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Insights into Tendon Damage from a Diabetic Rat Model

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Abstract

Tendinopathy is one of the most common musculoskeletal injuries in humans (Walden et al., 2017). It is also, due to the essential role that tendons play in everyday movement and locomotion, one of the most impactful. The need to prevent, mitigate and treat its occurrence is thus clear although, in order for this possibility to exist, greater insight is first needed into the mechanistic causes of tendon damage and the structural responses to it. While it is known that repeated overloading and, alternatively, fatigue loading of a tendon will increase its susceptibility to damage there are a number of other factors that can prove influential. One of these is diabetes - a disease that is correlated with an increased incidence of tendinopathy (Abate et al., 2013) and can be considered as equivalent to an increase in age (Monnier et al., 1984) - which is often investigated experimentally via animal models. The mechanism behind the effect that diabetes has on tendon is thought to be advanced glycation end-product (AGE) presence, an increase in which can occur in both diabetic and older patients (Thorpe et al., 2010; Eriksen et al., 2014). Another factor is the functional role of the tendon in question: those that are involved in locomotion or are loaded heavily in vivo behave in a spring-like manner - with the ability to store and then release energy - while those that are ‘positional’ and used for fine-motor control do not. In both cases these properties aid the performance of the respective functions and the tendons have structural differences associated with these (Ker et al., 2000). The aim of this dissertation was thus to explore tendon damage - its mechanisms and markers - and the method of investigation included using rat tendons from both a model of diabetes (‘STZ’) and from healthy controls with energy-storage and positional functional roles both being considered (tail tendons and Achilles tendons, respectively). Following a literature review (which, among other things, explored the definition of ‘damage’ and how it is modelled) two different mechanical tests were performed: static fatigue testing and ‘interrupted’ static fatigue testing. The first of these enabled the measurement of phenomenological markers related to damage, including the ‘steady-state strain rate’ (SSSR) which showed strong, significant differences with

STZ treatment ($p = 0.000001$ in tails and $p = 0.04$ in Achilles tendon) and functional role ($p = 0.01$ in STZ samples and $p = 0.0001$ in controls). The second mechanical test allowed the relationship between SSSR and a generally-accepted marker of tendon damage - a decrease in loading modulus - to be established in tail tendons ($p = 0.24$ in STZ samples and $p = 0.02$ in controls). It was thus concluded that SSSR is indeed a quantification of tendon susceptibility to damage. Finally, spectrofluorimetry was performed on STZ-treated and control samples in order to measure AGE presence. This revealed no significant difference ($p = 0.15$), leading to the conclusion that other factors related to STZ treatment must necessarily be occurring. Possibly, these include AGE-related stiffening of bonds within the tendon's non-collagenous matrix.

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Contents

Abstract	i
Acknowledgements	iii
1. Introduction	1
1.1. Problem Identification	1
1.2. Current Knowledge	2
1.2.1. Literature Review	3
1.2.2. Previous Investigations	3
1.3. Overview	5
1.4. Aim	7
1.5. Structure	7
2. Literature Review	9
2.1. Collagenous Structures	10
2.1.1. Collagen Types	10
2.1.2. Molecular Level	11
2.1.3. Fibril Level	13
2.1.4. Fibre Level Upwards	13
2.2. Non-Collagenous Components	14
2.2.1. Tendon Cells	14
2.2.2. Elastin	15
2.2.3. Proteoglycans	15
2.2.4. Crosslinks	16
2.2.5. Ground Substance	17

2.2.6. Other ECM Components	17
2.3. Non-Permanent Deformation	17
2.3.1. Viscoelasticity	17
2.3.2. General Deformation Behaviour	18
2.3.3. Specific Deformation Behaviour	23
2.4. Permanent Deformation	24
2.4.1. Yield and Rupture	24
2.4.2. Fatigue Behaviour	25
2.4.3. Phenomenological Definitions of Damage	28
2.5. Deformation Mechanisms	33
2.5.1. Molecular Level	34
2.5.2. Fibril Level	37
2.5.3. Fibre Level	41
2.5.4. Fascicle Level	43
2.5.5. Summary	45
2.6. The Effect of Functional Role	47
2.6.1. Tendon Types and Functional Roles	47
2.6.2. Effect on Collagenous Structures	49
2.6.3. Effect on the Non-Collagenous Components	50
2.6.4. Effect on Deformation and Damage Properties	50
2.7. The Effect of Diabetes	51
2.7.1. Mechanism Behind Diabetic Changes	51
2.7.2. Effect on Collagenous Structures	52
2.7.3. Effect on Non-Collagenous Components	52
2.7.4. Effect on Deformation and Damage Properties	53
2.8. Diabetes	57
2.8.1. Pathophysiology	57
2.8.2. Prevalence and Impact	58
2.8.3. Experimental Models for Diabetes	59

3. Investigating the Susceptibility of Tendon to Damage 61

3.1. Abstract	61
3.2. Introduction	61
3.2.1. Problem Identification	62
3.2.2. The Effect of Diabetes	62
3.2.3. The Effect of Functional Role	62
3.2.4. Mechanical Damage	63
3.2.5. Static Fatigue Testing	64
3.2.6. Murine Models	65
3.2.7. Aim	65
3.2.8. Hypotheses	66
3.3. Materials and Methods	66
3.3.1. Sample Collection	66
3.3.2. Dissection	67
3.3.3. Sample Preparation	68
3.3.4. Apparatus	69
3.3.5. Sample Loading	69
3.3.6. Static Fatigue Testing	69
3.3.7. Calibration	71
3.3.8. Data Analysis	72
3.3.9. Statistical Analysis	74
3.3.10. Uncertainty Analysis	76
3.4. Results	77
3.4.1. STZ Model vs Healthy Controls	77
3.4.2. Energy-Storage vs Positional Functional Roles	78
3.4.3. Mechanistic Markers	78
3.4.4. Wistar vs Sprague-Dawley	78
3.5. Discussion	82
3.5.1. STZ Model vs Healthy Controls	82
3.5.2. Energy-Storage vs Positional Functional Roles	84
3.5.3. Implications	84

3.5.4. Limitations and Future Research	86
3.6. Conclusions	87
4. Steady-State Strain Rate as a Measure of Damage Accumulation	89
4.1. Abstract	89
4.2. Introduction	90
4.2.1. Problem Identification	90
4.2.2. Steady-State Strain Rate	90
4.2.3. Material Properties	91
4.2.4. Phenomenological Markers of Tendon Damage	92
4.2.5. Fatigue Testing	93
4.2.6. Streptozotocin Treatment	94
4.2.7. Aim	94
4.2.8. Hypothesis	94
4.3. Materials and Methods	95
4.3.1. Sample Collection	95
4.3.2. Sample Preparation	95
4.3.3. Apparatus and Sample Loading	96
4.3.4. Interrupted Static Fatigue Testing	96
4.3.5. Calibration	97
4.3.6. Data Analysis	97
4.3.7. Statistical and Uncertainty Analyses	100
4.4. Results	101
4.4.1. Phenomenological Markers	101
4.4.2. Mechanistic Markers	102
4.5. Discussion	109
4.5.1. Steady-State Strain Rate as a Material Property	109
4.5.2. Phenomenological Markers	109
4.5.3. Mechanistic Markers	112
4.5.4. Implications	113
4.5.5. Data Analysis	113

4.5.6. Limitations and Future Research	115
4.6. Conclusions	116
5. Quantification of Advanced Glycation End-Products	117
5.1. Abstract	117
5.2. Introduction	117
5.2.1. Problem Identification	117
5.2.2. The Effect of STZ and of Diabetes	118
5.2.3. The Streptozotocin Rat Model of Diabetes	118
5.2.4. Spectrofluorimetry	119
5.2.5. Aim	119
5.2.6. Hypothesis	119
5.3. Materials and Methods	120
5.3.1. Sample Collection	120
5.3.2. Apparatus and Materials	120
5.3.3. Day One	121
5.3.4. Day Two	121
5.3.5. Day Three	121
5.3.6. Concentration Standard Curve	121
5.3.7. Analysis	122
5.4. Results	122
5.5. Discussion	123
5.5.1. Quantification of AGEs	123
5.5.2. Concentration of Hydrochloric Acid	124
5.5.3. Implications	124
5.5.4. Future Research	126
5.6. Conclusions	126
6. Discussion and Conclusions	127
6.1. Steady-State Strain Rate	127
6.1.1. Correlation Between SSSR and $\frac{d}{dt}$ Loading Modulus	128

6.2. The Effect of STZ	129
6.3. Tendon Damage	130
6.4. Limitations and Future Research	131
6.5. Conclusions	133
6.6. Implications	134
Bibliography	136
Appendices	155
A. Viscoelasticity Models: Derivations	157
A.1. Kelvin-Voigt Model	157
A.2. Maxwell Model	160
A.3. Standard Linear Solid Model - Stress-Based Configuration	163
A.4. Standard Linear Solid Model - Strain-Based Configuration	168
A.5. Sliding and Pullout Model	168
B. Rat Dissection: Video Protocol	171
C. The Susceptibility of Tendon to Damage: Software Protocol	173
D. The Susceptibility of Tendon to Damage: Written Protocol	177
E. The Susceptibility of Tendon to Damage: Mechanistic Markers	181
E.1. Materials and Methods	181
E.2. Results	183
E.2.1. STZ Model vs Healthy Controls	183
E.2.2. Energy-Storage vs Positional Functional Roles	183
E.3. Discussion	186
E.3.1. The Standard Linear Solid Model	186
E.3.2. STZ-Model vs Healthy Controls	189
E.3.3. Energy-Storage vs Positional Functional Roles	189
F. The Susceptibility of Tendon to Damage: Effect of Rat Strain	191

G. Steady-State Strain Rate as a Measure of Damage: Video Protocol	193
H. Quantification of AGEs: Written Protocol	195
H.1. Preparation	195
H.2. Day 1	196
H.3. Day 2	197
H.4. Day 3	197
I. Quantification of AGEs: Video Protocol	199

1. Introduction

This chapter provides a broad overview and a background to the project.

1.1. Problem Identification

Healthy tendon exhibits exceptional mechanical fatigue resistance properties. This ensures it can perform its biomechanical functions over many millions of cycles per year; transmitting tensile force between muscle and bone, absorbing shock and storing/releasing energy during locomotion. However, the overuse and overloading of tendons - such as may be experienced over time in a work environment that requires repetitive movements and/or manual labour, or acutely during a contact, jumping or running sport - will result in pain and, in extreme cases, rupture (Gomoll et al., 2004; Killian et al., 2012; Gross and Nunley II, 2016). As many as half of all sport-related injuries involve tendons (Docheva et al., 2015). The presence of additional risk factors such as advanced age (Moller and Westlin, 1996; Dymment and Galloway, 2015) and a lack of regular moderate exercise (Woo et al., 1980) (often exacerbated by obesity) can result in everyday movements also becoming problematic: lifting, bending and twisting. A particularly common cause of injury is accidental trips and falls, especially amongst the elderly and, with the proportion of people over 60 years of age set to almost double by 2040 (World Health Organisation, 2015) this problem will become increasingly relevant. Already, up to one third of people aged over 50 experience tendon disorders (Tempelhof et al., 1999) and 25% of the adult population will suffer from tendon injury at some point in their lives (Walden et al., 2017).

The effects of a tendon injury are considerable. They may include discomfort, a loss in locomotive ability and a decrease in joint function often leading to disability, pain, an affected quality of life and a reduced ability to work (Allenmark, 1992; Järvinen, 1992; Praemer et al., 1992; Dunkman et al., 2013). Indeed, the most common occupational

injuries requiring days away from work in the United States are tissue sprains, strains and tears (36% of total) (US Bureau of Labor Statistics, 2016). Tendon injury costs the United States healthcare system approximately \$30 billion annually (Butler et al., 2010). Although tissue is able to self-repair, this ability is limited. Treatment is thus often costly, invasive, slow and not always effective (Sharma and Maffulli, 2005) with many tendon problems becoming chronic. Reducing the degree to which tendon is susceptible to damage is therefore particularly important from a clinical perspective.

The motivation to investigate the problem of tendon damage is thus clear. Unfortunately, however, the mechanisms behind damage accumulation are poorly understood. New insights are needed; ones that will provide a greater understanding of how tendon behaves under load and why. Such knowledge would directly inform:

- Research into new ways of preventing, minimising and treating tendon damage
- Investigations related to the factors that put certain people at risk of tendon injury (e.g. research with subjects who are diabetic, physically active, elderly, etc)
- The design of bioreactors that use mechanobiological principles (e.g. applying load to samples to facilitate growth): loads that induce a healing response rather than a damaging one are of particular importance
- Tissue engineers creating scaffolds for regenerative medicine

Tissue engineers especially have been challenged by this lack of mechanical knowledge in the past; scaffolds that do not accurately mimic all aspects of tendon physical properties are limited in their usefulness (Han et al., 2013; Duncan et al., 2014; Patel et al., 2018).

1.2. Current Knowledge

Previously published journal articles and experimental reports were considered when analysing what is already known in the field of tendon damage.

1.2.1. Literature Review

In order to address the problem of tendon injury it is important to first take cognisance of the existing knowledge within this area. The anatomy, histology and physical structure of tendon, for example, is particularly important due to its influence over how the material reacts to load (Figure 1.1a). Secondly, the way in which a load is applied has relevance (Figure 1.1b & c): a large force applied quickly (e.g. a ramp-to-failure test) will elicit different behaviour than a smaller one for which time-dependent (viscoelastic) behaviour should be considered. Likewise, additional response behaviours can be induced through the different types of fatigue loading (static and dynamic) which are often more physiologically relevant. The mechanisms behind all of these must be understood in order to explain how tendon damages and, more importantly, explain how damage accumulates and leads to injury and tendinopathy (Figure 1.1c). This task is complicated by a lack of clarity within the literature as to the exact definition of ‘tendon damage’, and this question should be addressed by looking at both phenomenological and mechanistic markers that have been used to investigate damage in the past. Lastly, an investigation has greater insight if it contains experimental variables, and so it is beneficial to consider factors that influence a tendon’s mechanical properties (Figure 1.1d). Two of the most impactful variables are the presence of diabetes - which is known to predispose patients to tendinopathy (Holmes and Lin, 2006) - and tendon functional role - ‘spring-like’ energy-storing tendons used during locomotion are known to be stronger but less stiff than those used for positioning and fine motor control (Shadwick, 1990). These aspects of the field’s current knowledge are explored through a Literature Review included in Chapter 2.

1.2.2. Previous Investigations

Tendon damage, in accordance with its high clinical importance, has been the topic of a multitude of previous studies. These often included ramp-to-failure tests wherein a sample was loaded at a constant rate until rupture (e.g. Veres and Lee (2012)) while static fatigue tests (Wang and Ker, 1995) and dynamic fatigue tests (Abrahams, 1967; Wang and Ker, 1995; Wang et al., 1995; Misof et al., 1997a; Fung et al., 2010; Veres et al., 2013a,b; Herod and Veres, 2018), both of which allow for the investigation of damage

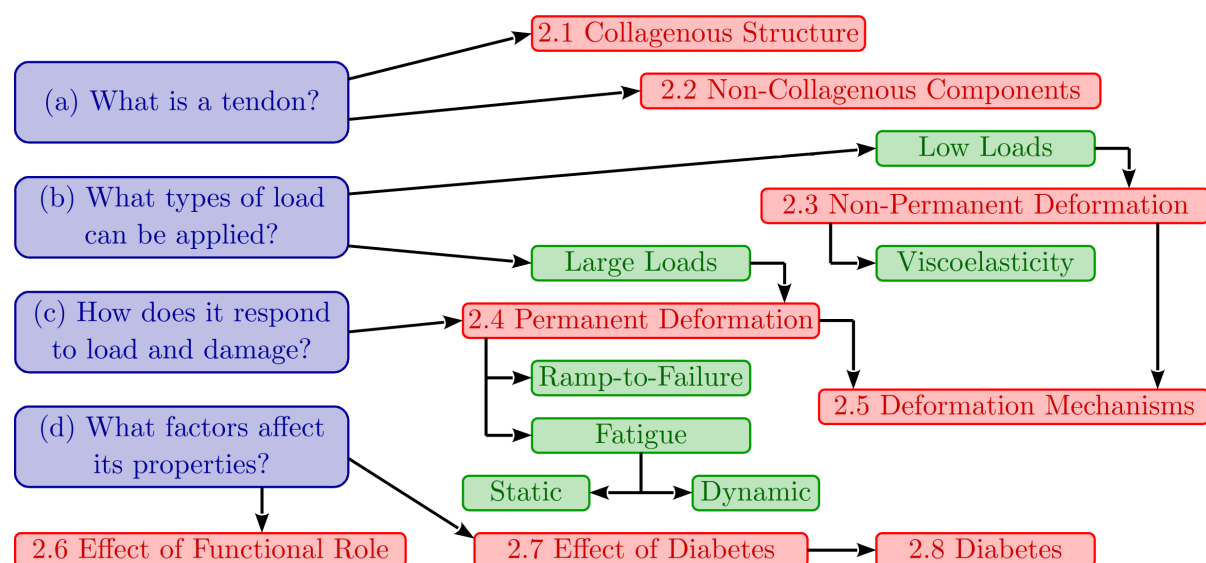


Figure 1.1: How the Literature Review in Chapter 2 is structured so as to best investigate the Current Knowledge. The review questions are shown in blue and the eight sections that make up the chapter are shown in red. The main sub-themes within each section are shown in green.

accumulation, were also commonly performed. Multiple different types of tendon have been studied in the past and as such it is possible to compare their properties. This can be used to investigate the aforementioned effect of functional role. Studies that have tested and compared tendons of multiple types directly include Birch (2007) (ramp-to-failure), Ker et al. (2000) (static fatigue) and Shepherd et al. (2014b) (dynamic fatigue). Contrastingly, the other aforementioned factor - the effect of diabetes (or of animal models of diabetes) - has only been investigated through ramp-to-failure tests (although dynamic loading has been performed on ligaments (Duquette et al., 1996)). 14 such examples are detailed in Table 2.18. Additionally, ‘creep rate’ - a measurement taken during a static fatigue test - has not been reported for tendon in any of the reviewed literature. This is despite its importance being recognised in other materials, such as metals, where its role in fatigue life considerations has been accepted (Hearn, 1997). As such, there exists a gap in the literature: the effects of diabetes on the fatigue properties of tendon, including creep rate, which may be different in functionally-distinct types.

