In contrast, it has also been reported that cells from the CDET synthesise significantly less collagen (Young *et al.,* 2009b) and collagen degrading enzymes than those from the SDFT (Hosaka *et al.,* 2010). However, these experiments were performed at either passage 2 or 3; it is possible that phenotypic differences between cells from flexor and extensor tendons are present at low passage numbers but are lost after further passaging. These studies also obtained cells by out-growing from tendon explants; therefore these contrasting findings are probably not as a result of the method used to obtain cells. Loss of phenotypic differences between SDFT and CDET tenocytes *in vitro* suggests that the distinct phenotype these cells exhibit in their native tissue is not an inherent property of the cells but instead is likely to be due to differences in the mechanical and physiochemical environment they experience *in vivo*.

7.4.4. Effect of Culture on Markers of Tenocyte Phenotype

Scleraxis, tenascin-C and tenomodulin are all potential markers of tendon cell phenotype (Taylor *et al.,* 2009). However, none of these genes are expressed exclusively by tenocytes and scleraxis is expressed at similar levels *in vivo* in other connective tissues such as bone and muscle (Jelinsky *et al.,* 2010), therefore it is of limited use as a marker of tendon cell phenotype. Tenomodulin is expressed in tendon at levels approximately 5-fold greater than in other tissues, but expression decreases rapidly in culture; it could not be detected in equine SDFT tenocytes at passage 1 or passage 5 (Taylor *et al.,* 2009), and was strongly repressed when rat tenocytes were grown in 2D primary culture and in organ culture (Jelinsky *et al.,* 2010). This is similar to the low levels of tenomodulin expressed by SDFT and CDET tenocytes reported in this chapter. It would be of value to assess tenomodulin expression by cells from the SDFT and CDET in native tendon tissue to be able to compare this with the *in vitro* data presented in this thesis. Previous work has found that tenocytes from the equine SDFT express relatively high levels of tenomodulin, both in developing and mature tendons (Taylor *et al.,* 2009). Tenomodulin is a modulator of tenocyte proliferation and fibril formation (Docheva *et al.,* 2005) and so the low levels reported in culture may have a detrimental effect on cell activity. Scleraxis expression was significantly down-regulated in 2D and 3D culture conditions, scleraxis is a transcription factor (Schweitzer *et al.,* 2001) that is able to modulate collagen synthesis (Lejard *et al.,* 2007); therefore the loss of scleraxis and tenomodulin expression provides further evidence to show that cell culture results in a loss of tenocyte phenotype.

This thesis proposes that the ratio of collagen to decorin expression can be used as a marker of phenotype to differentiate between cells from functionally distinct tendons. The data presented in chapter 5 identifies a difference in this ratio between the functionally distinct SDFT and CDET when *in vivo* levels were assessed; however, when cells from these tendons are placed in culture, they de-differentiate rapidly. In 2D culture, there is an increase in synthesis of type I collagen and a decrease in decorin synthesis. When cells are placed in 3D constructs, the ratio decreases but still remains significantly greater than it is in native tendon tissue. It is not surprising that cell phenotype alters when the cells are placed in culture; as they are in an environment very different to that which they would experience *in vivo*. They are no longer embedded within a collagenous matrix and therefore there will lack cell matrix interactions, a factor which has been shown to alter gene

expression *in vitro* (Lavagnino and Arnoczky 2005). Cell to cell interactions will also differ; in culture cells will be in contact with many more cells than they would be in native tendon, which has a relatively low cell density. It has been shown that alterations in cell communication pathways results in the perturbation of tenocyte proliferation and collagen synthesis (Banes *et al.,* 1999b). Further, culture of cells in the absence of mechanical load for a significant period of time may result in programmed cell death (Egerbacher *et al.,* 2008).

In addition, the physiological environment is likely to have an important effect on cell phenotype; cells are likely to experience different levels of oxygen, temperature and nutrients *in vitro* than in native tendon tissue. *In vitro* experiments maintain cells at 37 ºC with 21% O_2 and 5% CO_2 and bathe the cells in nutrient rich media, whereas it has been shown that *in vivo* tenocytes, particularly those in the SDFT, are exposed to a wide range of temperatures (Wilson and Goodship 1994), and are likely to experience low oxygen levels due to poor blood supply (Birch *et al.,* 1997a). It is possible that differences in oxygen levels are enough to alter cell phenotype; it has been shown that culture of porcine tenocytes in hypoxic conditions enhances proliferation capacity and decreases MMP-1 expression (Zhang *et al.,* 2010). The poor blood supply may also result in low nutrient levels in the core of the tendon, a factor which may also affect cell proliferation and collagen synthesis (Schwarz *et al.,* 1976). It has also been shown that culturing SDFT explants in growth factor rich media resulted in increased expression of collagen and COMP (Schnabel *et al.,* 2007). High density cell culture has been shown to partially maintain tenocyte phenotype, possibly as a result of low oxygen levels due to the high cell numbers (Schulze-Tanzil *et al.,* 2004; Stoll *et al.,* 2010). In support of this, exposure of the MAET to high CO_2 levels resulted in increased expression of decorin and a trend towards decreased MMP-3 expression, which may result in increased proteoglycan levels within the matrix. The increased decorin expression in response to increased CO_2 levels suggests that low oxygen levels may cause tenocytes to shift towards a phenotype associated with high strain energy storing tendons; it is likely that *in vivo* cells from the SDFT experience lower oxygen levels than their counterparts in the CDET as the SDFT has a larger CSA than the CDET.

7.4.5. Tendon Organ Culture

Tendon organ culture maintains tenocytes within their extracellular environment, meaning that they are less likely to alter their phenotype. In addition, when strain is applied the amount of cell deformation that occurs is likely to be similar to that which occurs *in vivo* due to the maintenance of matrix structure. However, it is impossible to maintain whole tendons in culture as tendon diameter is too great to allow diffusion of oxygen and nutrients to the core, which would result in cell death. For this reason, studies often involve loading isolated tendon fascicles, groups of fascicles or explants of tendon tissue (Abreu *et al.,* 2008; Dudhia *et al.,* 2007; Maeda *et al.,* 2009; Maeda *et al.,* 2007; Screen *et al.,* 2005b). However, fascicle removal is technically difficult and risks physically damaging the structure or allowing the tissue to dry. Explant culture is analogous to wounding the tendon and so the cells may exhibit a damage response and alter their gene expression. Fascicle and explant culture potentially result in changes in cell phenotype as a result of removal rather than loading conditions. Furthermore, the multi-composite structure of tendon results in a heterogeneous distribution of strain throughout the tendon (Screen and Evans 2009), meaning that it is very difficult to establish strain levels that are physiologically relevant as not all cells within a tendon will experience the same strain. Studies indicate that the majority of cells experience deformations lower than the overall strain that is applied to the tendon, and strains generally do not exceed 2% (Screen *et al.,* 2003). Studies that have exposed tendon fibroblasts to strain of up to 12% *in vitro* (Wang *et al.,* 2003) may therefore be exposing cells to strains that they would never experience in their native environment.

Loading of the MAET therefore represents a novel method for investigating the effect of mechanical load on tenocyte phenotype as the MAET can be isolated without the need for longitudinal incisions to be made in the tendon. As expected, cells in the MAET exhibit a phenotype that is similar to that seen in the CDET. Further, the ratio of Col1A2 to decorin expression is similar in the MAET and CDET, and approximately fourfold greater than that in the SDFT. However, expression of scleraxis is lower in the MAET than in the SDFT, whereas expression of tenascin-C and MMP-3 is higher. The low levels of scleraxis are not surprising as expression of this gene is modulated by loading; the MAET is not likely to experience high strains as it is a vestigial tendon. The higher levels of MMP-3 and tenascin-C suggest that cells in the MAET may be more metabolically active than those in the SDFT and CDET. Although previous work has shown that gene expression in tendon mid-

substance may be affected by clamping the ends of the tendon (Rempel and Asundi 2007), culture of the MAET within the loading cassette in the absence of load did not result in significant alterations in gene expression when compared to MAETs cultured under normal culture conditions. Therefore, MAETs cultured under normal culture conditions were used as paired controls for the loading experiments.

Maintenance of the MAET in culture does not result in the significant alterations in gene expression that were identified when culturing SDFT and CDET tenocytes in 2D or 3D. Expression of tenascin-C and tenomodulin were not altered in either loaded or unloaded MAETs compared to *in vivo* data; both these genes have been proposed as markers of tenocyte phenotype and therefore these results suggest that tenocyte phenotype is maintained. In contrast, previous studies have reported that tendon explant culture results in alterations in gene expression, including decreased tenomodulin expression (Jelinsky *et al.,* 2010) and increased MMP-3 and -13 expression (Leigh *et al.,* 2008). These studies used tendon fascicles; it is possible the changes in gene expression are as a result of the dissection process rather than unloading. However, these studies also used culture periods of up to 48 hours; longer periods of loading are likely to result in greater alterations in gene expression. The only gene that was affected by the loading protocol applied in this study was scleraxis; it is well established that expression of this gene can be modulated by mechanical load (Eliasson *et al.,* 2009; Farng *et al.,* 2008; Kuo and Tuan 2008). Scleraxis has been shown to up-regulate collagen synthesis (Lejard *et al.,* 2007) and cell proliferation (Shukunami *et al.,* 2006), and so it is possible that exposing the MAET to cyclic load for a longer period of time would result in increased cell proliferation and collagen synthesis. Other studies have reported that relatively short loading periods (10 minutes) result in significant alterations in the expression of type III collagen and degradative enzymes (MMP-3 and -13) in tendon fascicles (Maeda *et al.,* 2009), whereas loading fascicles for 24 hours resulted in increased collagen synthesis (Maeda *et al.,* 2007). However, these studies used fascicles from rat tail tendons which are not load bearing and therefore only likely to experience very low strains, therefore their response to applied load may be different from that of the MAET.

7.4.6. Effect of Mechanical Load on Cells from Functionally Distinct Tendons

The SDFT and CDET experience different strain levels and rates and so tenocytes from these tendons may respond in a different manner to similar levels of mechanical load. One of the aims of this work was to determine the if high strains caused cells in the MAET to shift towards a phenotype normally associated with cells from the SDFT; however due to low sample numbers no difference was apparent and therefore data from the low and high strain groups were combined for statistical analysis. Therefore it was only possible to determine the effect of strain, rather than different levels of strain on cell phenotype. Several studies have investigated the effects of mechanical load on monolayer tenocyte cultures. One study reported that tenocytes cultured from the SDFT and CDET respond differently to mechanical load; strain caused an increase in collagen oligomeric matrix protein (COMP) production by cells from both tendons, but the level of the increase was greater in cells from the SDFT than in those from the CDET (Goodman *et al.,* 2004). It has also been shown that specific tendons exhibit a distinct response to stress deprivation; collagenase expression was increased in both stress deprived rat Achilles and supraspinatus tendons, but the magnitude of upregulation was greater in the supraspinatus tendon (Thornton *et al.,* 2008). However, it has also been reported that exposing human flexor and extensor tenocytes to load elicits a similar response in terms of cell proliferation and collagen synthesis (Evans and Trail 2001). These contrasting results show that further research is required to determine if cells from functionally distinct tendons respond differently to the same levels of strain. These experiments need to be undertaken in an environment similar to that of native tendon which allows physiologically relevant strains to be applied. The MAET would be a suitable scaffold for this; cells could be implanted into decellularised MAETs and subsequently loaded.

The data presented in this chapter show that both the physiochemical and mechanical environment have a significant effect on cell phenotype. It may therefore be a combination of differences in strain levels, oxygen, temperature and pH that result in the difference in phenotype seen between the cells from the high strain SDFT and low strain CDET. To confirm the hypothesis that tenocyte phenotype is due to the *in vivo* environment rather than inherent differences in cell type further experiments are required to determine the effect of applying different levels of mechanical load to SDFT and CDET tenocytes.

7.5 Conclusions

- Cells from the SDFT and CDET de-differentiated rapidly in monolayer culture and no longer exhibited distinct phenotypes as assessed by the ratio of collagen type I to decorin expression.

- Culture of tenocytes in 3D collagen gels resulted in a shift in phenotype towards that seen in native tendon tissue, but did not restore the ratio of collagen type I to decorin expression.

- The different cell phenotype exhibited by cells from functionally distinct tendons appears to be as a result of the distinct environment these cells experience *in vivo* rather than being pre-set during development.

- The MAET is a suitable scaffold to expose tenocytes to mechanical load *in vitro*, while maintaining the cells in an environment similar to that which tenocytes would experience *in vivo*.