1.3. Overview

The literature reports a disconnect between what is seen in the clinic and what is seen *ex vivo*: it is generally accepted that diabetes increases the risk of tendon injuries in patients (Holmes and Lin, 2006; Abate et al., 2013) yet, in the majority of mechanical tests, tendon strength is actually greater in diabetic models than in controls (see Figure 2.18 and Table 2.1). No conclusive explanation for this has yet been developed and as such this is an area open for investigation. Part of the effects may be due to diabetes-induced peripheral nerve damage (Kumar et al., 1994) which has the dual-effect of altering gait patterns (and thus tendon loading) (Salsich et al., 2000) and dulling pain feedback (increasing the risk of injury and poor healing through neglect) (Harrison and Faris, 1976). Additionally, the decreased innervation may couple with decreased vasculature (and thus decreased oxygen supply) (Benjamin et al., 2008) to inhibit repair, allowing damage to accumulate and leading to injury. Another part of the effects may be due to metabolic disorders: tendon cells from diabetes sufferers have been seen to be permanently buckled (Pingel et al., 2014) which would disrupt their mechanobiological functioning (Waggett et al., 2006). Affected cells would slow the metabolic repair process down (Magnusson et al., 2010).

With regards to the exact mechanism(s) through which ultimate tensile strength and loading modulus is increased by diabetes an often-used hypothesis is that the disease increases the presence of advanced glycation end-products (AGEs) (Fessel et al., 2014). This comes after it increases the level of glucose within the extracellular matrix. These intermolecular crosslinks are stronger and stiffer than those in healthy tendon and thus they are thought to be responsible for the changes seen in diabetic tendons *ex vivo* (Eriksen et al., 2014). However, it is not known if experimental models of diabetes (e.g. rat models) will reproduce the mechanisms of clinical human tendinopathy - consider the vastly greater lengths of time over which diabetes affects humans (many years) vs laboratory rats (approximately eight weeks). Also, when clinical observations are made the tissue in question is living whereas when mechanical tests are performed it is on dead material. Thus, AGEs cannot be assumed to be entirely responsible for any results

derived from models: other factors, such as increased viscosity of the ground substance and/or altered proteoglycan (GAG-GAG) crosslinking, may be relevant to a greater or lesser extent than in humans. It may be that if tests were performed on both ex vivo human tissue and on dead rat tissue then the same general mechanical results would be seen but different quantities of AGEs and/or proteoglycans would be measured.

Thus, ideally, multiple studies would be performed on both human and rat tendon - both in vivo and ex vivo and with different experimental parameters - in order to elucidate the various mechanisms that are relevant to each. However, this study will only be concerned with ex vivo rat tendon, and will seek to add a material science perspective on the mechanisms behind this structure's resistance to mechanical damage. With regards to testing parameters, while it is true that the tendinopathy seen clinically is usually caused by dynamic fatigue (as this is what is induced by physiological, cyclic movement), this has already been studied (Wang and Ker, 1995), although not extensively. The knowledge gap that exists thus calls for an investigation involving creep loading in order to investigate *static* fatigue resistance, and the effect of diabetes on this.

One challenge posed by this suggestion is that one of the previously-used definitions of tendon damage - a decrease in loading modulus - cannot be measured during static fatigue as it requires the applied load to be changed. While this has not been addressed in previous literature, textbooks in the field of solid mechanics make mention of tertiary creep rate as a measure of fatigue resistance (Hearn, 1997). This could thus be re-defined for biological material and used as a measure of damage accumulation, although the correlation between creep rate and damage susceptibility would need to be verified.

In summary, it is likely that diabetes acts to both increase tendon's strength and stiffness and decrease its ability to self-repair. This would thus make the structure more damage resistant but, at the same time, less able to repair any microdamage that did occur and so more prone to damage accumulation and injury overall.

1.4. Aim

The aim of the project was to gain insight into the mechanisms behind, and the markers of, tendon damage and its causes. This was done by considering the effects of a rat model of diabetes on the properties of tail and Achilles tendon samples in order to achieve three objectives (the relevant experimental approaches are included in brackets):

- Compare the tendons' susceptibilities to mechanical fatigue damage (static fatigue test)
- Establish the utility of steady-state strain rate/creep rate as a descriptor of this susceptibility (interrupted static fatigue test)
- Confirm the presence of diabetes-induced advanced glycation end-products, a possible mechanism for the differences that were measured (spectrofluorimetry)

1.5. Structure

Four chapters follow this introductory section, including a review of the literature and three experimental chapters (each corresponding to one of the above bullet points in order). The dissertation is then concluded by an overall discussion.

2. Literature Review

Diabetes is known to affect biological tissue, causing functional deterioration, increased scarring, structural abnormalities (Grant et al., 1997; Abate et al., 2014), tendonitis (Deshmukh and Akarte, 2017) and degenerative features (Abate et al., 2010, 2016) whilst inhibiting healing (Greenhalgh et al., 1990; Ahmed et al., 2012). It is correlated with Achilles tendinopathy in men younger than 44 years (Holmes and Lin, 2006) and the elderly (Abate et al., 2012, 2016) and rupture of this tendon is more likely as a result. Diabetic Achilles tendinopathy is also, together with motor and sensory neuropathy (which affects 42% of diabetic patients (Kumar et al., 1994)) and weakened tissue, believed to contribute to the development of foot ulcers ('diabetic foot') by altering patients' gait (Salsich et al., 2000; Batista et al., 2008; Cronin et al., 2010) and sensory feedback (Harrison and Faris, 1976; Giacomozzi et al., 2005; Diabetes UK, 2015). These ulcers affect between 5.3% and 15% of diabetic patients (UK and US data respectively (Kumar et al., 1994; Reiber, 1996)) and have an especially high risk of infection and subsequent amputation (Girod et al., 2003). Two-thirds of patients experience symptoms of diabetic foot (Rosenqvist, 1984). There is thus a pressing clinical need to investigate the differences in properties between diabetic and healthy tendons; a better understanding of the mechanisms behind these adverse effects can lead to better methods of treatment and prevention.

This literature review will start by looking at the structure of tendons and similar biological tissues (Sections 2.1 and 2.2) before considering this structure's behaviour under relatively small loads (Section 2.3) and relatively large loads (Section 2.4). The current understanding of the mechanisms behind tendons' response to these will then be considered (Section 2.5) as well as how the type of tendon (Section 2.6) and the presence of diabetes (Section 2.7) affect this response. Finally, diabetes will be discussed in the context of research (Section 2.8).

2.1. Collagenous Structures

Collagen is the primary structural component of connective tissue and it is probably the most abundant protein in the animal kingdom (Gross, 1963) (see Figure 2.1). Specifically, it is found in the ‘extracellular matrix’ (ECM) of tissue which is the structure into which cells are embedded. Here is where collagen serves its primary function: providing strength and resilience to the material. Within the ECM, it arranges itself into a number of hierarchical levels (see Figure 2.2) which transmit force between each other and other tissue components. It is these interactions, along with the composition of the collagen in terms of what types are present (see Section 2.1.1), that determine the material’s overall mechanical properties. In this section, the collagenous structure within one type of connective tissue - tendon - will be discussed, although the general principles apply to all types.

2.1.1. Collagen Types

There are 28 types of collagen (Types I to XXVIII) (Ricard-Blum, 2011), all of which are structural in nature. Each type responds differently to load (Baer et al., 1987), and thus anatomy will consist of different combinations of collagen types according to the individual functional loading characteristics (Liu et al., 1995). If the loading pattern changes, the structure will adapt its composition of collagen types accordingly (Liu et al., 1995; Reeves, 2005). Of the collagen found in tendon, 97-98% is Type I with a small amount of Type II in the cartilaginous zones, Type III in the walls of blood vessels and in scar tissue (Józsa and Kannus, 1997) and trace amounts of Type V, XII and XIV also present (Liu et al., 1995). Type III is thought to help form attachments to bone and aid in healing processes (by rapidly forming crosslinks to stabilise the repair site) while Types V, XII and XIV may control the fibre diameters (Killian et al., 2012) and/or the initiation of fibril assembly (Wenstrup et al., 2004).

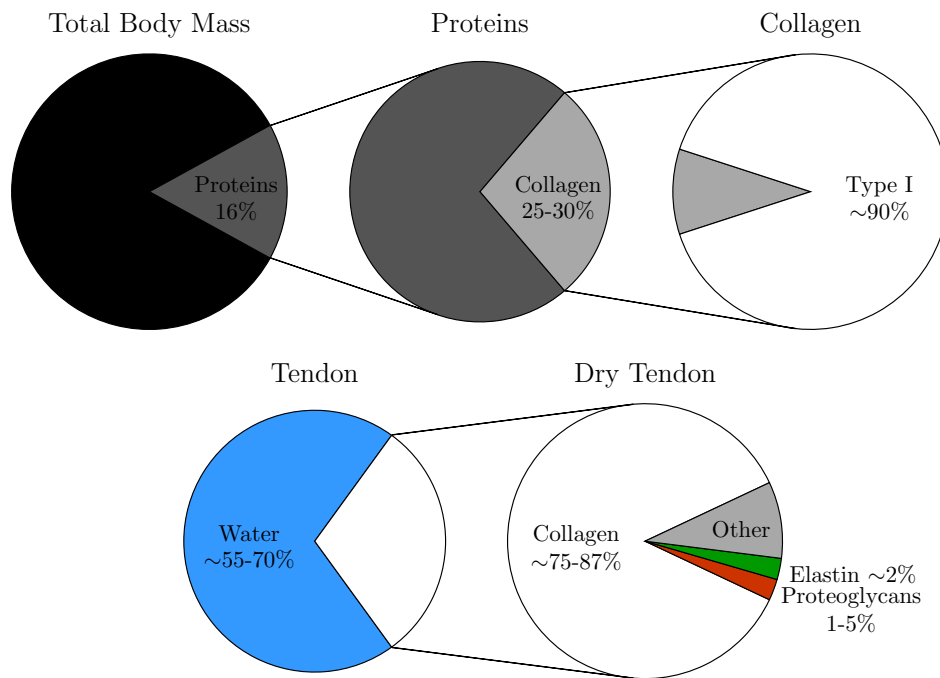


Figure 2.1: Protein constitutes 16% of humans by mass (Brožek, 1966), around 25-30% of this protein is collagen (Neuberger, 1955) and it is estimated that 90% of this collagen is Type I (Di Lullo et al., 2002). Regarding tendon specifically, 55-70% is water (Aparecida de Aro et al., 2012) while the dry mass is between 75% (Lagier and Exer, 1960) and 87% (Amiel et al., 1984) collagen. Of the remainder, ~2% is elastin and 1-5% is proteoglycans (Józsa and Kannus, 1997) with inorganic compounds making up about 0.2% (Spadaro et al., 1970).

2.1.2. Molecular Level

The collagen molecule - also known as ‘tropocollagen’ - consists of three polypeptide strands, each made up of 1055 amino acids (Bucci, 1994). The differences between the aforementioned 28 types of collagen lie in which amino acids are present and in what order they exist in each of the three strands (Baer et al., 1987; Lodish et al., 2000). The molecules arrange themselves in such a way as to have a characteristic stagger of 67 nm (the ‘D-period’, see Figure 2.2) regardless of collagen type (Baer et al., 1987). This occurs because some of the amino acids are polar and hydrophobic and thus interact with neighbouring molecules; a 67 nm offset happens to result in these interactions being strongest (Baer et al., 1987).

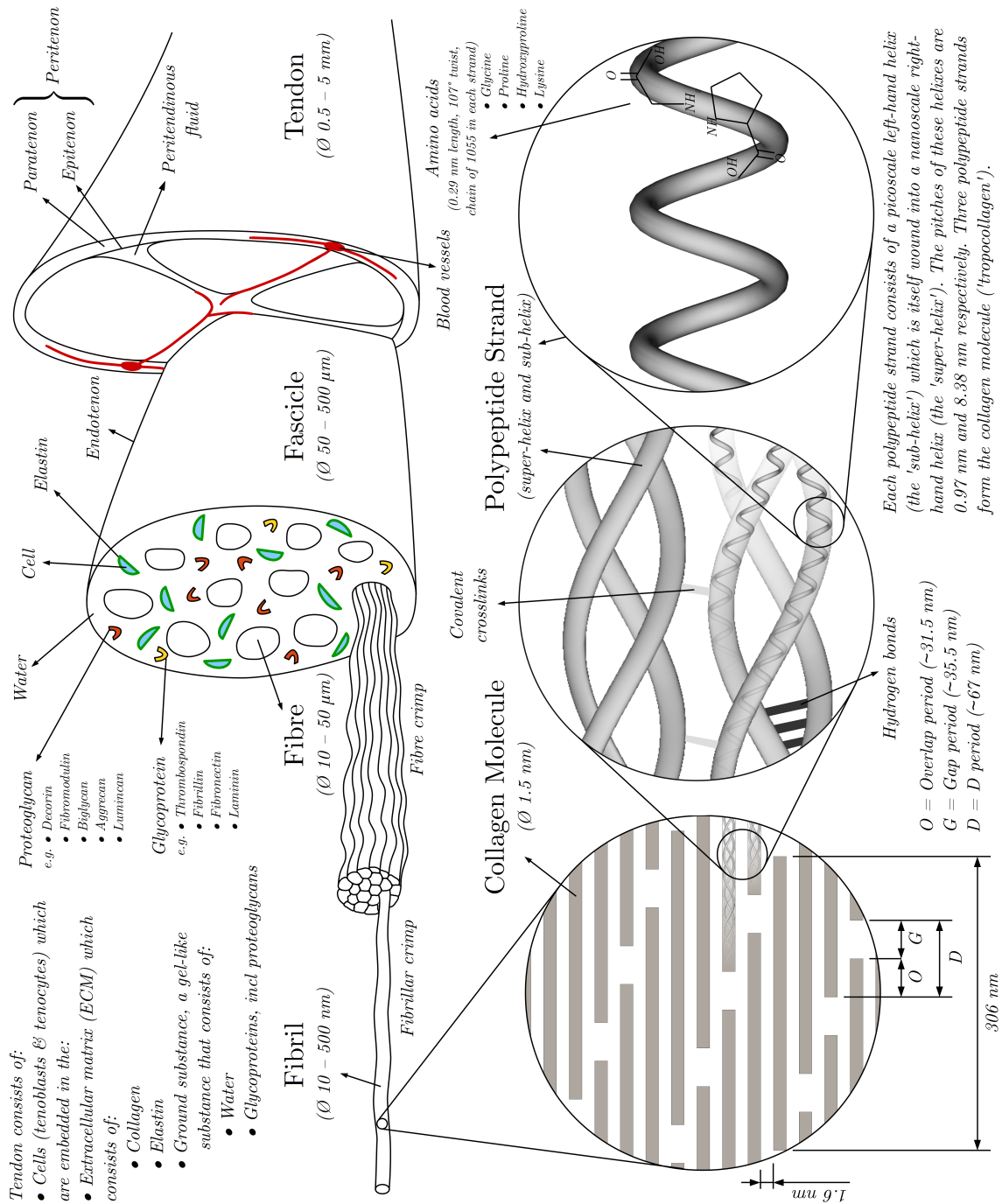


Figure 2.2: There is no consensus as to the exact details or nomenclature of this model; see Handsfield et al. (2016) for a review and Kastelic et al. (1978), O'Brien (1997), Kannus (2000), Wang (2006), Screen (2009), Szaro et al. (2009), Edama et al. (2015), Bojsen-Møller and Magnusson (2015) and Handsfield et al. (2016) for individual models and sets of nomenclature. Additional information was taken from Petruska and Hodge (1964), Fraser et al. (1979), Bucci (1994), Orgel et al. (2006), Tresoldi et al. (2013) and Fessel et al. (2014). Not to scale.

2.1.3. Fibril Level

On a more macro level, the bundled-together molecules are given the functional term ‘fibrils’ - the fundamental building blocks of connective tissue (Parry and Craig, 1978). When new collagen is synthesised by tendon cells, it is fibrils (as opposed to molecules) that are produced. They are generally thought to run the entire length of the tendon or, at least, they are long enough to be considered continuous for all practical purposes (Provenzano and Vanderby, 2006) although there is no universal agreement on this (Rigozzi et al., 2013). This implies that they are the chief load bearing unit. In unloaded tendon, fibrils have a regular, wavy (‘crimped’) shape (Yahia and Drouin, 1989) of period $\sim 100\text{ }\mu\text{m}$ (Grytz and Meschke, 2009). They are also slightly helical in shape, similar to man-made rope (Bozec et al., 2007), and the inner region is less stiff than the outer ‘shell’ (Gutsmann et al., 2003). While fibrils generally run co-axial to the longitudinal axis of the tendon, parallel to one another, it is more precise to say that fibril alignment follows the direction of load (and this may actually be haphazard) (Cooper et al., 1970). The fibril alignment, in turn, influences the mechanical properties of a tendon and can adapt so as to best suit the way in which it is loaded - similar to collagen type composition as mentioned in Section 2.1.1. Both of these factors are controlled by the body through collagen turnover: healthy fibrils are constantly (and at a relatively high rate (Eliasson et al., 2007)) replaced by newly synthesised collagen, the type and alignment of which is determined by the functional requirements placed on the tissue (Liu et al., 1995; Snedeker and Gautieri, 2014). Note that this is only true for a minority of the collagen - most of the human Achilles tendon tissue present at the age of 17 will remain there permanently (Heinemeier et al., 2013). Only a small amount will be subject to turnover around this ‘core’.

2.1.4. Fibre Level Upwards

Bundles of fibrils make up collagen ‘fibres’ (Nimni and Harkness, 1988). These, along with tendon cells, proteoglycans and certain other structures, make up the ‘fascicle’ (Baer et al., 1987) which is surrounded by a sheath of connective tissue - the endotenon - that helps it slide relative to other fascicles (Thorpe et al., 2015b). This sliding is aided by

peritendinous fluid which acts as a lubricant (Kannus, 2000). Collectively, the fascicles are then bounded by a thin ‘epitenon sheath’ which, in turn, in some tendons, is covered by the fatty areolar tissue of the ‘paratenon’ (although the naming convention for these layers is inconsistent (Benjamin et al., 2008)). This outermost connective tissue contains blood vessels, nerves and mesenchymal cells which the structure otherwise generally lacks (Dunkman et al., 2013).

2.2. Non-Collagenous Components

The remaining components of tendon include other structural elements such as proteins and proteoglycans as well as cells and the gel-like ‘ground substance’.

2.2.1. Tendon Cells

The majority - 90-95% (Abate et al., 2009) - of the cells found within tendon are fibroblasts (cells which synthesise molecules - in this case collagen - in order to construct the fibres that make up connective tissue) (Benjamin et al., 2008) and are known as ‘tenocytes’ (Kannus, 2000). Indeed, their main functions are to build, maintain and repair the structural matrix (i.e. the ECM) that surrounds them (Józsa and Kannus, 1997). Attachment to this matrix is via molecules called ‘integrins’ (Wall et al., 2007). They are long, thin (2-10 μm in diameter (Kannus, 2000)) and found in parallel rows sandwiched between the collagen fibres that likewise run longitudinally along the tendon (see Figure 2.3) (Screen et al., 2004a; Benjamin et al., 2008). While this fibre organisation gives the tendon strength (Ross et al., 2003) and a resistance to tension (Liu et al., 1995) it means that the rows of tenocytes are relatively far away from each other and scarce (Ross et al., 2003). The functioning of tenocytes is influenced by mechanical stimuli and the local strain environment (Waggett et al., 2006); indeed, Birch (2007) ascribed the differences seen between functionally distinct tendons (see Section 2.6) to variations in physiological strain causing differences in cell behaviour between tendons.

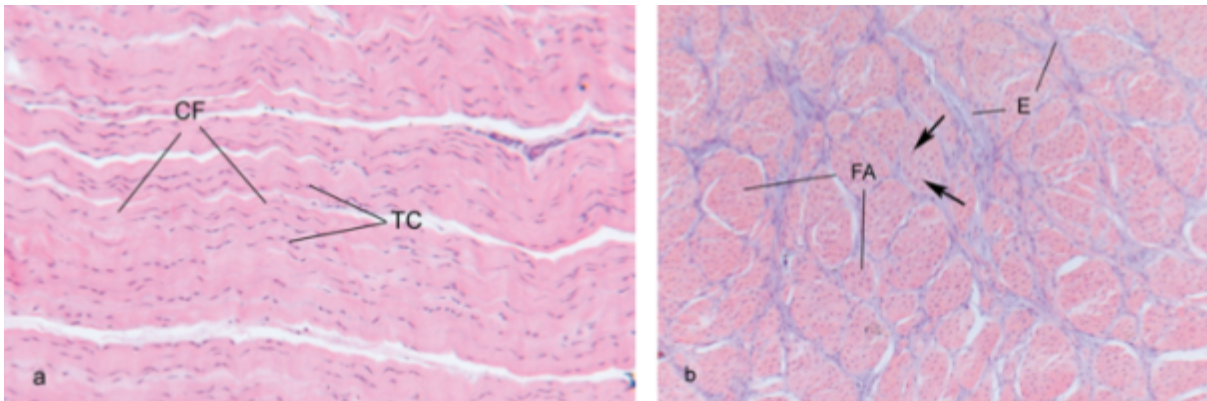


Figure 2.3: Low-power longitudinal (a) and transverse (b) sections through the limb tendon of a calf, stained with Haematoxylin and Eosin. Rows of elongated tenocytes (TC and arrows) can be seen between collagen fibrils (CF). Notice the characteristic crimp pattern in (a). Fascicles (FA) are shown, separated by endotenon (E).

Image credit: Benjamin et al. (2008)

2.2.2. Elastin

A protein - also produced by the tenocytes - that forms fibres which are thinner and less stiff, but otherwise similar, to those of collagen (Ross et al., 2003). These are relatively scarce (0.1-2% of tendon's dry mass (Abate et al., 2009)) but are able to withstand significantly larger strains (up to 100-150% (Abate et al., 2009)) than collagen (~21% (Shen et al., 2008)) with little hysteresis loss (Baldock et al., 2011), high fatigue resistance (Gosline et al., 2002) and the capacity for spring-like energy storage (Gosline et al., 2002). They thus increase the structure's compliance and recoil considerably, especially in highly-strained tendons which are involved in locomotion (and elastin is more abundant in these) (Thorpe et al., 2015c). They are found running longitudinally through the tendon, located particularly between fascicles (Grant et al., 2013) and around the tenocytes (suggesting that elastin has an effect on tenocytes' mechanical environment and thus mechanosensitive cellular functions (Grant et al., 2013)). It may also play a role in maintaining macroscopic crimp (Parry et al., 1978).

2.2.3. Proteoglycans

Proteoglycans (~1-5% of the tissue's dry mass (Koob and Vogel, 1987)) are proteins onto which glycosaminoglycan (GAG) chains - highly anionic carbohydrate molecule(s)

- have covalently attached in a process known as glycosylation (Yanagishita, 1993). Glycosaminoglycans (also known as mucopolysaccharides) are a type of glycan (also known as polysaccharides) which are a type of carbohydrate (also known as saccharides). Proteoglycans are thus a specific type of ‘glycoprotein’ - a generic term for a protein bonded with a carbohydrate (Sharon, 1988). The polar GAG chains (along with osmotic pressure and intermolecular hydrogen bonds) attract water molecules which create a swelling pressure inside the tissue, absorbing force (especially shock) (Baer et al., 1987) and greatly affecting the viscoelastic properties (Thornton et al., 2001; Screen, 2008; Legerlotz et al., 2013) although proteoglycans are thought to not carry considerable load themselves (Screen et al., 2005b). One of the most important proteoglycans in tendon is decorin which controls the rate of fibril production, affects collagen fibril assembly, forms links between Type I fibrils (Scott, 1988; Yanagishita, 1993) and maintains the interfibrillar distances (Danielson et al., 1997). It is also the smallest, with only one GAG chain (Rigozzi, 2011).

2.2.4. Crosslinks

The chief source of mechanical strength and stiffness (Thompson and Czernuszka, 1995) in Type I collagen is the peptide bond that links neighbouring collagen molecules (Puxkandl et al., 2002) and fibrils (Eriksen et al., 2014) together. Indeed, if the crosslinks are artificially removed from a tendon it will behave more like a liquid than a solid (Misof et al., 1997b). In other words, there will be more viscous sliding between molecules and fibrils than elastic stretching of these structures (Gautieri et al., 2013). The formation of these covalent bonds is normally controlled by the lysyl oxidase enzyme, although if a sugar molecule (usually glucose) is present it may also occur non-enzymatically. The latter process is termed ‘glycation’ and the resulting crosslink an ‘advanced glycation end-product’ or AGE (Avery and Bailey, 2006). Whereas the enzymatic process is essential to matrix development, strengthening and repair, non-enzymatic glycation happens more haphazardly and is instead associated with ageing and disease of the tissue (Fujimoto et al., 1978; Monnier et al., 1992; Avery and Bailey, 2005; Snedeker and Gautieri, 2014).

2.2.5. Ground Substance

The majority of the remaining space within the tendon will be filled up by a gel, also produced by the tenocytes, known as the ‘ground substance’ (Puxkandl et al., 2002). This consists of water (60-80% (Abate et al., 2009)) together with the proteoglycans and loose GAG chains. Chief amongst these chains is hyaluronan, thought to contribute to structural integrity and maintenance of fibrils (Baer et al., 1987) and fibrillogenesis (Scott, 1988). Additionally, adhesive glycoproteins will be present, connecting the ground substance to the collagenous matrix and cells (Ivatt, 1984).

2.2.6. Other ECM Components

There will be nerves and blood vessels present in the ECM. However, the former mainly exist in the paratenon (Ackermann et al., 2001) while the latter only penetrate as far as the endotenon (O’Brien, 1997). This lack of vasculature means that the blood supply to the fascicles is poor and, as a result, tendon is vulnerable to degeneration and poor healing (Benjamin et al., 2008). Traces of calcium, magnesium, cadmium, copper, zinc, lead, nickel, fluoride, phosphor and silicon are present, making up 0.2% of the dry mass (Spadaro et al., 1970).

2.3. Non-Permanent Deformation

Tendon’s response to load is complex and influenced by a number of different factors. To begin with, its non-permanent response (i.e. deformation which is recovered when the force is removed) will be discussed. As with all sections, only tensile forces will be considered.

2.3.1. Viscoelasticity

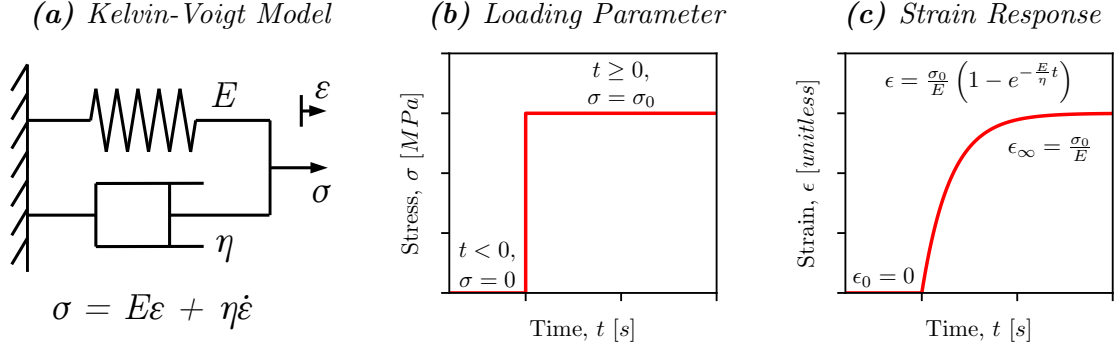
As detailed previously, tendon is comprised mostly of a collagenous matrix (which behaves elastically) and water (which is virtually incompressible) (Reese and Weiss, 2013). This combination is termed ‘viscoelasticity’ and results in three main phenomena: creep strain, stress relaxation and hysteresis. These can be explained most simply through the Kelvin-

Voigt and Maxwell models of tendon (Puxkandl et al. (2002); Meyers and Chawla (2009), see Figure 2.4) although the Standard Linear Solid (SLS) Model (Roylance (2001), see Figure 2.5) and its generalised form, the Wiechert model (Vincent (2012), see Figure 2.6) are more accurate but more mathematically complex. In all cases, the models are heuristic; linear springs and dashpots (dampers) are used to represent the elastic and viscous behaviour of the collagen matrix and of the ground substance (Takács et al., 2015). López-Guerra and Solares (2014) note that non-linear components should be substituted in if a better fit of real-world experimental data is desired. See Appendix A for the derivations of the mathematical descriptions. Note that, because tendon is poroelastic (the matrix has interstitial spaces/voids in it which the water can permeate into, creating the initial circumstances under which the viscoelastic behaviour can take place (Hannafin and Arnoczky, 1994)), it is also possible to use poroelasticity models (biphasic theory) to investigate behaviour (Detournay and Cheng, 1993). Another model that could be considered is the Quasi-Linear Viscoelastic (QLV) theory (Woo et al., 1982; Provenzano et al., 2001; Fung, 2004) which separates out the elastic and the time dependent components of the response. However, this theory is derived in terms of stress relaxation and is thus not immediately applicable to other manifestations of viscoelasticity.

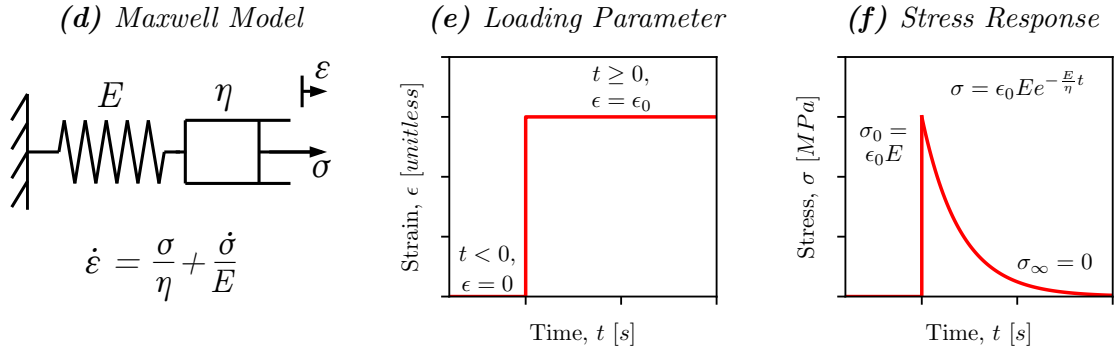
2.3.2. General Deformation Behaviour

Overall load response is determined by the aggregate of all of the deformation mechanisms of each sub-structure and hierarchical level combined (Han et al., 2013). Sasaki and Odajima (1996a) investigated three of these levels - molecular, fibril and macro - and found the first two to be approximately Hookean while the third exhibited a more complicated response (with distinct ‘toe’, ‘heel’ and ‘linear’ regions which are characteristic of tendon). These are shown in Figure 2.7a. The implication is that a simple superposition of molecular and fibrillar elongation is not sufficient to describe tendon’s full response to load; other deformation mechanisms must necessarily be occurring.

Strain Creep



Stress Relaxation



Hysteresis

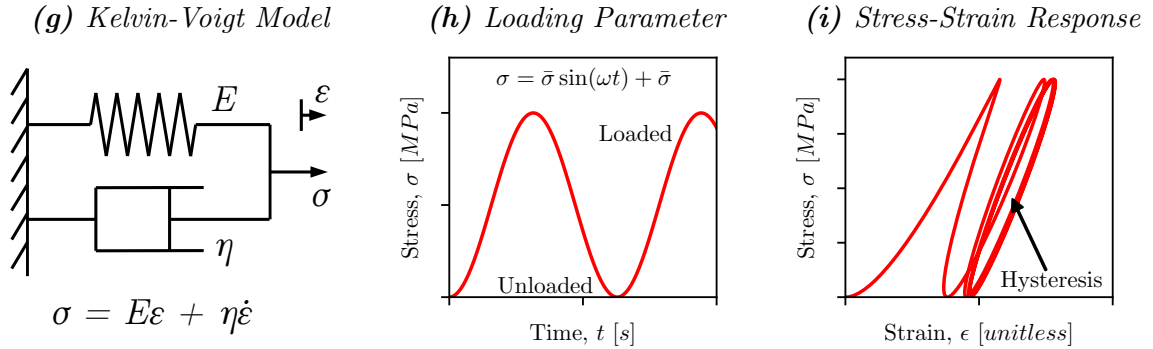
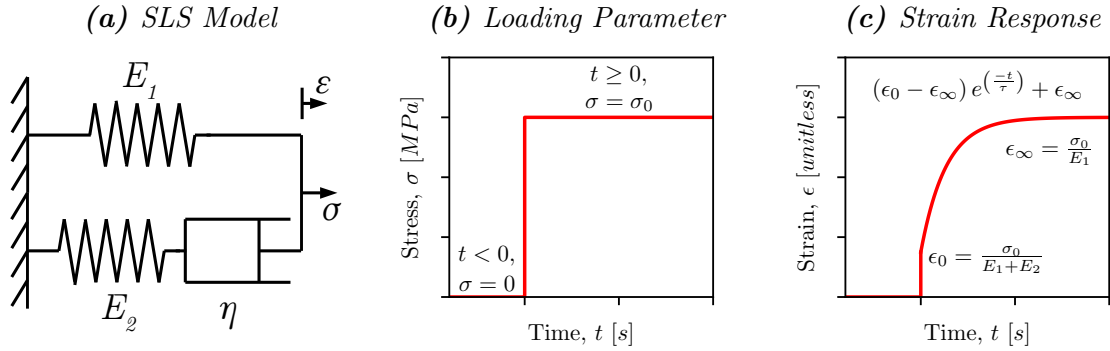
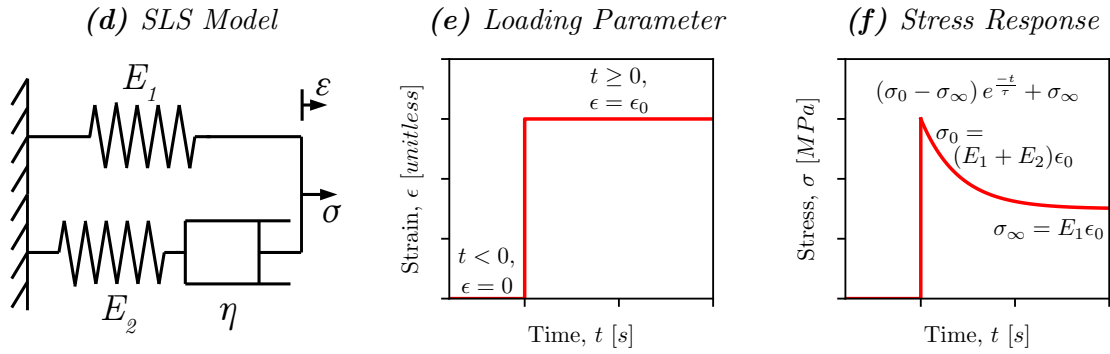


Figure 2.4: Strain creep: modelled most simply by (a) a spring and dashpot in parallel which, when subjected to (b) a constant load, will (c) stretch over time. Stress relaxation: modelled most simply by (d) a spring and dashpot in series which, when subjected to (e) a constant stretch, will (f) relax over time. Hysteresis: better modelled by Kelvin-Voigt (g) when the applied load is small and by Maxwell when the load is large. When a Kelvin-Voigt is subjected to a cyclic tensile load (h), the stress-strain response (i) will form loops that initially increase in strain but become more consistent. Note that the application of stress and strain in (b) and (e) will not, in reality, be instantaneous (although this is deemed inconsequential if the loading time $\ll 10\tau$ where time constant τ equals $\frac{\eta}{E}$).