CHAPTER EIGHT

8. General Discussion and Conclusions

8.1. Introduction

The data presented in this thesis have improved the understanding of normal matrix turnover in functionally distinct tendons and provided a novel insight into the changes that occur with ageing. It is well established that tendon injury occurs as a result of gradual accumulation of micro-damage to the tendon matrix, resulting in an increased risk of tendon injury in aged individuals (Clayton and Court-Brown 2008; Fung *et al.,* 2010b; Hess 2010; Riley 2004) although the nature of the damage is not clear. Specific tendons are known to be more susceptible to injury than others; high strain energy storing tendons such as the equine superficial digital flexor tendon (SDFT) and human Achilles tendon are injured far more frequently than low strain positional tendons (equine common digital extensor tendon (CDET), human anterior tibialis tendon) (Ely *et al.,* 2009; Hess 2010). Other tendons are also prone to injury; the patellar tendon, which may also function as an energy store, is subject to overuse injury in the human often as a result of sporting activity (Tan and Chan 2008). There is also a high incidence of pathological changes to rotator cuff tendons such as the supraspinatus; this tendon does not contribute to energy storage and injury is thought to occur due to compression of the tendon rather than repetitive tensile loading (Thornton *et al.,* 2008). However, even though the mechanism of injury is likely to differ between tendons, the pathological changes that occur within the matrix are similar between the Achilles, patellar and supraspinatus tendons (Riley 2005; Tan and Chan 2008).

It would be logical to assume that tendons exposed to high stresses and strains would have a greater capacity for matrix turnover in order to repair micro-damage, however previous work suggests that the matrix in the high strain SDFT is turned over at a slower rate than in the low strain CDET (Birch *et al.,* 2008b). The reasons for this low rate of matrix turnover have not been established previously, and it is not clear why specific tendons are more prone to injury than others or why the incidence of injury increases with subject age. This thesis tested the hypothesis that tenocyte metabolism is programmed by the strains the cells experience *in vivo* meaning that high strain energy storing tendons have a lower rate of matrix turnover than low strain positional tendons and that this declines further with increasing age in both tendon types. In support of the hypothesis the data presented in this thesis have shown that turnover of the collagenous fraction of tendon matrix is greater in

the CDET than in the SDFT. However turnover of the non-collagenous matrix components occurs at a faster rate in the SDFT than in the CDET. In contrast to the hypothesis tested, this work has also shown that the ability of cells to synthesise key matrix proteins and degradative enzymes is not decreased in aged tendons. Rather, older tendons may be more susceptible to injury as they have reduced mechanical integrity which is due to an accumulation of partially degraded collagen within the matrix of energy storing tendons. This may be due to an increased resistance to degradation caused by age related modifications to the matrix such as glycation and racemization.

8.2. Matrix Turnover Differs between Functionally Distinct Tendons

The rate at which extracellular matrix is synthesised and degraded varies according to tissue type; collagen in soft tissues such as the heart and lung is turned over at a relatively rapid rate (Gineyts *et al.,* 2000; McAnulty and Laurent 1987). In contrast, collagen within bone and tendon is metabolised at a much slower rate (Gineyts *et al.,* 2000; Neuberger *et al.,* 1951). This is not altogether surprising as tendon has a relatively low cellularity compared to other collagenous tissues. However it might be expected that tendons with a greater cellularity, such as the SDFT, would have a higher turnover rate than those with a lower cell number, such as the CDET, but the data presented previously (Birch *et al.,* 2008b) and in this thesis show that the opposite is true. Determination of matrix age within a tissue is difficult; previous studies have used the incorporation rate of radioactive proline or hydroxyproline into collagenous-rich tissues as a marker of the rate of matrix turnover (Laurent 1987; Neuberger *et al.,* 1951). However this is a very invasive and costly procedure and experiments have to be undertaken over a considerable period of time. Further, it is difficult to use this method to determine tendon matrix turnover as the proline would be incorporated at a very low rate. More recent studies have assessed matrix age by measuring the accumulation of spontaneously occurring matrix modifications; this requires tissue samples from subjects with a wide age range. Pentosidine is a well established marker of matrix age but it is not possible to estimate matrix protein half-life by measuring the concentration of pentosidine within a tissue. However, matrix half-life can be estimated by determining the rate of aspartic acid racemization. The work presented in this thesis has shown for the first time that the turnover of matrix components differs between functionally distinct tendons.

Assessment of the age of the tendon matrix as a whole by measuring pentosidine accumulation and the rate of D-aspartate racemization (see chapter 4) showed that there was no difference in matrix age between the functionally distinct SDFT and CDET. This does not support the hypothesis tested in chapter 4, which predicted that the accumulation of these age related matrix modifications would be greater in the energy storing tendons, reflecting a lower rate of turnover in these tendons. The deep digital flexor tendon (DDFT) had the highest pentosidine and D-aspartate levels, and correspondingly matrix half-life was the longest in this tendon. It was not possible to group the positional DDFT or energy storing suspensory ligament (SL) with the CDET or SDFT respectively in terms of matrix composition, age or gene expression. Tenocytes from the DDFT and SL exhibit a phenotype intermediate between tenocytes from the SDFT and CDET. When the matrix of the SDFT and CDET was separated into its collagenous and non-collagenous components a difference in the rate of matrix turnover was identified; with greater collagen turnover in the positional CDET and a faster rate of turnover of the non-collagenous matrix in the energy storing SDFT. This gives an insight into function of the different components of the matrix within functionally distinct tendons.

8.2.1. Collagen is Turned Over More Rapidly in Positional Tendons

As the most abundant component of tendon matrix, collagen is the major determinant of tendon mechanical properties. The hierarchical structure of the collagen within tendon facilitates the transfer of large forces; the majority of tensile load is transmitted through the tendon by the collagen fibrils and studies have shown that collagen content, fibril number and fibril diameter are the main determinants of tendon stiffness and strength (Derwin and Soslowsky 1999; Parry 1988; Rigozzi *et al.,* 2009; Robinson *et al.,* 2004a). Further, studies have shown that collagen fibrils experience heterogeneous strains throughout the tendon, which can markedly exceed the strain experienced by the whole tendon in some areas (Cheng and Screen 2007; Snedeker *et al.,* 2006), suggesting that some regions within the tendon are more prone to injury than others. Therefore the ability of cells to turnover the collagenous fraction of tendon extracellular matrix has important consequences for tendon repair and maintenance of tendon mechanical properties.