Strain Creep



Stress Relaxation



Hysteresis

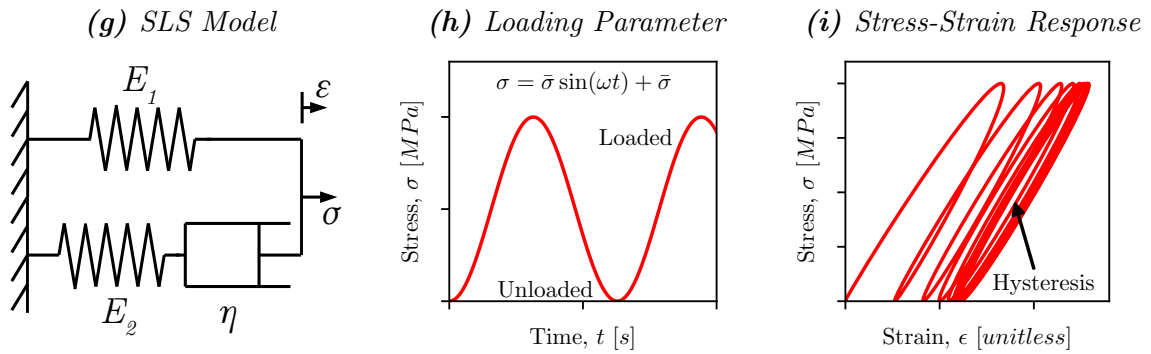


Figure 2.5: A compromise between simplicity and accuracy can be achieved by using (a, d and g) a spring and dashpot in series with a spring parallel to both (i.e. the Kelvin-Voigt and Maxwell Models in combination). Applying (b) a constant load will result in (c) an initial elastic response followed by strain creep. Applying (e) a constant stretch will result in (f) initial stress relaxation tending towards an elastic response. Applying (h) cyclic tensile load will result in (i) closed loops in the stress-strain plot, representing energy lost from the system as internal friction.

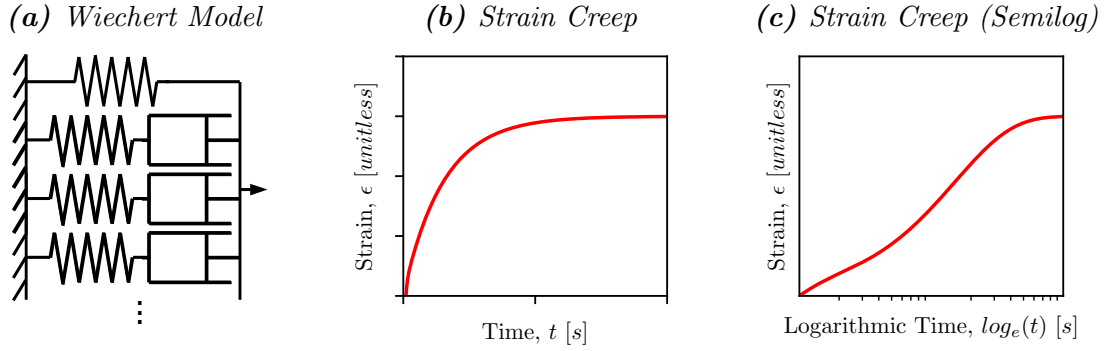


Figure 2.6: The Standard Linear Solid Model can be generalised by adding additional Maxwell elements in parallel (a), each representing an individual collagen fibre. If two Maxwell elements are used (as Gautieri et al. (2013) and Shen et al. (2011) did to model isolated fibrils) the strain response (b) to a constant load does not initially appear to differ from that of a Kelvin-Voigt or of an SLSM (compare (b) with Figure 2.4c and Figure 2.5c), but plotting that same response on linear-logarithmic axes (c) reveals that there are in fact now two creep processes taking place concurrently, each with a time constant derived from one of the Maxwell elements. This fits experimental observations well - it appears as if two, separate viscoelastic responses take place at once; one fast and one slow (Shen et al., 2011). Another method of generalisation would be to replace the dashpot in the SLSM with another SLSM - the Nafion model (López-Guerra and Solares, 2014) - but this does not add insight beyond the Wiechert model.

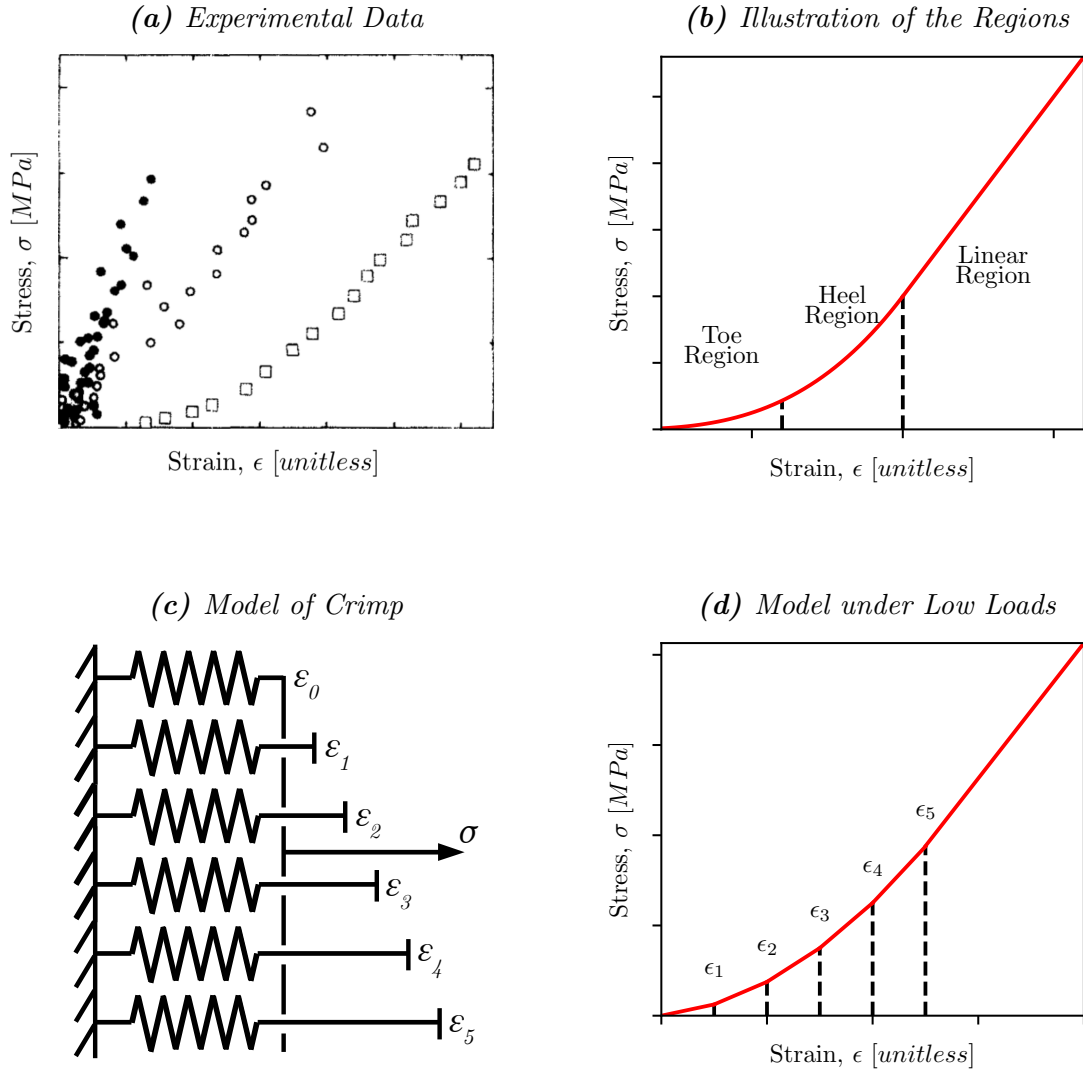


Figure 2.7: (a) The stress-strain response of collagen molecules (black circles), fibrils (white circles) and tendon as a whole (white squares) (Sasaki and Odajima, 1996a). Note that the first two of these are approximately linear whereas the third exhibits the classic toe, heel and linear regions illustrated in (b). Also, the fibril- and macro-level stiffnesses are approximately equal once the linear region is reached. Fibril recruitment and molecular unkinking can be modelled as (c), a set of springs in parallel which each engage at different strains (Viidik, 1966; Frisén et al., 1969; Hollister, 2007). The response of this model to load is shown in (d), for comparison with (b). Each spring in (c) represents a fibril or molecule which adds to the stiffness of the material once recruited or unkinked (Roy et al., 2011), hence the gradually increasing gradient in (d).

2.3.3. Specific Deformation Behaviour

Deformation can be analysed in more detail by considering loads of different magnitudes.

Small Loads: When a gradually increasing tensile force is applied to a tendon, the first mechanism of deformation to occur is the straightening out of the fibrils' and fibres' crimp (Franchi et al., 2007), known as 'recruitment'. This occurs at relatively small strains - below 3-4% (Diamant et al., 1972; Hansen et al., 2002; Screen et al., 2004a) - and creates the aforementioned toe region (see Figure 2.7) (Miller et al., 2012). It also has the effect of being a shock absorber, increasing the amount of time over which a rapidly applied load is taken up by the material (Riley, 2008). Additionally, elastin may stretch in the toe region (Gosline et al., 2002), increasing the capacity for recoil (Smith et al., 2011) whilst also aiding fascicle sliding (Smith et al., 2011). Beyond $\sim 3\%$ strain (Fratzl et al., 1997), it is likely that the long tropocollagen molecules begin to unkink. This results in the 'heel' region of the plot (Fratzl et al., 1997).

Medium Loads: In the 'linear' region, the fibrils themselves elongate (Fratzl et al., 1997). This elongation is a combination of three mechanisms: molecular sliding (Fratzl et al., 1997), G-period increase (Sasaki and Odajima, 1996a) and molecular elongation (Fessel et al., 2014). Sasaki and Odajima (1996a) suggest that the elongation - which is due to an increase in the molecular helix pitch (Cowan et al., 1955; Sasaki and Odajima, 1996b) - is solely responsible for $\sim 85\%$ of the strain that occurs in the linear region, although Fratzl et al. (1997) and Puxkandl et al. (2002) claim that both molecular elongation and sliding together account for only 40%. The remainder possibly coming from sliding between fibrils, between fibres and/or between fascicles (Screen et al., 2005a; Screen and Evans, 2009). Measurements from Screen et al. (2004a) show fascicle sliding to account for $\sim 49\%$ with local strain within fascicles making up a further 15%. Alternatively, the elongation could result from fibril translation, unfolding and slippage (Provenzano and Vanderby, 2006) or from deformation of the proteoglycan matrix (Puxkandl et al., 2002). Gautieri et al. (2013) note that elastic behaviour is due to molecular elongation whilst viscous behaviour is molecular and fibrillar sliding. In other words, viscoelastic behaviour is caused by two mechanisms: the movement of water

molecules and the sliding of collagenous structures.

2.4. Permanent Deformation

If the load continues to increase the deformation will no longer be entirely reversible: if the load were to be taken off after this point - and sufficient relaxation time then given to allow viscoelastic effects to dissipate - the tendon would still not return to its original state. Instead, it would retain a certain amount of strain and fibril disorganisation (which could be called ‘damage’ although there is no precise definition of this term, see Section 2.4.3). Of course, *in vivo*, this ‘permanent deformation’ would not be permanent in an absolute sense as the body has the ability to repair itself and reverse damage over time. The timescale relevant to this section, therefore, is shorter than this ‘turnover time.’

2.4.1. Yield and Rupture

A tendon will be in the linear region until about 4% (Abate et al., 2009) or 5% (Screen et al., 2004a) strain after which the fibres that are sliding start pulling free from one another (Fratzl et al., 1997) as the intermolecular crosslinks start to break (Puxkandl et al., 2002). This will cause permanent stretch and/or a loss in structural organisation (Fessel et al., 2014). The first bonds to break will be sacrificial in nature; they will be of a type that heals relatively easily (Thompson et al., 2001). Under further load, the decreasing effective load-bearing area of the tendon (due to this fibre pullout but also due to the Poisson effect) will lead to an increase in true stress and an eventual rupture. This occurs at 8-15% elongation (Bennett et al., 1986; Sasaki et al., 1999) although, more generally, the strength of a tendon is proportional to the amount of stress it experiences in life (Wang and Ker, 1995). The exact mechanisms of this are likely tensile failure of the fibres and shear failure between the fibrils (Abate et al., 2009). Some authors (Gutsmann et al., 2003) believe that the fibrils rupture surface-first whilst others (Wenger et al., 2008) believe that it happens uniformly. This lack of consensus stems from disagreement about whether fibrils are homogeneous or not: Gutsmann et al. (2003) found evidence that collagen molecules at fibrils’ surfaces are more crystalline in arrangement compared to

those in the more disordered core, which suggests a ‘tube-like’ structure. It is hypothesised that the harder, more brittle shell will plastically deform first if an overload is applied, preserving the softer, less dense centre of the fibril. Material might then be transported down this fluid-like core during a subsequent healing process, restoring said shell. This theory would also explain ‘buckling’ - the kinking of fibrils which have been subjected to a large load that was then removed, see Figure 2.8.

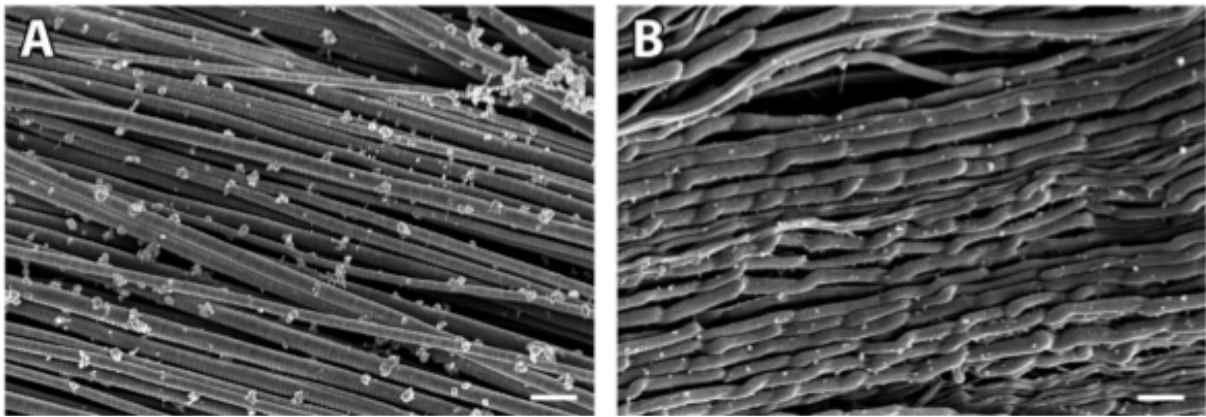


Figure 2.8: *Fibrils of healthy tendon that has not been loaded are straight (A) whereas those of tendon that has been loaded to rupture and then unloaded display buckling (B).*

Fibrils likely deform plastically at regular nodes along their lengths when overloaded, meaning that these areas do not elastically return to their original shapes when unloaded whilst the regions between them do.

Image credit: (Veres and Lee, 2012)

2.4.2. Fatigue Behaviour

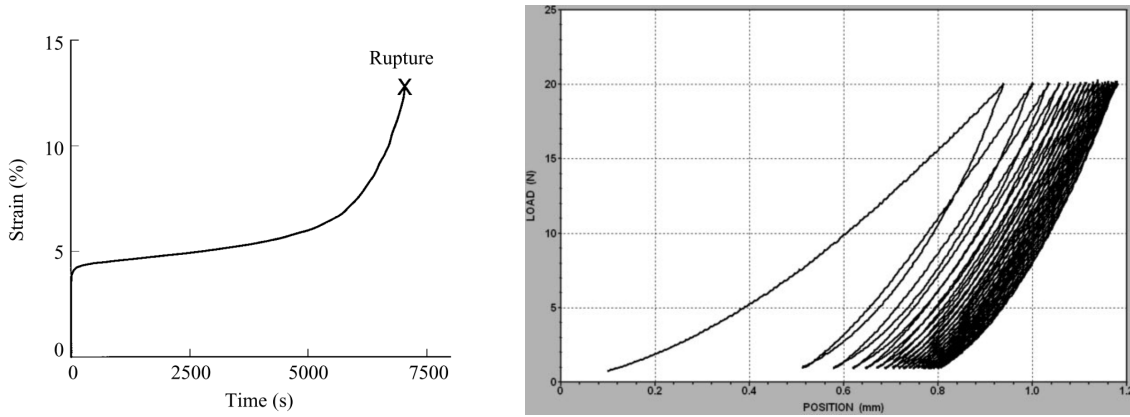
Whilst Sections 2.3.3 and 2.4.1 detailed the response of tendon to acute loading, this Section will address fatigue loading. The former is relevant when characterising the elastic and *total* damage properties of a material but the latter is relevant when the post-yield and damage *accumulation* properties are of interest. These are more representative of clinical tendon problems - tendon injury usually occurs during periods of prolonged overloading (e.g. playing sport/running) when plastic deformation accumulates (Kannus and Jozsa, 1991; Tallon et al., 2001).

Fatigue can be induced by applying a static load or a dynamic load to a sample. These

are the same loading parameters that were discussed in Section 2.3.1 and in Figures 2.4, 2.5 and 2.6 with regards to inducing viscoelastic strain creep and hysteresis except that in this instance the loads are larger (sufficient to induce permanent deformation). Stress relaxation is the odd-one-out of the viscoelastic phenomena in that it does not have an analogous fatigue behaviour, although this type of loading can still result in permanent deformation and damage (Legerlotz et al., 2011) which Shepherd and Screen (2013) classify as fatigue damage.

Static Fatigue: Insight into a material's post-yield (plastic) behaviour can be provided by performing 'creep' testing (Tang et al., 2007): loading a sample with a constant stress and monitoring its increase in length over time. Primarily, this allows the strain rate to be measured. Given that microtears and permanent fibre elongation are markers of pre-rupture behaviour (see Section 2.4.1) it may be that permanent strain (i.e. that beyond $\sim 5\%$) is a quantification of material damage. This would imply that the steady-state strain rate is a measure of the rate of damage (see Chapters 3 and 4). Additionally, the time to failure can be measured in creep tests, a potential measure of total capacity for damage which correlates with the amount of stress the tendon usually experiences in life (Ker et al., 2000). A handful of past experiments that used static fatigue testing to measure mechanical properties in tendon have been found (Wang and Ker, 1995; Ker et al., 2000; Wren et al., 2003; Shen et al., 2011; Gautieri et al., 2013) and an example of one of these, showing creep behaviour, is shown in Figure 2.9a.

Dynamic Fatigue: A more physiologically-relevant loading scenario can be achieved through 'cyclic' testing: applying an oscillating stress in a similar manner to how a tendon would experience load in life (consider an Achilles tendon during walking, for example). A number of past experiments have used this technique on tendon (Wang et al., 1995; Schechtman and Bader, 1997; Pike et al., 2000; Eliasson et al., 2007; Fung et al., 2009, 2010; Veres et al., 2013b; Thorpe et al., 2014b,a); one of these is shown in Figure 2.9b. Note that a tendon is a flexible material and must therefore always remain taut; it cannot be loaded in compression longitudinally. In other words, the applied stress must always be positive and, thus, it must have a positive mean. This implies that it



(a) Strain vs time data from a creep ('static fatigue') test on wallaby tail tendon (Wang and Ker, 1995). Note the initial 'strain creep' viscoelastic response in the first ~2500 seconds (similar to Figures 2.4c and 2.5c) followed by a 'damage response' as strain increases until rupture

(b) Load vs position data from a cyclic ('dynamic fatigue') test on rat Achilles tendon (Eliasson et al., 2007). Note that, initially, strain increases with each cycle and hysteresis is large, but these effects disappear and cycles become more consistent over time (note the similarities with Figures 2.4i and A.4). If a larger average load were applied the response would likely bear more resemblance to Figure A.2; strain would not stop increasing until rupture occurred.

Figure 2.9: Examples of fatigue behaviour.

can be separated into a constant component and an alternating component which, in turn, implies that dynamic fatigue necessarily includes static fatigue. However, static fatigue does not include dynamic fatigue and, therefore, it could be considered the more fundamental of the two despite being the less physiological (Ker et al., 2000). Note that this does not mean that dynamic fatigue can be modelled as a simple addition of a static load and an alternating load: the two types of fatigue are different phenomena. Data from wallaby tail tendons shows that dynamic fatigue life is shorter than would be predicted by a model that used accumulation of creep damage alone (Wang et al., 1995), and thus the act of applying a load and then removing it causes damage in addition to that caused by the load itself.

Modelling: Whilst the constitutive models introduced in Section 2.3.1 are accurate in describing non-permanent tendon behaviour - i.e. that under relatively small loads (Shen et al., 2011) - they are insufficient when considering permanent deformation. Thus, alterations need to be made in order to incorporate this. A good starting

point is the SLSM (see Figure 2.5) which explains all viscoelastic behaviours without the mathematical complexity of the Wiechert model. It is also mechanistic rather than phenomenological: it offers explanations of the mechanisms behind the behaviour (the springs represent the collagen matrix stretching whilst the dashpot represents the movement of water molecules) rather than just describing it, and thus this model relates the structure to the function. Alterations would need to be made to this SLSM in order to model the creep and cyclic behaviour seen in Figure 2.9 (note that stress relaxation no longer needs to be modelled as it is generally not a behaviour linked with fatigue damage) and it is known that this is caused by molecular, fibril and fibre sliding (this is the other mechanism behind viscoelasticity, as mentioned in Section 2.3.3). This new mechanism can be incorporated by adding a number of dashpots in parallel (see Figure 2.10). These dashpots would have static friction associated with them - no sliding would take place below a certain load - and thus this altered model would be exactly the same as an SLSM below a certain force threshold. This ensures that behaviour at low loads is still correctly modelled, and separates this model from similar ‘Maxwell-Kelvin’ models that have been used in the past (Takács et al., 2015). This modified SLSM (Figure 2.11) will be used for modelling during this dissertation. Note that while it is made up of components that have all already been seen in the literature, this specific arrangement is novel. Note also that this model is still relatively simple; a number of more complicated models (beyond the scope of this dissertation) have been reviewed by Fang and Lake (2016).

2.4.3. Phenomenological Definitions of Damage

There is no definitive consensus as to what exactly tendon damage is. One definition is that a region within the tissue is considered damaged if it no longer contributes to the stiffness and strength of the material (Wang and Ker, 1995). Alternatively, ‘damage’ is an irreversible change in the microstructure that alters the macroscopic mechanical properties (Lee and Elliott, 2017). Specifically, such changes would occur on the nanoscale fibril-level as this is where cells interface directly with the collagen matrix (Herod and Veres, 2018). Another definition might be ‘that which compromises the tendon’s ability to perform its function.’ As the function of a tendon is to connect a muscle to a bone

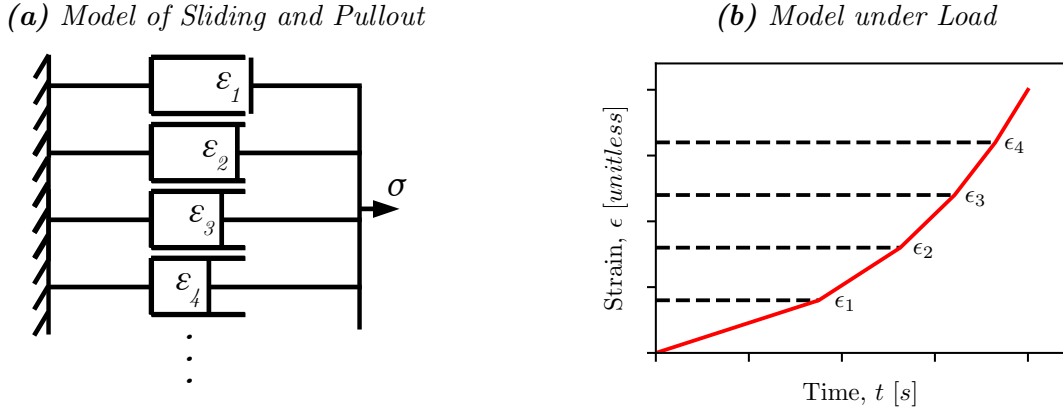


Figure 2.10: Molecular, fibril and fibre sliding can be modelled by dashpots in parallel (a), with each dashpot representing the interface between two sliding elements. Under a constant load σ , the dashpots will expand at a constant rate as shown in (b) up until ϵ_1 . At this point, the piston of the first dashpot will pull out of its cylinder (representing a microrupture: a fibril pulling away from its neighbour), causing the effective viscosity of the system to decrease (as shown in Equation A.32 in Appendix A.5). The rate at which the remaining dashpots will expand after ϵ_1 is thus faster - although still constant - until ϵ_2 at which point the second dashpot likewise pulls out and the rate increases again. This will continue for all dashpots, resulting in the full curve shown in (b). This curve is well approximated by a combination of a linear and an exponential function, representing the sliding and the ‘fast fracture’ behaviour, respectively.

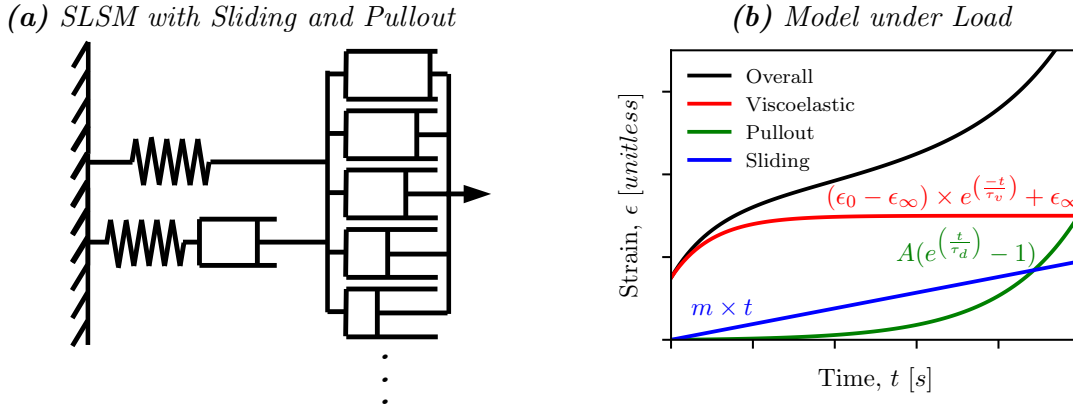


Figure 2.11: Combining the SLS model of viscoelasticity from Figure 2.5 with the model of sliding and pullout from 2.10 yields the complete model that will be used in this dissertation (a). The strain vs time response of this model is shown in (b): the viscoelastic response of the SLSM appears in red and the linear and exponential responses of the sliding and pullout model are shown in blue and green, respectively. The mathematical description of each response is shown alongside its curve. The overall response (i.e. the superposition of all three responses) is shown in black and should be compared with the experimental data in Figure 2.9a.

and transmit force between them, anything that causes this to be impossible (i.e. total rupture) or difficult (painful; often labelled ‘tendinopathy’) could be considered damage in a clinical sense (Kaux et al., 2011). However, this definition may prove limiting as investigations into tendon are often conducted in vitro where pain cannot be detected, and thus other phenomenological markers of damage must be used. Some candidates are introduced here.

Stiffness Loss: A reduction in the loading modulus has been associated with tendons that are nearing rupture during mechanical tests (Beaumont, 1989; Wang and Ker, 1995; Wang et al., 1995; Schechtman and Bader, 2002; Wren et al., 2003; Fung et al., 2010; Freedman et al., 2014) and with fragmented collagen (Thorpe et al., 2010). However, exercise (Józsa and Kannus, 1997), ageing (Bailey et al., 1974) and diabetes (Volper et al., 2015) all *increase* the modulus. More heuristically, it is known that loading modulus is linked to functional role (see Section 2.6), and so a change in this property would cause a tendon to be less optimal at performing its function. Note that, during a static fatigue test, stiffness initially increases and then decreases until failure (Freedman et al., 2014) which mirrors the behaviour in ramp-to-failure tests wherein stiffness increases in the toe-heel region, remains constant in the linear region and decreases in the plastic region (Gusachenko et al., 2012). Note that changes in the tendon’s cross-sectional area as a result of damage might mask true changes in stiffness: Freedman et al. (2014) note that cross-sectional area increases significantly in injured tendon.

Increased Permanent Strain: Also correlated with tendons nearing rupture (Wren et al., 2003) but associated with lower levels of damage as well (Fung et al., 2009).

Percentage Increase in Peak Strain: Herod and Veres (2018) reported that, during tendon dynamic loading, the cycle-on-cycle percentage increase in peak strain was a good predictor (i.e. significant with low dispersion in the data) of the total number of cycles before failure. No other mechanical parameters (loading modulus, hysteresis) were found to predict the number of cycles.

Decreased Ultimate Tensile Strength: An injured tendon will experience a decrease in UTS proportional to its decrease in loading modulus (LaCroix et al., 2013). This would impact its ability to perform its physiological function.

Decreased Viscoelasticity: Viscoelastic behaviour - which absorbs energy so as to protect the tendon (Bonifasi-Lista et al., 2005) - decreases in damaged and diseased samples (Abramowitch et al., 1999; Woo et al., 2000). Viscoelastic properties can also change in tendinopathic and ruptured tendons, as these cause increased cross-sectional area, increased hydration and an abnormal collagen-fibre structure (Soslowsky et al., 2000; Lin et al., 2004).

Disrupted Mechanobiology: It is known that there is both ‘healthy’ and ‘unhealthy’ loading of tendon; loads experienced during moderate exercise, for example, are beneficial (Kjær et al., 2005; Abate et al., 2009). These will stretch the Achilles tendon and the extracellular matrix within it (strain of 5-6% is induced by physiological loads (Screen et al., 2004a)) causing the fibroblasts to experience force and, in turn, strain. This will induce a mechanobiological response: the mechanical loading causes the cells to produce new collagen and proteoglycans (anabolism) (Vailas et al., 1985; Maeda and Ohashi, 2015). This collagen turnover replaces old collagen and, in this way, the structure is kept healthy. Under too large a load, however, microruptures start to form. These disrupt the tenocytes’ physical environment, buckling them (Pingel et al., 2014), which disrupts the mechanobiology process as well (leading to excessive catabolism) (Magnusson et al., 2010; Maeda and Ohashi, 2015). This is supported by evidence of cellular changes taking place even before the development of tendinopathy (Cook et al., 2004). Given that Achilles tendons experience loads close to their ultimate tensile strengths during normal running (Komi, 1990), and that even physiological loads are large enough to cause microdamage (Screen et al., 2004a), it is likely that unhealthy loading only starts beyond this point. Note that ‘too little exercise’ and ‘too much exercise’ are both risk factors for tendon injury: both increase the likelihood of overload. In the case of the former the tendons are not adapted to large strains whilst in the latter insufficient time has been allowed for damaged collagen to be replaced. Given this argument, it appears as if damage is likely

a result of a build-up of permanent changes to the structure affecting cell reactions. This is opposed to it being a result of more short term effects, such as would be measurable by changes in mechanical properties (increased permanent strain, decreased UTS, etc).

Biochemistry: Following on from the previous paragraph, it may be that loads which no longer invoke a healthy mechanobiological response but rather a ‘healing’ response are what should be considered damaging (Gibbon et al., 1999). Markers of a healing response include inflammation, the presence of matrix metalloproteinases (MMPS; enzymes which degrade the matrix during catabolism) (Thorpe et al., 2015a) and increased collagen III production which is associated with scar formation (and which has inferior mechanical properties) (Pingel et al., 2014). The way in which inflammation plays a role is still debated because tendinopathy can occur with or without this response (Yeung et al., 2018); it possibly only appears when the matrix damage is severe (Shepherd and Screen, 2013). Jones et al. (2013) note that tendinopathy has been seen to be associated with a decrease in collagen content and an increase in turnover rate and protease activity. Kannus and Jozsa (1991) mention cellular proliferation and lipid accumulation as signs of damage accumulation resulting from repeated overuse in older patients.

Disrupted Homeostasis: As discussed, an equilibrium exists between the rate of anabolism and the rate of catabolism of collagen with activity such as exercise increasing both (Magnusson et al., 2010). Indeed, cells’ deformation environment is vital for homeostasis (Malaviya et al., 2000) and the mechanosensitivity creates a feedback loop which maintains the equilibrium (Vailas et al., 1985). It may be that cells are programmed to function specifically at the strain levels experienced by their anatomical location (Snedeker and Gautieri, 2014) and if a disruption to their physical environment occurs they then behave so as to counteract it (Birch, 2007). In this context, damage would only occur as a result of an overload/unhealthy levels of loading. However, it may be that damage occurs even during normal, everyday activities (Wang and Ker, 1995) and that the turnover (‘repair’) process merely prevents it from accumulating. This would imply that it is a disruption of the equilibrium that causes clinical problems, either an increase in the rate of damage or a decrease in the rate of repair (or both), and that it is thus

damage *accumulation* that is relevant (Archambault, 2003) together with a cell response (Thorpe et al., 2015a). Indeed, tendinopathy can often develop without a traumatic event having occurred, implying that it is caused by an accumulation of microinjuries (Kannus and Jozsa, 1991; Wang, 2006).

Fibril Diameter: A greater ratio of small-diameter to large-diameter fibrils are found in tendinopathic regions (Pingel et al., 2014). This is likely because a greater proportion of the fibrils are newly-made and thus have not yet matured to full size. Additionally, the regions of discrete plasticity would decrease in cross-sectional area due to the Poisson effect and also dissociation.

Net Loss of Collagen after Exercise: Collagen turnover occurs in healthy tendon: synthesis and degradation processes are constantly creating new fibrils and breaking down old ones, respectively (Dyment and Galloway, 2015). Both these processes are elevated after exercise, with this probably being a mechanobiological response of the tenocytes to strain (Abate et al., 2009; Magnusson et al., 2010). In any case, this may imply that insufficient rest after overloading (especially in the first 24 hours) may disrupt the balance between anabolism and catabolism, leading to a net collagen loss and thus a weaker structure (Arnoczky et al., 2007).

Fibril Disorder: The usually parallel alignment of fibrils, as discussed in Section 2.1.3, is more fragmented and chaotic in tendinopathic samples (Kannus and Jozsa, 1991; Pingel et al., 2014).