At the transcriptional level, cells from the functionally distinct tendons in the equine forelimb exhibit differences in their ability to synthesise and degrade collagenous matrix

proteins. In support of the hypothesis tested in chapter 5, the data show that the cells in the SDFT produce less message for synthesis of collagen and collagen degrading enzymes than their counterparts in the rarely injured CDET. This difference is also apparent at the translational level; the data presented in chapters 5 and 6 show that tenocytes in the CDET synthesise approximately four times more pro-collagen than those in the SDFT, and CDET tenocytes also synthesise greater amounts of the collagenase MMP-13. This results in an older collagenous matrix in the SDFT, with a half-life almost six times greater than that in the CDET (chapter 4). Although at first these data appear counterintuitive, it is possible that the collagenous fraction of the matrix comprising the SDFT is protected from extensive remodelling after maturity; it has been suggested previously that continual turnover of collagen fibrils in tissues with a supportive role would be a disadvantage as this may weaken the tissue transiently and increase the risk of injury (Laurent 1987). Further, energy storing tendons require specific mechanical properties for efficient energy storage and return; significantly increasing the stiffness of the SDFT in response to exercise would decrease its energy storing capacity. However, this relatively low rate of collagen turnover would mean that once micro-damage has occurred to the collagen fibrils the rate of repair is likely to be slow and therefore more micro-damage may accumulate, resulting in an increased risk of injury with ageing. In addition, the high collagen half-life will result in the accumulation of age related modifications to the collagenous matrix, including increases in the concentration of advanced glycation end products and the extent of amino acid racemization.

The rate of collagen turnover depends not only on the ability of cells to synthesise collagen and matrix degrading enzymes, but also on the resistance of the matrix to degradation. Fibrillar collagen is more resistant to degradation than collagen in solution (Slatter *et al.,* 2008). Differences in fibril diameter between tendons may therefore affect the susceptibility of the collagen molecules to degradation; the SDFT has a lower mass average fibril diameter than the CDET (Birch 2007), suggesting fibrils within this tendon would be more susceptible to degradation than those in the CDET. However, the data presented in this thesis indicate that collagen degradation occurs more rapidly in the CDET. The resistance of matrix collagen to degradation may also be affected by the levels of enzymatic crosslinks; although pentosidine levels were similar between tendons there may be other enzymatic crosslinks present at higher levels within tendon. However, the precise effect of

specific enzymatic crosslink concentration on the susceptibility of fibrillar collagen to degradation has yet to be determined.

8.2.2. Turnover of Non-Collagenous Proteins is Greater in Energy Storing Tendons

Although the main protein that comprises tendon extracellular matrix is type I collagen, tendons also contain varying amount of non-collagenous proteins, of which proteoglycans are the most abundant. Proteoglycans are hydrophilic and their role in tissues such as cartilage is to increase hydration to enable tissues to resist compression (Roughley 2006). Proteoglycans within tendon also play an important role in determining tendon water content (Screen *et al.,* 2006), are important regulators of collagen fibrillogenesis (Scott 1995) and are also thought to contribute directly to tendon mechanical properties. While tendon mechanical strength is determined mainly by the collagen content and organisation, it is thought that proteoglycan content affects the rate of stress-relaxation and may aid in the transfer of strain between fibrils (Brent *et al.,* 2003; Gupta *et al.,* 2010; Robinson *et al.,* 2004b). Studies have shown that a large degree of deformation occurs outside the collagen fibrils (Puxkandl *et al.,* 2002), suggesting that the non-collagenous tendon matrix also experiences significant levels of strain and therefore is likely to be damaged if high strains are experienced by the tendon.

The energy storing SDFT has higher glycosaminoglycan levels than the positional CDET, indicating a greater concentration of proteoglycans in this tendon. In contrast to the hypothesis tested in chapter 5, assessment of gene expression showed that the cells from the SDFT produced more mRNA coding for proteoglycan synthesis and degradation than those in the CDET. Correspondingly, there was also greater potential for degradation of the non-collagenous matrix in the SDFT at the protein level, with greater concentrations of enzymes able to degrade proteoglycans and glycoproteins in this tendon. This leads to a difference in the half-life of the non-collagenous matrix in functionally distinct tendons; the non-collagenous proteins in the SDFT had a half-life of 2.2 years whereas those in the CDET had a half-life of 3.5 years (see chapter 4). It has previously been established that proteoglycans are metabolised more rapidly than collagen in tendon, and it is thought that a certain level of proteoglycan turnover is important for tissue homeostasis (Rees *et al.,* 2000; Rees *et al.,* 2009; Smith *et al.,* 2008), but no previous studies have investigated the turnover

of non-collagenous proteins in tendons with different functions. It is likely that the difference in the turnover of the non-collagenous matrix between the SDFT and CDET is related to the different functions of these tendons. The SDFT and CDET are loaded at different strain levels and rates; proteoglycans are likely to play a more important role in transfer of strain between fibrils at high strain levels as experienced by the SDFT, at which the extension mechanism is dominated by fibre sliding relative to one another rather than fibre extension (Puxkandl *et al.,* 2002; Screen *et al.,* 2004) and therefore proteoglycans within the matrix of the high strain energy storing SDFT are more likely to be damaged, and require repair, than those in the low strain positional CDET.

Interestingly, other studies have shown a decrease in the potential for degradation of the non-collagenous matrix components in tendinopathy, with decreased expression of the stromelysins (Ireland *et al.,* 2001; Jones *et al.,* 2006). It is possible that the cells prioritise remodelling of the collagenous matrix in tendons that exhibit pathology; possibly to the detriment of the non-collagenous matrix. This is likely to have important consequences for the integrity of high strain energy storing tendons; accumulation of proteoglycan fragments in this tendon may decrease the efficiency of strain transfer between collagen fibrils and contribute to the poor quality of matrix repair.

8.3. Effect of Ageing on Tendon Matrix Turnover

In general, it is thought that cell activity declines with ageing in many tissues. It is well established that ageing results in a gradual decline in muscle mass (Lexell *et al.,* 1988; Narici *et al.,* 2008). Myosin heavy-chain synthesis rate has been shown to decrease with increasing age suggesting cells within aged muscle are less able to remodel this contractile protein (Balagopal *et al.,* 1997). Studies have also shown that ageing results in a decrease in the myosin content of heart muscle but an increase in collagen content, which results in fibrosis and inefficient function (Varagic *et al.,* 2001); this may be as a result of alterations in cell phenotype with ageing. Chondrocytes have also been shown to exhibit alterations in phenotype with ageing; cells express lower levels of type II collagen and aggrecan (Acosta *et al.,* 2006) and become senescent in cartilage from aged individuals (Martin and Buckwalter 2002). It is well established that the risk of tendon injury increases with increasing age (Clayton and Court-Brown 2008; Kasashima *et al.,* 2004), suggesting that

there is a decrease in tendon mechanical integrity in aged individuals. However, no previous studies have determined if this is due to an age-related decline in cell activity.