2.5. Deformation Mechanisms

While much previous work has been done around the changing behaviour of tendon as it is loaded with force, the exact physical mechanisms behind these changes are yet to be fully elucidated. This section will look at what is currently known in this area.

2.5.1. Molecular Level

At the lowest hierarchical level are the long, thin tropocollagen molecules arranged in an ordered structure approximately parallel to the axis of the fibril which they comprise.

Molecular Unkinking: When unloaded, the gap region (G-period) between molecules often creates kinks in the structure (Wess et al., 1998b). Under load, these are soon (in the ‘heel’ region of the stress-strain graph) straightened out (Fraser et al., 1983; Folkhard et al., 1987; Misof et al., 1997a).

Triple Helical Microunfolding: While usually twisted into a triple-helix shape, an applied tensile load can cause individual molecules to unwind and straighten out, breaking the intra-molecular hydrogen bonds that exist between polypeptide chains (Sasaki and Odajima, 1996a). This takes place at loads much lower than those traditionally associated with damage (Bourne et al., 2014) and is recoverable (Veres and Lee, 2012). Gautieri et al. (2013) attribute viscous behaviour to the breaking and re-forming of these H-bonds.

Molecular Denaturation: While microunfolding is a pre-yield phenomenon, the post-yield region sees a further structural change taking place. This is described as an uncoiling of the triple helix and is again attributed to the breaking of intra-molecular hydrogen bonds (Veres and Lee, 2012). Possibly, this mechanism may be a consequence of the disruption of the matrix that takes place at high loads (fibril dissociation) creating more lateral space and thus allowing more microunfolding to occur. It may be permanent and increases in tendons that have been overloaded (Steven et al., 1975; Willett et al., 2007). Denaturing occurs first at the sites where the fibrils were kinked (indeed, discrete plasticity, fibril kinking and denaturation are probably all directly related), see Section 2.5.2, with further denaturing then occurring on the surface of some disrupted fibrils (which suggests that fibrils are ‘tube-like’ with different properties on the surface compared to in the core) (Veres and Lee, 2012).

Molecular Elongation: In the linear region, the now-straight molecules stretch as an affine lengthening which is usually non-permanent (Mosler et al., 1985; Folkhard et al., 1987). However, when a large enough strain is applied (>5%) the molecules become

stiffer, possibly due to the triple helix tightening and reduced microunfolded and/or reduced amino-acid mobility (Bianchi et al., 2016).

Molecular Rearrangement: The fibril D-period increases with load, partly due to molecular sliding (whereas, on the other hand, molecular elongation does not change the D- or O-periods) and partly due to rearrangement of the molecular matrix (Sasaki and Odajima, 1996a; Fratzl et al., 1997). In healthy tendons, this D-period lengthening is due to elongation and rearrangement in equal parts, but in ‘damaged’ (pre-strained) tendons the latter is more prominent (and also happens marginally later). This suggests that damage involves a degradation of inter-molecular crosslinks which exist all along molecules’ lengths but are most highly concentrated in the overlap regions. This, despite the molecular stiffening mentioned previously, results in a net loss in stiffness for the fibril (Bianchi et al., 2016). Gautieri et al. (2013) cite molecular rearrangement as one of the reasons for viscoelastic behaviour. Eventually, the D-banding (which is visible under scanning electron microscopy) will disappear completely from the surface fibrils (which were the first to be recruited and also the first to plastically deform), signalling a disrupted packing arrangement and further denaturation (Veres et al., 2013b).

Lateral Molecular Motion: While it is initially possible for molecules to undergo a small amount of lateral movement, this ability disappears at the same time as fibre uncrimping occurs (i.e. at very low loads, in the toe region) (Misof et al., 1997a).

Molecular Sliding: Molecules are connected to each other by both covalent crosslinks (specifically, lysine bonds (Barnard et al., 1987)) and Van der Waals forces (Hooley et al., 1980). Under increasing load, these bonds shear and allow molecules to slide (Folkhard et al., 1987). Initially, this sliding is mostly reversible (Mosler et al., 1985) and results in the linear region of the stress-strain plot (along with molecular and fibril elongation) (Misof et al., 1997a) and the viscoelastic regions of the stress- and strain-time plots (along with the movement of water molecules (Screen et al., 2013)). Under larger forces, however, the sliding is permanent and results in the plastic region of the stress-strain plot. A possible explanation for these two behaviours is detailed in Figure 2.12.

Discrete Plasticity: The first molecules to exhibit permanent sliding will be those that were recruited first, resulting in ‘discrete plasticity’ when some regions are plastic and others not. This non-uniform response suggests that either load is not distributed evenly or local regions do not have equal mechanical properties (Veres and Lee, 2012). Increasing loads and/or fatigue will cause a greater density of plasticity (Fung et al., 2010). Plastic deformation disrupts the gap period in the molecular structure, and thus if load were to be removed at this point the plastic regions of fibrils would show greater kinking (Fung et al., 2009). Wiens et al. (2016) and Veres et al. (2013b) mention that the molecular sliding and denaturation associated with discrete plasticity are mechanisms for absorbing excessive strain in overloaded tendons, protecting it. Veres et al. (2013b) go on to note that discrete plasticity does not decrease load bearing capacity.

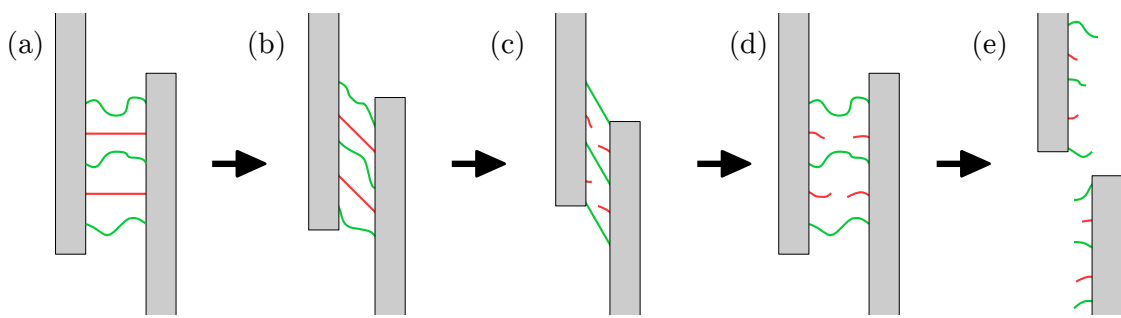


Figure 2.12: *It may be that some of the inter-molecular crosslinks are longer than others, represented in green and red respectively, with collagen molecules in grey (a). Under a small amount of load (i.e. in the heel region, once the fibril in question has been recruited) only a small amount of sliding is possible (b). In the linear region, however, the smaller, ‘sacrificial’ bonds break which allows greater sliding and delays the onset of damage (c) (Smith et al., 1999). If load was removed at this point, the Van der Waals forces between the molecules (due to polar amino acids) would result in a return to the original configuration (d). The sacrificial bonds would then self-heal relatively easily (Thompson et al., 2001). If, however, more load were to be applied such that the tendon entered its plastic region then even the long crosslinks would break (e). A microtear would be created, forming a region of ‘discrete plasticity’ and the permanent deformation would be measurable as an increase in the gap period. Beyond the UTS point, the build-up of microtears would start to affect the tendon’s structural integrity and lead to total rupture. It is not clear how broken crosslinks are dealt with: they might be repaired or replaced, or the entire fibril might be repaired or replaced as part of a homeostasis or a healing response.*

Molecular Pullout (Microrupture): If molecules continue to slide/have their inter-molecular bonds broken, eventually they will pull away from one another ('pullout'). This is also described in Figure 2.12.

2.5.2. Fibril Level

Evidence suggests that tendons' sub-rupture characteristics are due to mechanisms that occur on this level (Svensson et al., 2012). This may be because the cellular processes of tenocytes are found here, implying that collagen-cell interactions are most influenced by fibril-level structures (Orgel et al., 2011; Herod and Veres, 2018).

Movement of Water Molecules: The gel-like 'ground substance', containing hydrophilic proteoglycans (among other components), exists in the spaces between fibres (bundles of fibrils). Water molecules are held by the proteoglycans which creates a swelling pressure and absorbs force (especially shock) in the toe region (Baer et al., 1987). If a load is applied for an extended period of time the stress induces diffusion of free fluid molecules from the intra-fibril space into the extra-fibril space. This is a viscoelastic response and is thus one of the mechanisms behind the creep, stress relaxation (although the direction of flow is opposite in this case (Screen et al., 2011)) and hysteresis properties of collagenous material. Specifically, it is responsible for the longer-duration viscoelastic behaviour as it occurs more slowly than fibril sliding (Gautieri et al., 2013); this is because there is friction that hinders the water's flow. Strain energy that would otherwise be conserved in the spring-like collagen matrix is instead lost due to this movement (Veres and Lee, 2012). If a dry tendon that has permanent strain is rehydrated, that strain will disappear (Gautieri et al., 2013) suggesting that water molecules 'push' their way back into the interstitial locations rather than just passively responding to load. It may be that molecular sliding is only possible because the water molecules have the ability to move out of the way (Sarkar et al., 1987).

Osmotic Pressure: Changes in the distribution of water within the tendon (essentially, small amounts of relative 'drying' occur in the regions that water flowed out of while the other regions remain 'wet') causes stresses to be set up due to the pressure differences

(Masic et al., 2015).

Fibril Uncrimping: The first deformation mechanism to occur when a tendon is loaded - in the toe region - is the straightening out of fibrils (which have a wavy shape under no load) (Viidik, 1972; Bruehlmann et al., 2004). This absorbs shock and may add to the ability of a spring-like tendon to store energy by increasing its elastic recoil (Screen et al., 2013). Once a fibril has become straight and aligned with the loading axis it starts to bear load directly and elongate; it has been ‘recruited.’ Fibrils do not recruit uniformly: those nearer the surface of the fibre will straighten and start to be stretched first whilst those in the core are still uncrimping. Those that are recruited first will start elongating first (discrete plasticity). When overloaded (but not ruptured) and then unloaded, some of the crimp will return whilst other fibrils will remain straight (Wiens et al., 2016). Also, cyclic loading increases crimp amplitude (Freedman et al., 2015). This kink morphology following overload may be what induces the cellular healing response to injury (Veres et al., 2013b).

Fibril Rearrangement: Unloaded fibrils are slightly twisted, similar to rope. When subjected to a force they will untwist, straighten and align, becoming more parallel (Gusachenko et al., 2012). If they are loaded to the plastic region and then unloaded, however, they will become distorted and lose alignment (Veres and Lee, 2012). As with stiffness, fibrils are not uniformly twisted: those near the outer surface of fibres will have a more oblique torsion angle. This contributes to the lack in consistency of the fibril loading modulus (Yahia and Drouin, 1989; Benjamin et al., 2008).

Fibril Elongation: When deformation occurs on the molecular level the fibril level will also deform. Specifically, molecular elongation will cause elastic strain of the fibril whilst molecular sliding will cause plastic strain. The latter is evidenced by a loss of visible D-banding on the fibril surface, thought to be caused by disruption of the packed molecular structure as a result of sliding and rearrangement (Veres et al., 2013b).

Fibril Kinking: As fibril elongation does not happen uniformly within the fibre, areas of plastic strain develop in patches (Gupta et al., 2004; Veres et al., 2013b; Fessel et al.,

2014) and, specifically, those fibrils that are recruited first will strain first. If load is subsequently removed from the tendon - as happens during cyclic loading to zero (or near-zero) load - these patches of plastic strain form ‘kinks’ whilst the rest of the tendon recoils elastically (Fung et al., 2009, 2010; Shepherd et al., 2014a), as described by the ‘Compression of Unrecovered Elongation’ (CUE) model (Herod and Veres, 2018). These *fibril* kinks are not to be confused with *molecular* kinks which form in the gap regions of the molecular structure (Fratzl et al., 1997; Wess et al., 1998a) and were discussed earlier, although molecular kinks cause fibril kinks. Their presence increases in tendinopathic tissue (Pingel et al., 2014) and at low levels of damage in proportion with the amount of permanent strain (Fung et al., 2009). They develop transversely across the tendon (Fung et al., 2010). It has been noticed that molecular denaturation occurs primarily at kinking sites (Veres and Lee, 2012) and thus denaturation, molecular sliding, molecular kinking, fibril kinking, fibril elongation and cell-kink interactions are likely all related. Also, kinks will appear at regular intervals all the way along a fibril’s length (see Figure 2.8B) (Veres et al., 2013b). This suggests that plastic deformation (i.e. molecular sliding) strengthens that particular patch of fibril (strain hardening): if the opposite was true, the fibril would plastically deform at its weakest point and then simply rupture there before further kinking had a chance to occur (Veres et al., 2013b). However, the evidence is conflicted: stress-strain curves from mechanical tests of single fibrils do not always display strain hardening and, indeed, often show perfectly plastic deformation or strain softening (Shen et al., 2008).

Fibril Dissociation: Beyond the yield point, fibrils dissociate into substructures (see Figure 2.13) (Torp et al., 1975; Kastelic et al., 1980; Knorzer et al., 1986; Fung et al., 2009; Veres et al., 2013b). However, this may only occur in certain, poorly crosslinked tissues (Nemetschek et al., 1977).

Fibril Rupture: Fibrils are the first structures to rupture, which they do in a brittle manner (Gautieri et al., 2013), with these ruptures merging to form larger tears (Pierre-Jerome et al., 2010; Calleja and Connell, 2010). The damage area fraction increases with fatigue (Fung et al., 2009). It is presumed that the mechanism of fibril rupture

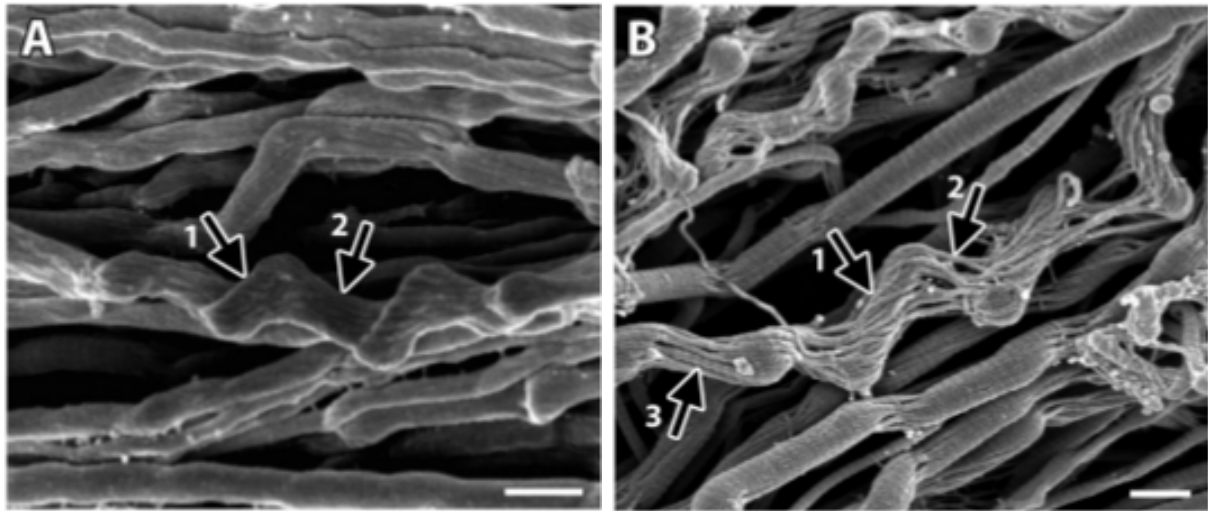


Figure 2.13: Kinked fibrils without (A) and with (B) dissociation into ‘sub-fibrils’

Image credit: (Veres and Lee, 2012)

is molecular pull-out (sliding until they lose contact with one another). Tears develop independently throughout the material (Nakama et al., 2005) and grow until total tendon rupture occurs. In vivo, healthy tendons seldom rupture and it is more likely that an accumulation of microtears over time (i.e. fatigue (Magnusson et al., 2010)) leads to tendinopathy (Frost et al., 2002) or degenerative changes which increase the ease with which further microtears develop and spread from then on (and this would then contribute to the risk of total rupture) (Nakama et al., 2005; Hodgson et al., 2012).

Fibril Sliding: Fibrils are connected to each other via covalent crosslinks between their surface molecules (Sims et al., 2000) and to proteoglycans in the ground substance via hydrogen bonding with glycosaminoglycans (GAGs) (Gupta et al., 2010). Note that the crosslinks formed by GAGs between fibrils are only incidental, they do not serve a major mechanical roles and the main function of GAGs is rather to facilitate fibril sliding via their hydrophilic properties (Rigozzi et al., 2013). Fibrils are also connected directly via fibril branching (small offshoots from a main fibril will form physical bridges to neighbouring fibrils, thus creating a continuous fibril ‘network’) (Szczeny et al., 2017). Initially, a small amount of sliding can happen easily because the proteoglycans readily deform (Screen et al., 2011; Rigozzi et al., 2013). Over time, further sliding can occur as the proteoglycans release their water molecules and unravel under stress (Sarkar et al.,

1987; Screen et al., 2011). Eventually, fibril-fibril (Puxkandl et al., 2002; Cheng and Screen, 2007) and fibril-ground substance (Fessel and Snedeker, 2009; Svensson et al., 2011; Szczesny and Elliott, 2014b; Pensalfini et al., 2014) shearing occurs. However, the latter ('shear-lag') may be negligible in dissipating load compared to the former (Szczesny et al., 2017) and both might be negligible compared to other deformation mechanisms; the major component of fibril deformation is likely molecular deformation (i.e. fibril elongation) as opposed to fibril sliding (Gonzalez et al., 2014). Regardless, Rigozzi et al. (2011) claim that this mechanism is relevant up to 5% strain and Svensson et al. (2012) found tendon strain-at-failure to be greater than that of individual fibrils (implying that a significant amount of fibril sliding occurs).

2.5.3. Fibre Level

This hierarchical level is responsible for the majority of tendon extension (Puxkandl et al., 2002; Sasaki and Odajima, 1996a).

Fibre Uncrimping: Similar to fibrils, unloaded fibres display a crimping pattern which also disappears in the toe region as they get recruited (Ciccone et al., 2006).

Fibre Reorganization: Fibres reorganise themselves under small loads - becoming more aligned and increasing in stiffness - with this happening to a greater degree in tendons that have a load-bearing function as opposed to a positional function (Screen et al., 2013). During stress relaxation, transverse contraction occurs possibly due to the flow of water out of the structure. There is also a small amount of fibre rotation due to the structure having a slight helical shape (Screen, 2008).

Fibre Disorganization: While healthy fibres are continuous, well-aligned and crimped, tendinopathic fibres are fragmented, disordered and less structured (Khan et al., 1999; Maffulli et al., 2004).

Fibre Sliding: Inter-fibre sliding has been identified as one of the chief mechanisms behind stress relaxation (Screen, 2008; Screen et al., 2013) although, in contrast to the fibril level, the forces connecting neighbouring fibres are negligible (Haraldsson et al.,

2008). It is the chief mechanism of failure for fascicles, occurring at strains between 8 and 14% (Screen et al., 2004b).

Fibre Elongation: Strain due to individual fibre extension is 6-8% smaller than that due to fibre sliding (Screen, 2008). During the first 4% of macro strain it contributes to fascicle elongation, but after this point only fibre sliding does (Screen et al., 2004b).

Cell Disruption: Tenocytes in tendon that has tendinopathy are found to be permanently buckled, rounded and shortened (Pingel et al., 2014; Thorpe et al., 2015a). The cause might be fibril kinking. This affected mechanical environment likely results in disrupted mechanobiology, as mentioned previously.

Ground Substance: While it may be true that proteoglycans form bridges between neighbouring fibres, it is thought that the load carried by these is negligible (Szczeny et al., 2012). For example, the loading modulus of a tendon in the transverse direction is orders-of-magnitude lower than that in the direction of load (Lynch et al., 2003). The proteoglycans merely have an indirect affect in that they attract water molecules. Deformation of the ground substance happens easily as it requires only relatively weak intermolecular forces to be overcome (Screen et al., 2004a).

Total Rupture: It is thought that a build up of microtears on the fibril level (due to molecular pullout) will lead to macro level tendon rupture (Fung et al., 2010). Indeed, a ruptured tendon will often show degenerative changes, implying that it had accumulated damage before failure (Kannus and Jozsa, 1991; Tallon et al., 2001). A ruptured fibril will not contribute to load-bearing, and thus the effective cross-sectional area of the tendon will decrease and more load will be transferred onto the remaining fibrils. The other fibrils will be more likely to rupture as a result and a snowball effect will develop. For this reason, a tendon as a whole is only marginally stronger than its weakest fibril, which is backed up by Svensson et al.'s (2012) findings that a tendon's stress-at-failure is lower than that of individual fibrils. This also takes into account the fact that tendons contain components which do not carry significant load - cells, elastin, proteoglycans, etc - while fibrils do not (they are entirely collagen and water).

2.5.4. Fascicle Level

Although it is a build up of fibril level microtears that causes failure, the rupture on a macro level will occur via fascicle pullout, see Figure 2.14.

Fascicle Sliding: In some cases, a tendon will originate from more than one muscle: the Achilles tendon, for example, forms out of both heads of the gastrocnemius and also out of the soleus (see Figure 2.15). The fascicles from each muscle fuse to form the tendon proper. However, each of these individual fascicle groups within the single tendon will experience a slightly different loading pattern because each of the three muscle endings contract with a slightly different force. Thus, a large amount of inter-fascicle sliding occurs - more than on any other hierarchical level (Handsfield et al., 2016) - and each fascicle can be considered as a functionally independent structure bonded only weakly to its neighbours (Haraldsson et al., 2008; Franz and Thelen, 2016). The superficial region of the human Achilles tendon, for example, elongates 16-29% more than the deep region with faster walking creating greater non-uniformity (Franz et al., 2015). This difference in fascicle movement allows the muscles to act more independently and thus perform their specific functions more effectively (Thorpe et al., 2012): the gastrocnemius's chief function is forward propulsion whilst the soleus's is vertical support (Franz and Thelen, 2016).

Fascicle Twisting: In the Achilles tendon, the fascicles from the three origins twist together as they fuse and, as such, the tendon has a helicity to it. This twist is approximately 90° - although a large amount of natural variation exists - with fascicles which are on the posterior surface at the proximal end of the tendon inserting onto the lateral side of the calcaneal tuberosity (Edama et al., 2015). When load is applied this twist tightens, similar to the strands of a rope, causing part of the load to be carried in compression and thus more efficiently. It also reduces the need for fascicle sliding (Roukis et al., 1996). However, it emphasises the heterogeneity of fascicle strain and muscle force dissipation - albeit to a lesser extent than fascicle sliding - which may be a contributing factor to injury (Edama et al., 2015; Handsfield et al., 2016).

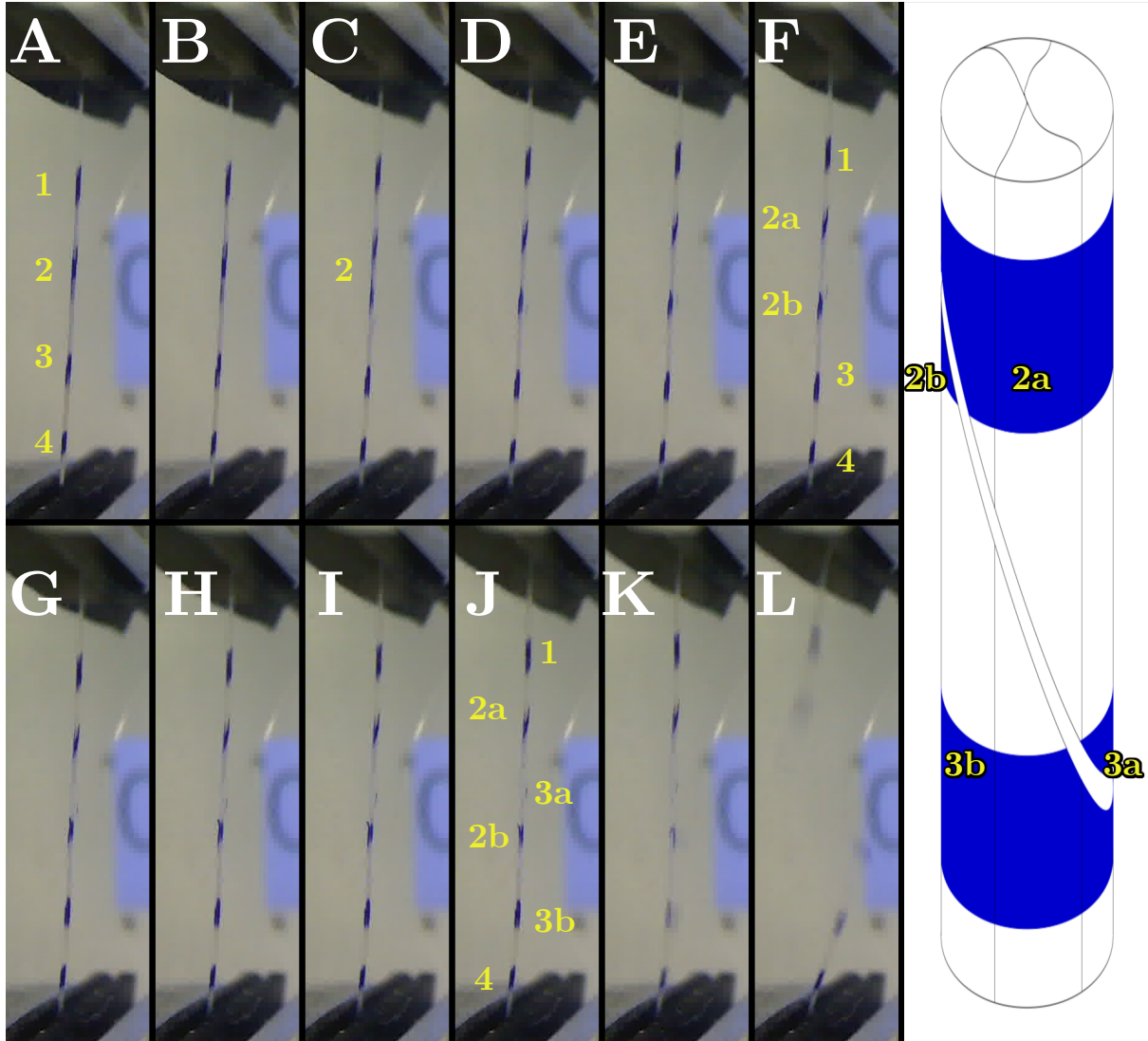


Figure 2.14: Still frames from a video depicting rat tail tendon rupture that was captured as part of the experiment detailed in Chapter 3. Four blue dots were drawn on the tendon, labeled as in (A). Notice that by (C) the second dot has split. In (F) these two pieces are explicitly labeled as dots 2a and 2b. Later, dot 3 also splits - albeit less clearly - and forms pieces 3a and 3b in (J). This implies that macro failure occurs along the tendon - rather than at a certain point - via the mechanism of fascicle sliding and pullout; this is depicted in the diagram on the right. A similar observation was made by Ker (2007).

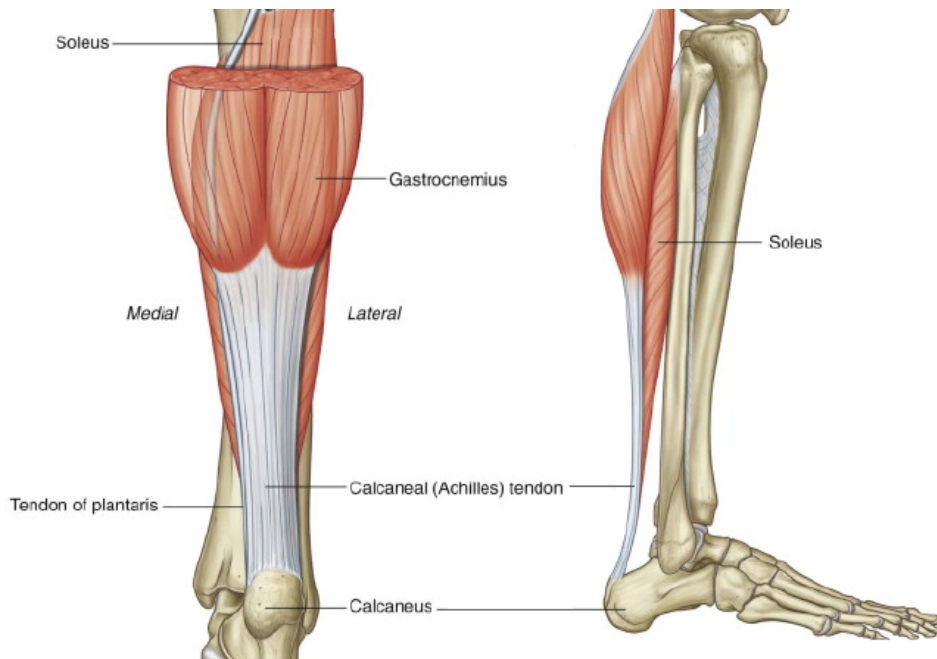


Figure 2.15: The Achilles tendon originates from the medial and lateral heads of the gastrocnemius as well as from the soleus.

Image credit: (Drake et al., 2015)

2.5.5. Summary

The chief mechanisms that govern tendon response are summarised here and in Figure 2.16.

Ramp-to-Failure: If an increasing load is applied to an unloaded tendon quickly, there will be uncrimping, molecular elongation and then fibril and molecular sliding as the bonds between these structures are broken. On a fibril level this will form discrete plasticity (microdamage) then molecular-level pullout (microrupture) and fascicle-level pullout (macrorupture). Mechanisms will appear first in those fibrils that are recruited first.

Static Fatigue: A constant load, sufficient to cause damage but not rupture, will trigger the same deformation mechanisms as a ramp-to-failure test while, at the same time, it will also trigger mechanisms that are time-dependent. As discussed in Section 2.4.2 and Figure 2.11, creep appears to comprise of a non-permanent viscoelastic response, a linear response and an exponential failure response. The mechanisms behind these are likely

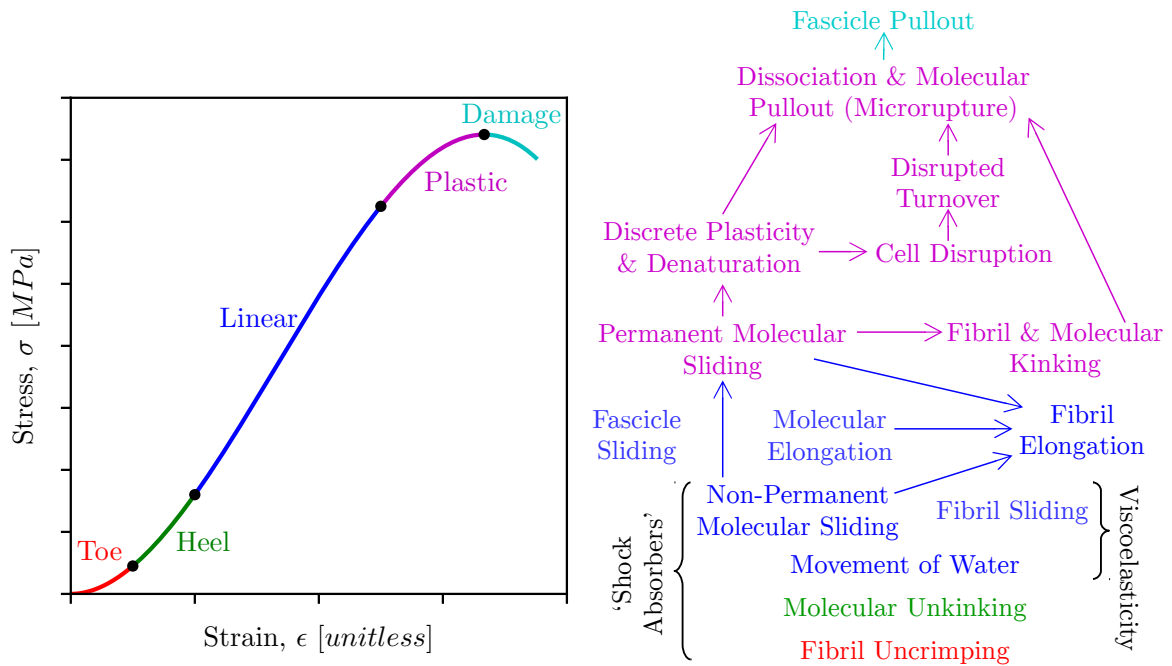


Figure 2.16: A summary of Section 2.5. The stress-strain response of a tendon can be divided into regions according to the load response behaviour in each, as shown on the left. The mechanisms that cause these various behaviours are shown on the right, colour-coded to correlate with the region in which they occur and linked with arrows to show causality.

movement of water molecules and fascicle sliding (viscous), non-permanent molecular and fibril elongation (elastic), molecular sliding and discrete plasticity (linear) and molecular pullout (exponential) which accumulates and leads to fascicle pullout (failure). Note that the two linear processes do not decrease the load-bearing capacity of the tendon (Veres et al., 2013b) as the effective cross-sectional area remains unchanged.

Dynamic Fatigue: It has previously been mentioned that cyclic loading consists of a static load combined with an alternating load. The static load will cause the mechanisms mentioned above while the alternating load will additionally cause molecular and fibril kinking and increased fibril crimp during the unloading (Fung et al., 2010). Kinking is likely a damage mechanism in and of itself, which explains why cyclically loaded samples

accumulate damage faster and fail sooner than would be predicted by creep alone (Wang and Ker, 1995; Thornton et al., 2007).

Long-Term: Mechanisms of damage that only appear in vivo include a disrupted physical cell environment as a result of discrete plasticity and/or microtears, affecting the mechanobiology and collagen production. This impairs the healthy turnover, homeostasis and healing processes, and thus allows microdamage to accumulate. It is this accumulation that causes injury and tendinopathy.

2.6. The Effect of Functional Role

As discussed previously, the functional role of a tendon - whether it is energy-storage for use in locomotion or positional for use in fine-motor control - will have an impact on its properties. This is because tendons have to be strong enough to handle the loads they experience in life but, at the same time, small enough to be efficient (Ker et al., 2000) and so a ‘one-type-fits-all’ structure would not be feasible. To a certain degree, mechanical properties are pre-programmed into tendons even before birth (the mechanical properties of embryonic tendons start to develop before they experience load for the first time (Birch, 2007; Schiele et al., 2015)) although a large amount of adaptation can take place in response to the physical stresses experienced during use (Shadwick, 1990; Ker et al., 2000). This is analogous to how bone adapts under Wolff’s law.

2.6.1. Tendon Types and Functional Roles

The types of tendon that have relevance to this project are the energy-storing Achilles tendon and the positional tail tendon.