In contrast to the hypothesis tested in this thesis, the gene expression data show that synthesis of key matrix proteins and degradative enzymes does not decrease with increasing horse age at the transcriptional level. Measurement of collagen synthesis and levels of matrix degrading enzymes at the protein level show that synthesis of proteins required for matrix synthesis and degradation is maintained in aged tendons in the equine forelimb. However, cells must also be able to respond appropriately to cytokines and growth factors in order to increase metabolic activity if an injury does occur; it is possible that this response is decreased in aged tenocytes and further work should be undertaken to assess this. Assessment of actual collagen degradation showed that partially degraded collagen accumulates within the matrix of the SDFT with increasing age, suggesting that the collagenous matrix becomes more resistant to degradation in aged tendons. Taken together, these data suggest that the increase in collagen matrix half-life with increasing age is not due to a decrease in cell activity but appears to be due to increased resistance of the matrix to degradation, which may be attributed to the accumulation of spontaneous matrix modifications. This is likely to reduce tendon mechanical integrity and may account for the increased risk of tendon injury in aged individuals.

8.4. Effect of Age Related Matrix Modifications

Age related modifications to the matrix may affect tendon mechanical integrity by a variety of pathways. The accumulation of AGEs has been shown to have a significant effect on tendon mechanical properties, resulting in increased stiffness and strength (Reddy *et al.,* 2002; Reddy 2004). The data presented in chapter 4 shows that the AGE pentosidine and the percentage of D-Aspartic acid accumulate linearly with age in both energy storing and positional tendons, however previous studies have not identified an increase in strength or stiffness of the SDFT with increasing horse age (Birch 2007). AGEs may have a deleterious effect on tendon mechanical properties in aged individuals as their presence within the matrix increases the resistance of collagen to enzymatic degradation (DeGroot *et al.,* 2001; Mott *et al.,* 1997; Schnider and Kohn 1981; Verzijl *et al.,* 2000a), therefore inhibiting the repair of damaged collagen and leading to accumulation of partially degraded collagen within the matrix. There is also evidence to suggest that glycation has an inhibitory effect

on collagen fibrillogenesis; it has been reported that soluble collagen incubated with glucose forms unstable fibres with low levels of enzymatic crosslinks *in vitro* (Guitton *et al.,* 1981). While newly synthesised collagen molecules would not be glycated it is likely that incorporation of new collagen into fibrils as part of the repair process would be hampered if high levels of glycated crosslinks were present in the mature fibril. Little is known about the mechanisms of matrix turnover within tendon; it has not been established whether whole fibrils are removed and replaced or if damaged collagen molecules can be repaired without remodelling of the entire fibril, therefore it is not possible to fully determine the extent of the effect of age related changes to the matrix on tendon mechanical properties. However, these studies suggest that both collagen degradation and subsequent repair can be inhibited by age related matrix modifications, resulting in an accumulation of partially degraded collagen within the matrix which would have important consequences for matrix integrity and resulting mechanical properties.

Accumulation of damaged collagen within the matrix may also alter cell-matrix interactions, resulting in localised unloading of cells within damaged fibrils. Cells require a certain strain threshold or 'set point' to function normally and therefore stress deprivation may result in an abnormal cell phenotype; cells in stress deprived tendons express higher levels of degradative enzymes (Arnoczky *et al.,* 2008b; Gardner *et al.,* 2008), exhibit altered morphology and may undergo apoptosis (Egerbacher *et al.,* 2007). AGEs can also directly affect cell matrix interactions, resulting in a decrease in the ability of cells to attach to the collagenous matrix (Liao *et al.,* 2009; Paul and Bailey 1999). AGE crosslinks are able to form at lysine and hydroxylysine residues throughout the collagen triple helix, but form preferentially on hydroxylysines 434 on the α1(I) chain and 453, 479 and 924 on the α2(I) chain (Sweeney *et al.,* 2008). Hydroxylysine 479 is in close proximity to the cell interaction domain on the collagen fibril and modelling studies have shown that glycation at this site would be likely to affect collagen-ligand interactions (Sweeney *et al.,* 2008). AGEs that form between arginine residues, such as pentosidine, are also likely to influence cell matrix interactions, as the binding site Arg–Gly–Asp is recognised by the α1β1 and α2β1 matrix integrins, which form a link between the cell and collagen molecules (Tuckwell and Humphries 1996). In support of this, it has been reported that increased glycation of arginine *in vitro* results in a decrease in cell adhesion and proliferation (Paul

and Bailey 1999). Alterations in cell phenotype combined with micro-damage accumulation will further increase the risk of gross injury with increasing age.

In addition to altering fibrillogenesis and cell-matrix interactions, there is also evidence to suggest that synthesis and degradation of matrix molecules can be directly modulated by the presence of advanced glycation end products (AGEs). It has been reported that articular chondrocytes synthesise lower levels of type II collagen and collagen degrading enzymes in artificially glycated cartilage when compared to non-glycated controls (DeGroot *et al.,* 2001). Another study has reported that human dermal fibroblasts (HDFs) cultured in glycated collagen lattices produce lower levels of MMP-1 compared to HDFs cultured in non-glycated collagen lattices, and while MMP-2 production was not affected by glycation, inhibition of MMP-2 by TIMPs-1 and -2 was significantly increased (Rittie *et al.,* 1999). In addition, cells were less able to contract glycated collagen lattices, presumably due to increased stiffness as a result of glycation (Rittie *et al.,* 1999). In contrast, a more recent study using an *in vitro* model of glycated skin found that glycation of type I collagen with ribose caused an increase in collagen, collagenase and MMP-2 and -9 synthesis (Pageon *et al.,* 2007). These results indicate that, in addition to increasing the resistance of the matrix to enzymatic degradation, the presence of AGEs within aged tendon matrix may also have a direct cell effect on the synthesis and degradation of matrix proteins.

8.4.1. Effect of Age Related Matrix Modifications on Cell Activity

Interestingly, it has been shown that young and old fibroblasts cultured in an *in vitro* skin model showed no differences in histological and functional properties (Michel *et al.,* 1997). In a comparable manner fibroblasts from UV damaged skin, which has high levels of partially degraded collagen, have a similar capacity for type I pro-collagen production to cells from sun protected skin (Varani *et al.,* 2001). However, culture of fibroblasts on partially degraded collagen resulted in decreased cell proliferation and type I collagen production (Varani *et al.,* 2001). Furthermore, it has been shown that collagen extracted from the tail tendons of young and old mice has different structural properties; fibril formation occurred at a slower rate in collagen from aged tendons, and the resulting fibrils were thinner and less organised that those formed by collagen extracted from the tail tendons of younger mice (Damodarasamy *et al.,* 2010). In addition, culture of fibroblasts within 3-D gels formed by the polymerisation of collagen from young and old tendons

resulted in the differential expression of several genes (Damodarasamy *et al.,* 2010). This study suggests that the decreased ability of aged collagen to polymerise readily *in vitro* may be due to defects in collagen at the molecular level. An *in vitro* study has found that mature tenocytes cultured in 3D constructs produce homogeneous collagen fibrils similar to those formed by embryonic tendon cells (Bayer *et al.*, 2010), suggesting that the poor regenerative potential in mature tendon is as a result of factors other than a decrease in intrinsic cell function. Taken together, these data suggest that the accumulation of micro-damage and resultant decrease in matrix integrity that is a common feature in aged collagenous tissues including tendon is due to changes in the structure and properties of the extracellular matrix. These changes may cause alterations in cell activity rather than ageing causing a decrease in the intrinsic ability of the cells to synthesise and degrade the matrix. These results may also partially explain the large variations in tendon mechanical properties between individuals. Tendon mechanical properties are not correlated with parameters such as horse body weight or exercise history (Birch 2007); it would be of interest to determine if these mechanical properties are related to the extent of partially degraded collagen within the matrix.

The presence of AGEs within the matrix does not only affect turnover of the collagenous proteins, pentosidine has been shown to form in cartilage proteoglycans (Pokharna and Pottenger 1997). DeGroot *et al*. (1999) also identified an inverse relationship between pentosidine levels and synthesis of proteoglycans by articular chondrocytes *in vitro*, suggesting that increased AGE levels may decrease cell synthetic capacity. It is likely that the non-collagenous matrix of the CDET contains a higher concentration of AGEs in older tendons than that of the SDFT due to the differences in non-collagenous protein half-life between these tendons, but this requires further investigation. Studies have reported that glycation decreases the fidelity of collagen proteoglycan binding as proteoglycan binding regions overlap with preferred glycation sites on the collagen molecule (Reigle *et al.,* 2008).

It is likely that glycation plays a larger role in the increased resistance to enzymatic degradation than amino acid racemization. While the racemization of amino acids is a useful marker of protein age the effect on mechanical properties is likely to be small. Molecular dynamics simulations suggest that the intermediate formed in the racemization

reaction is energetically unfavourable within the constraints of the collagen triple helix and therefore racemization is likely to be confined to the telopeptide regions of the molecule (van Duin and Collins 1998). In any case, the amounts of the D form of aspartic acid compared to the L form are very small. Studies have demonstrated that if racemization did occur in the collagen triple helix it could have a significant destabilising effect (Punitha *et al.,* 2009; Shah *et al.,* 1999); however it should be noted that these experiments were conducted on small collagen-like helices in a dilute solution which represents a completely different chemical environment to fibrillar collagen in tendon. Therefore the magnitude of triple helix destabilisation is likely to be different in a collagen fibril, where collagen molecules interact strongly with the neighbouring molecules, than in solution where interactions between collagen molecules will be much weaker. Racemization of the aspartic acid residues in the telopeptide region may however alter the conformation of enzyme cleavage sites within the region (Ritz-Timme and Collins 2002). This could result in a decrease in the ability of MMP-3 to unwind the C-terminal telopeptide, a process which is essential for cleavage of triple helical collagen (Chung *et al.,* 2004). Racemization also occurs in non-collagenous proteins and the rate constant of racemization is greater in aggrecan than collagen (Maroudas *et al.,* 1998). It is likely there are fewer stereochemical restrictions within the non-collagenous matrix proteins, and so the succinamide intermediate would be able to form more readily in proteoglycans than in collagen. A greater level of D-Asp accumulation in proteoglycans may also result in perturbations in the rate of proteoglycan turnover, such that proteoglycan fragments also accumulate within the matrix. This is an area that requires further investigation.

The accumulation of partially degraded collagen within tendon matrix is likely to decrease mechanical integrity and therefore increase the risk of tendon injury by several pathways. It is logical to assume that the presence of collagen fragments within the matrix would result in decreases in tendon strength and stiffness as the transfer of strain between collagen fibrils would be impaired. This may also place additional strain on intact areas of the matrix, increasing the risk of further damage occurring. In addition, damaged collagen within the matrix may result in localised alterations in cell matrix interactions. This is likely to result in unloading of cells in areas of matrix where partially degraded collagen has accumulated. It is well established that stress deprived cells show alterations in their mechanostat set point and upregulation of degradative gene expression (Arnoczky *et al.,*

2008b; Arnoczky *et al.,* 2007; Lavagnino *et al.,* 2006). Alterations in cell phenotype may further hamper matrix repair and therefore also increase micro-damage accumulation. Although overall levels of pentosidine are similar between the SDFT and CDET, it is likely that, in the SDFT, a greater proportion of the pentosidine is present in the collagenous matrix as the half-life of this fraction of the matrix is significantly greater than in the CDET. While the levels of pentosidine are relatively low (1 per 70 collagen molecules in the tendons assessed) the recently discovered lysine-arginine crosslink derived from glucose; namely glucosepane, reaches levels comparable to those of the lysyl oxidase derived crosslinks (1-5 moles/mole collagen) (Sell *et al.,* 2005), and there may be other, as yet unidentified glycated crosslinks that accumulate with age in tendon.

8.5. Effect of Culture on Phenotype of Cells from Functionally Distinct Tendons

The data presented in chapter 7 show that maintaining cells from the functionally distinct SDFT and CDET in culture results in alterations in tenocyte phenotype. The differences in phenotype between tenocytes from the SDFT and CDET that were identified *in vivo* were lost when the cells were cultured in monolayer and in 3D collagen gels. In culture the tenocytes produced more message for collagen synthesis and downregulated decorin synthesis. This resulted in an approximate 300 fold increase in the ratio of type I collagen to decorin. It is likely the alterations in tenocyte phenotype in culture are in response to changes to the cells' mechanical and physiological environment. These results highlight the importance of maintaining cells within an environment that is similar to that which they would experience *in vivo* in order to obtain physiologically relevant results. Culturing SDFT and CDET tenocytes within a 3D collagen gel resulted in a decrease in the ratio of collagen type I to decorin expression towards normal levels, although the ratio was still approximately 50 fold greater than *in vivo*. These experiments were carried out in the absence of load, it is possible that mechanically loading these 3D constructs would result in a cell phenotype closer to that seen *in vivo*.

In order to maintain tenocyte phenotype *in vitro* cells need to be cultured in an environment that replicates their native tendon tissue both physiologically and mechanically. Therefore, the most rational approach would be to culture whole tendons under dynamic loading conditions. However, the cross sectional area of most tendons is too great to allow adequate

diffusion of nutrients to the centre, which would result in loss of cell viability. Tendons with a small cross sectional area tend to be too short to clamp at either end for mechanical loading. However, the medial accessory extensor tendon (MAET) has a small cross sectional area and is also long, making this tendon suitable as a scaffold for mechanically loading tenocytes in their native environment. The preliminary data presented in chapter 7 of this thesis indicates that maintenance of this tendon in the absence of load for a short period of time does not result in a significant alteration in cell phenotype. Further, loading of the MAET in organ culture does not cause alterations in gene expression, with the exception of the load inducible gene scleraxis. This suggests that this culture system maintains cells within an environment that is physiologically and mechanically similar to native tendon tissue and therefore is a more suitable system than culturing cells in monolayer or in artificial scaffolds. Interestingly, changes to the physiological environment in terms of carbon dioxide levels had a significant effect on decorin gene expression both in loaded and unloaded MAETs, a result that requires further investigation.

8.6. Tendon Cell Phenotype is Determined by *In Vivo* Strain Environment

Taken together, the results presented in this thesis indicate that tenocyte phenotype is determined by the strains the cells are exposed to *in vivo*, and therefore phenotype is altered in response to changes in strain experienced by the cells rather than being pre-set during tendon development. It is clear that cells from the functionally distinct tendons in the equine forelimb are phenotypically different, especially in terms of proteoglycan and collagen turnover. This corresponds to differences in the *in vivo* strain environment; the greatest difference in gene expression was seen between the SDFT, which experiences the highest strains and the CDET, which is exposed to the lowest strains. The DDFT and SL experience moderate levels of strain *in vivo*; correspondingly cells from these tendons exhibited gene expression patterns between those of the SDFT and CDET. This difference is maintained at the protein level, resulting in different rates of matrix turnover in the SDFT and CDET. Furthermore, culture of SDFT and CDET tenocytes in the absence of mechanical strain caused the cells to adopt a phenotype that was no longer different from cells from the other tendon, but was distinct from the phenotype measured *in vivo*. This evidence of phenotype plasticity lends support to studies that have shown that cell phenotype can be altered by exposing cells to varying mechanical and physiological

conditions. However to answer this question fully further work needs to be undertaken to elucidate the effect of altered strains on SDFT and CDET tenocyte phenotype.

8.7. Implications for Prevention and Treatment of Tendon Injury

The data presented in this thesis also have important implications for development of successful treatment programmes for tendon injuries in both the horse and human. The majority of non-surgical treatment protocols for tendinopathy target cell activity (see chapter 1) but the data presented in this thesis suggest that age related tendinopathy is not a cell mediated problem. Rather, the matrix becomes more resistant to repair due to age related modifications such that in aged individuals the degraded collagen cannot be removed completely. Areas of repaired matrix are generally disorganised; it is likely that partially degraded collagen is present alongside the new matrix as it cannot be fully degraded and removed. Preliminary studies have shown that cell-based therapies result in lower rates of re-injury than conservative treatment methods (Smith 2008; Young *et al.,* 2009a). These treatments may be effective in young animals with relatively low levels of AGE accumulation and fragmented collagen within the tendon matrix. However, it is likely the efficacy of these methods would decrease in aged tendons as matrix repair would be hampered by the presence of AGEs and partially degraded collagen, but as yet this has not been assessed. An alternative approach would be to develop treatments that prevent or reverse the accumulation of AGEs and partially degraded collagen; possibly by the use of AGE inhibitors or AGE-breakers. AGE inhibitors are currently being developed for use to prevent soft tissue dysfunction associated with diabetes, primarily targeting the vascular system (Huijberts *et al.,* 2008; Susic 2007). However, it has been reported that although AGE-breakers are able to cleave model AGEs *in vitro*, they are unable to cleave AGEs in glycated rat tail tendon *in vivo* (Yang *et al.,* 2003). In contrast, another study has reported that diabetic rats fed a supplement reported to reverse AGE accumulation had lower AGE levels and a higher percentage of soluble collagen within tail tendons when compared to diabetic controls (Jagtap and Patil 2010). These studies indicate that AGE inhibitors may be a potential treatment for tendinopathy but this area requires much further investigation. It is likely that methods aimed at preventing accumulation of micro-damage within the matrix will be more successful than attempting to reverse AGE formation and subsequent accumulation of damaged collagen. It would be difficult to target degradation of damaged collagen without detrimental effect to the healthy matrix. A greater understanding of tendon

matrix biology and mechanics, especially with regard to role of proteoglycans within the matrix and their interactions with collagen, is required to further understand the initiation and progression of tendinopathy. In general, research into tendon matrix biology has thus far been limited as it has been assumed by many that tendons are simple structures with the simple function of transferring force from muscle to bone. The data presented in this thesis and by others show that, contrary to these assumptions, tendon has a high degree of complexity and specificity in terms of function, matrix composition and turnover and cell phenotype.

8.8. Why is Collagenous Matrix Turnover Low in Injury Prone Tendons?

It seems counterintuitive that the horse has evolved to have a low rate of collagen turnover in the SDFT as this predisposes this tendon to injury, both in elite athletes and the general equine population. However, the mechanical properties of energy storing tendons must be maintained within a narrow range of strength and stiffness for efficient energy storage and return, therefore extensive remodelling of these tendons would be detrimental to their energy saving function. In addition, remodelling in response to micro-damage may weaken the tendon transiently during the repair process, further increasing the risk of damage to healthy matrix. The horse has evolved long limbs with correspondingly long tendons in order to be able to travel at high speeds to evade predators; use of the horse in racing over a pre-defined distance with additional weight in the form of tack and rider will place much higher stresses and strains on the tendons than would be experienced in the undomesticated horse. While it is clear that some degree of exercise is essential for development of optimum tendon properties, the additional demands placed on equine athletes expose the energy storing tendons to excessive forces. Exercise is therefore likely to accelerate the age related changes seen in equine tendon; it is possible that in addition to causing micro-damage, exercise also results in impaired tenocyte function due to changes in the cells' physiochemical environment such as hyperthermia and hypoxia. The possible causes and factors contributing to age related tendinopathy are shown in Figure 8-1.

SDFT

Extensive remodeling
would weaken tendon

Low rate of
collagen
turnover

Protective

Ageing

Exercise

Physiochemical
factors e.g.
hyperthermia,
hypoxia

Maintain optimal
mechanical properties
for energy storage

AGE accumulation

Micro-damage
accumulation

Changes in
cell phenotype

Altered cell matrix
interactions

Impaired
fibrillogenesis

Increased resistance
to degradation

Impaired repair
processes

Accumulation of
partially degraded
collagen

Further
mechanical
loading

Age related
tendinopathy

Figure 8-1: Flow chart showing the possible causes and factors contributing towards the development of age related tendinopathy in the SDFT.

234

8.9. Future Work

The data presented in this thesis have provided a valuable insight into the specialisation of functionally distinct tendons resulting from differences in the rate of turnover of matrix components and the nature of age related tendinopathy in energy storing tendons. However there is much more that is still to be determined in terms of tendon matrix turnover, tenocyte phenotype and the pathways that result in tendon degeneration. There are several ways in which the data presented here could be used as a starting point for further studies. Although the equine SDFT and human Achilles tendon have similar functions, the turnover of collagen in Achilles tendon has not been assessed and so further work should be undertaken to determine if this tendon has a lower rate of matrix turnover than positional tendons. It would be of value to determine the extent of advanced glycation end product accumulation in the collagenous and non-collagenous fractions of the matrix; this could be achieved by assessing pentosidine levels in the Guanidine-HCl extracted tendon tissue. In addition, levels of other AGEs present at greater concentrations in the matrix, such as glucosepane, could be assessed. The effect of AGE levels on the resistance of the matrix to degradation could also be assessed by incubating tendon explants with reducing sugars and correlating AGE levels with collagenase digestion time. The methods used in this thesis were only able to assess levels of mature crosslinks in equine tendon as immature crosslinks do not fluoresce naturally; in order to measure levels of immature crosslinks the crosslinks need to be derivatized with a compound such as ninhydrin (Sims and Bailey 1992) after separation of the crosslinks by ion exchange chromatography. Measurement of immature crosslinks in tendons from a group of horses with a wide age range would give an indication of changes in the rate of crosslink formation with age, and therefore contribute to the overall understanding of tendon matrix turnover.

The effect of ageing on cell activity could be assessed further by determining if cells within aged tendon become senescent by measuring telomere length and levels of β-galactosidase (Martin and Buckwalter 2002). It is also important to determine if aged cells are able to respond to increased levels of growth factors and cytokines as this will affect their ability to respond appropriately when an injury does occur.

Tendon mechanical properties show large variations between individual horses, and are not correlated with parameters such as horse age and body weight (Birch 2007). It is therefore

important to determine if tendon mechanical properties are correlated with half-life of the collagenous and non-collagenous matrix components or with the concentration of AGEs within the different fractions of the matrix. In addition, mechanical properties may be correlated with markers of collagen degradation; it would be logical to assume that tendons with high levels of partially degraded collagen within the matrix would be weaker than tendons in which collagen fragments had not accumulated to a great extent. Mechanical testing of tendon at the fascicular level could also be of use to determine where within the matrix micro-damage occurs and how it accumulates; this work is currently being undertaken.

The non-collagenous fraction of tendon matrix is also susceptible to age related glycation and racemization; therefore it is possible that proteoglycans within the matrix also become more resistant to degradation in aged tendons. This could be assessed by measuring the accumulation of decorin fragments within the matrix as a function of age. Although the half-life of the non-collagenous matrix is greater in the positional CDET this tendon has a lower GAG content than the energy storing SDFT and therefore the concentration of proteoglycan fragments may also be greater in the SDFT than in the CDET.

This thesis has validated the MAET for use as a scaffold *in vitro*. Further work is currently being undertaken to determine the effect of decellularising this strip of tendon using previously published methods (Ingram *et al.,* 2007), and implanting different tendon cell types by injecting cells into the centre of the tendon as well as seeding cells on the surface (Tischer *et al.,* 2007). This study will therefore determine if tenocytes from the SDFT and CDET respond in the same manner to different loading protocols, or if exposing cells from the SDFT to strains normally experienced by cells in the CDET would cause them to become similar in phenotype to native CDET cells and *vice versa*. Use of the MAET as an *in vitro* scaffold gives the opportunity to control both the cells' mechanical and physiochemical environment. Further studies could determine the effect of altering the temperature, oxygen and carbon dioxide levels during the loading programme; these are likely to have a significant impact on tenocyte phenotype although it is difficult to assess these variables *in vivo*. The effect of glycation on cell phenotype could also be assessed by incubating decellularised MAETs with reducing sugars before injecting cells into the tendon.

8.10. Conclusions

- The rate of matrix turnover differs between functionally distinct tendons. Although in all tendons the non-collagenous matrix is turned over more rapidly than the collagenous matrix, cells from high strain energy storing tendons have a greater ability to synthesise and degrade non-collagenous matrix proteins, whereas cells from low strain positional tendons have a greater ability to turnover the collagenous matrix than their counterparts in high strain energy storing tendons.

- The ability of tenocytes to synthesise matrix proteins and degradative enzymes does not decrease with increasing age, either at the transcriptional or translational level.

- Tendon extracellular matrix becomes more resistant to degradation with increasing age in injury prone energy storing tendons, resulting in an accumulation of partially degraded collagen within the matrix.

- Age related tendinopathy may therefore be due to an accumulation of collagen fragments within the matrix, which result in a decrease tendon mechanical integrity, rather than a decrease in cell activity with increasing age.

- Tendon cell phenotype is determined by the mechanical and physiochemical environment within the tendon milieu rather than being pre-set during tendon development.

- The medial accessory extensor tendon is suitable for use as a scaffold to study the effects of alterations in mechanical and physiological environment on cell phenotype *in vitro*.

- Tendon is a complex dynamic structure with properties that differ according to precise function, change in response to mechanical and physiological environment and are modified with increasing age.

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