Human and Rat Achilles Tendon: Connecting the muscles in the superficial posterior compartment of the leg to the calcaneus (calcaneal bone) in the heel, the calcaneal (Achilles) tendon is the largest, strongest and thickest in the body (Wren et al., 2001; Benjamin et al., 2008; Baravarian and Redkar, 2016) and enables plantar flexion of the foot. Tension is applied during concentric, eccentric and isometric contractions of the

soleus and gastrocnemius muscles with these occurring, respectively, during locomotion (in the ‘Terminal Stance’ phase of the gait cycle as the forefoot pushes against the ground, or when jumping up), landing (such as from a jump or when walking down stairs) and standing. The tendon thus experiences large amounts of load - up to four times body weight when walking and eight times when running and/or jumping (Giddings et al., 2000) - and, indeed, comes closer to its ultimate stress than any other tendon during normal use (Magnusson and Kjær, 2003). As a result - and in spite of its strength - it is the most commonly injured tendon in humans (Kannus and Jozsa, 1991; Maffulli et al., 1999). It is susceptible to both acute and chronic damage (Baravarian and Redkar, 2016) caused during either concentric or eccentric contraction or by sudden dorsi- or plantarflexion uncontrolled by muscle contractions. In humans, the weakest part is 2-6 cm above the insertion zone (the ‘watershed area’ (Carr and Norris, 1989)) where, due to a number of factors (concentrated torsional stress, small cross-sectional area, poorest blood supply, relatively high proportion of fibrocartilaginous material) 75% of ruptures occur (Abate et al., 2009; Gwynne-Jones et al., 2011). See Figure 2.17(a).

Rat Tail Tendon: The tail serves two purposes - thermoregulation (Vanhoutte et al., 2002) and balance (Hanson, 2004) - of which only the latter is relevant when considering tendons. Similar to a tightrope walker’s pole, the tail increases the rotational inertia of the animal to help it avoid toppling over when turning quickly. Additionally, it can be accurately positioned and held steady if needed, enabling fine control over the location of its centre of gravity (useful when traversing ropes and wires) and acting as a rudder when swimming or as a counterbalance when jumping. As a result, a single tail will contain many tendons of various lengths (but almost all relatively long) that can slide large amounts relative to the tail skeleton and skin in order to pull it to one side, see Figure 2.17(b). It shares the same hierarchical structure as the Achilles, including fibrils, fibres, fascicles, ground substance and endo-, epi- and paratenons which facilitate the sliding (Rowe, 1985). Unlike the Achilles, however, crimp exists even in situ (Rowe, 1985), indicating that tail tendons experience complete unloading (which the Achilles never does). Their long, thin shape, ease of isolation and abundance makes them a popular choice for tendon experiments.



(a) A rat Achilles tendon. Notice, from top (proximal) to bottom (distal), the flat aponeurosis sheath which interfaces with the gastrocnemius muscle, the individual fascicles before they have fused together, the tendon proper and, finally, the cartilaginous material surrounding the calcaneal bone.

(b) Pulling on a tendon from the proximal end of a rat tail will cause the distal end to curl dramatically, illustrating both the length of the tendon and the mechanism of function.

Figure 2.17: Rat Achilles and rat tail tendons.

2.6.2. Effect on Collagenous Structures

While total collagen content is the same, the proportional collagen content is greater in positional tendons (98% (McLennan et al., 1986)) compared to energy-storing ones (75% to 87% (Lagier and Exer, 1960; Amiel et al., 1984)). The former also have a larger average fibril diameter (Birch, 2007) and will be longer and thinner than the latter (Józsa and Kannus, 1997). The rate of collagen turnover is faster in tendons that are energy-storing in nature; the larger loads cause more microdamage to occur, resulting in more fibrils needing to be replaced more often (Ker et al., 2000; Birch et al., 2008; Abate et al., 2009). However, Birch (2007) reported the opposite (albeit in horses), postulating that a slower turnover rate is the reason why damage accumulates and leads to rupture more often in these tendons. Another explanation could be that remodelling weakens the structure and

so tendons subjected to high loads do not do so readily (Laurent, 1987).

2.6.3. Effect on the Non-Collagenous Components

Energy-storage tendons are larger and contain fewer nerve fibres and endings (Abate et al., 2009), but more water (Birch, 2007). They have more glycosaminoglycans (GAGs) (Batson et al., 2003) and, in particular, higher levels of sulphated GAGs (indicative of different types and quantities of proteoglycans being present) which likely has a regulating effect on fibril diameter (Birch, 2007). It is hypothesised that tension inhibits the catabolism of GAGs and proteoglycans (Arnoczky et al., 2004). Energy-storage tendons also have higher levels of DNA, which affects cellularity (Birch, 2007), and their inter-fascicular matrices behave more elastically (Thorpe et al., 2016) helped by a greater proportion of elastin (Thorpe et al., 2015c). The types of inter-molecular crosslinks present and the distribution of proteoglycans also differs (Thorpe et al., 2010).

2.6.4. Effect on Deformation and Damage Properties

Energy-storage tendons have been shown to have lower stiffness (loading modulus), more fibril reorganisation, less hysteresis, less stress relaxation and higher strains at failure and at the onset of plasticity compared to positional tendons, but there is no significant difference in ultimate tensile strength (UTS) (Shadwick, 1990; Birch, 2007; Shepherd and Screen, 2013; Screen et al., 2013; Thorpe et al., 2015b). They also have a greater fatigue life when compared directly to positional tendons (Batson et al., 2003; Shepherd et al., 2014a) but an equal fatigue life/rate of damage when testing is done at the stress levels experienced in vivo (Ker et al., 2000). The lower stiffness in energy-storage tendons is likely due to the smaller fibril diameters; there is a correlation between loading modulus and mass average fibril diameter due to inter-molecular crosslink concentration (Smith, 2006). Importantly, this means that functionally different tendons will adapt their loading moduli by changing their thicknesses; the intrinsic material property is *not* the controlled variable (Pollock and Shadwick, 1994; Ker et al., 2000). From a functional perspective, low stiffness decreases the amount of control a muscle has over the length of a tendon and thus the position of the anatomy it is attached to, which is why positional tendons are

more stiff. Similarly, the toe regions are smaller in these tendons (Screen et al., 2004a); they are larger in energy-storing ones where they are needed for shock absorption. In contrast to positional tendons, low stiffness is beneficial in energy-storage tendons as it increases their compliance, allowing muscles to act over a shorter distance (Gomes et al., 2017). For example, a short, powerful contraction of the gastrocnemius muscle creates a tensile force in the Achilles which is partly absorbed and partly transmitted to the foot. If the foot then starts to plantarflex in response to this, the tendon will shorten but still be able to maintain the same force due to the absorbed energy. Thus, the muscle does not need to contract as much in order to transmit that force, which is more efficient. The higher maximum strain in energy-storage tendons is due to increased fascicle sliding (Thorpe et al., 2012). Also, these tendons have a lower ‘safety margin’ - even physiological loading is enough to cause permanent deformation (Birch, 2007). While this is a necessary property given the functional requirement, it has been cited as the reason for the increased disposition of these tendons to degeneration and rupture (Rees et al., 2006). By definition, energy-storage tendons exhibit more resilience.

2.7. The Effect of Diabetes

Diabetes leads to changes in tendon which are associated with deteriorating function, increased propensity to damage and poorer healing (Lui, 2017).

2.7.1. Mechanism Behind Diabetic Changes

The cause of the effects detailed in this section is thought to be an increase in the formation of advanced glycation end-products (AGEs, which are stronger than regular crosslinks, see Section 2.2.4, and thus cause a stiffening of the structure (Eriksen et al., 2014)) (Fessel et al., 2014). This increase results from a higher presence of glucose in the tendon, it having perfused out of the vascular system due to its diabetes-induced high concentration in the bloodstream (Vogt et al., 1982). Note, however, that Couppé et al. (2015) and Batista et al. (2008) both reported that the effectiveness with which the diabetes is managed has no significant impact of the effect on tendon and Volper et al.

(2015) suspect that hyperglycaemia is not the sole cause of the mechanical changes seen. It is also known that AGEs increase naturally in people and animals as they get older (Thorpe et al., 2010) and a number of studies related to age (and unrelated to diabetes) have independently found AGEs to be correlated with increased loading modulus, failure stress and failure strain (Reddy, 2004; Peffers et al., 2014; Thorpe et al., 2015b). Diabetes can thus be considered to have the effect of accelerating age (Monnier et al., 1984).

2.7.2. Effect on Collagenous Structures

It is thought that fibrils pack together more tightly in diabetic tendon due to AGE crosslinks pulling them together (Abate et al., 2009): the diameters of individual fibrils decrease (Bai et al., 1992) and collagen density increases (Couppé et al., 2015) causing an increase in strength (Maffulli et al., 2003). However, tendon size increases in diabetic females (and males who also suffer from either retinopathy or neuropathy, both of these being established complications of diabetes) (Akturk et al., 2007), thickening (and scarring) of the Achilles can occur (Reddy et al., 2002) and total collagen content increases (de Oliveira et al., 2012). This would suggest that there are more fibrils overall. Paradoxically, however, diabetes decreases collagen production (Magnusson et al., 2010) which would suggest that collagen anabolism decreases too, by a greater amount. Additionally, changes in the remodelling and homeostasis processes of the collagen have been reported (Fox et al., 2011). Lastly, whilst the structure and arrangement of fibres remain the same (Volper et al., 2015) the disorganisation of fibrils (linked to tensile strength (Scott, 1988)) becomes more pronounced (Reddy et al., 2002).

2.7.3. Effect on Non-Collagenous Components

As already mentioned, more AGE crosslinks form. The altered deformation properties that result (see following paragraph) will mask mechanical stimuli and thus hinder cells' mechanobiological functioning (Snedeker and Gautieri, 2014). Neuropathy occurs in extremities (Diabetes UK, 2015), interfering with feedback responses and thus indirectly increasing patients' susceptibility to injury. Additionally, cell density and the density of proliferating cells increases (suggesting the presence of inflammation and thus increased

damage accumulation from everyday loads) (de Oliveira et al., 2013) leading to an inhibited capacity for repair and remodelling (Bedi et al., 2010). Indeed, cell proliferation levels at a rupture site will be less than those in a non-diabetic patient, indicating a decreased healing ability (Loots et al., 1999). Additionally, this healing will be fibrotic when it does occur (Ackerman et al., 2017). The levels of glycosaminoglycans do not change (Connizzo et al., 2014).

2.7.4. Effect on Deformation and Damage Properties

With a few exceptions, ultimate tensile strength (UTS) and stiffness/loading modulus (E) increases in diabetic samples, see Table 2.1 and Figure 2.18, with these increases being greater on average in rat tail tendons compared to rat Achilles tendons. While, *ex vivo*, these changes create a material that is more resistant to load it is suggested that, *in vivo*, they increase the likelihood of tendinopathy and rupture (Fox et al., 2011). A diabetic Achilles tendon that is stiffer and thicker may alter the gait pattern and thus the distribution of pressure on the foot, contributing to greater wear of the skin on the forefoot (Abate et al., 2013) leading to ‘diabetic foot’ (de Oliveira et al., 2011). There is also a considerable reduction in the viscoelastic response of the material: inter-fibre sliding and lateral fibre movement is drastically reduced whilst fibre stretching accounts for more of the strain (Snedeker, 2016). Note that Table 2.1 does not include any reports on tendon *fatigue* properties, a topic which - in general - has not been extensively researched and still constitutes a gap in the literature.

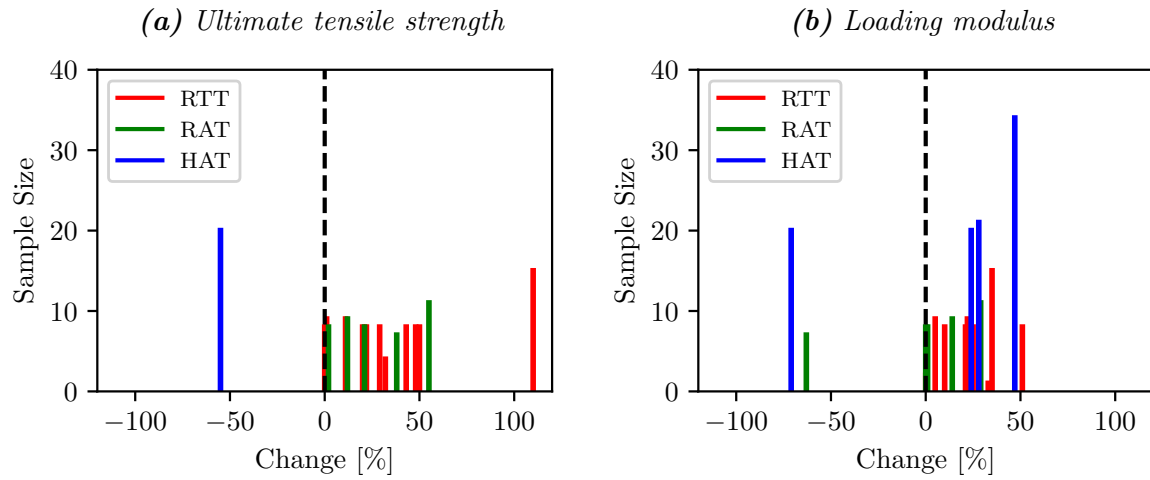


Figure 2.18: Summaries of the most relevant information from Table 2.1. Each vertical bar represents one diabetic experimental group from a study shown in the table with the height representing the number of samples involved and the horizontal position representing the increase or decrease in ultimate tensile strength (UTS) or loading modulus (E), relative to healthy samples. RTT = rat tail tendons, RAT = rat Achilles tendons, HAT = human Achilles tendons. As can be seen, most studies reported an increase in both UTS and E ; the exceptions to this are discussed in the caption of Table 2.1. When these exceptions are removed, it can be noted that rat tail tendons are affected more on average than rat Achilles tendons (the weighted mean increases are 42.1% vs 27.0% for UTS and 25.0% vs 16.2% for E , respectively)

Table 2.1: Summary of past studies related to the effect of diabetic models on tendon strength (UTS) and loading modulus (E). All reported an increase or no change in UTS and E with a few exceptions. Grant et al. (2005) reported decreases in UTS (-51.5%) and E (-70.7%) although the diabetic samples used in this instance were all sourced from patients with Charcot foot (neuropathic arthropathy); a comorbidity that may affect the Achilles to a greater degree than diabetes in and of itself. Boivin et al. (2014) also reported decreases in UTS (-41.0%) and E (-50.0%) in db/db mouse Achilles, however, their protocol included significant pre-conditioning, a strain-based (as opposed to displacement-based) loading rate and no method for keeping the samples hydrated during the experiment (dehydration is known to affect tendon mechanical properties (Haut and Haut, 1997)). de Oliveira et al. (2011) measured a decrease in E only (-63.3%) but, similarly, did not keep samples hydrated. Fox et al. (2011) also measured decreases in E only (-19.0% and -14.8%) but in rat tibia-patellar tendons.

Authors	Animal Tendon		n	Age at Start*	Trial Length*	Model	Result (vs Control)		Significance
							UTS	E	
Galeski et al. (1977)	Rat	Tail	1	8	28	Alloxan		0.1% ↑	
Galeski et al. (1977)	Rat	Tail	1	8	27	Alloxan		33.2% ↑	
Galeski et al. (1977)	Rat	Tail	1	8	38	Alloxan		0.01% ↓	
Andreassen et al. (1981)	Rat	Tail	8	21.3	1.3	STZ (70)	20% ↑	↑	2p < 0.05
Andreassen et al. (1981)	Rat	Tail	8	18.4	4.2	STZ (70)	50% ↑	↑	2p < 0.05
Andreassen et al. (1981)	Rat	Tail	8	18.4	4.2	STZ (70) ⁱ	=	=	2p < 0.05
Reddy et al. (2002)	Rabbit	Achilles	12	12-16	8	Ribose	110.7% ↑	108.4% ↑	p < 0.01
Reddy (2004)	Rabbit	Achilles	6	12-16	8	Ribose	159% ↑	161% ↑	p < 0.001
Grant et al. (2005)	Human	Achilles	20			Charcot	55.1% ↓	70.7% ↓	
Fox et al. (2011)	Rat	Tibia-patellar	8		2.5	STZ (65)	=	19.0% ↓	p < 0.02
Fox et al. (2011)	Rat	Tibia-patellar	8		1.5	STZ (65)	=	14.8% ↓	p < 0.04
de Oliveira et al. (2011)	Rat	Achilles	7	10	10	STZ (60)	38.3% ↑	63.3% ↓	p > 0.01
de Oliveira et al. (2012)	Rat	Achilles	11	10	10+	STZ (60) ^t	54.6% ↑	29.3% ↑	p < 0.01
Connizzo et al. (2014)	Mouse	Achilles (ins.)	8	8.4	N/A	db/db		↓	p < 0.025
Connizzo et al. (2014)	Mouse	Achilles (mid.)	8	8.4	N/A	db/db		=	p > 0.05
Connizzo et al. (2014)	Mouse	Patellar (ins.)	8	8.4	N/A	db/db		=	p > 0.05

Connizzo et al. (2014)	Mouse	S'spinatus (ins.)	8	8.4	N/A	db/db		=	p > 0.05
Connizzo et al. (2014)	Mouse	S'spinatus (mid.)	8	8.4	N/A	db/db		=	p > 0.05
Connizzo et al. (2014)	Mouse	Patellar (mid.)	8	8.4	N/A	db/db		=	p > 0.05
Boivin et al. (2014)	Mouse	Achilles	10	5	N/A	db/db	41.0% ↓	50.0% ↓	p/2 < 0.001
Gonzalez et al. (2014)	Rat	Tail	4	20	12	ZDSD	31.5% ↑	23.6% ↑	p = 0.016
Fessel et al. (2014)	Rat	Tail	15	17-24	N/A	MGO	110% ↑	35 % ↑	p < 0.03
Couppé et al. (2015)	Human	Achilles	21	~58 yrs	N/A	PCD		28 % ↑	p > 0.05
Couppé et al. (2015)	Human	Achilles	20	~60 yrs	N/A	WCD		24 % ↑	p > 0.05
Couppé et al. (2015)	Human	Achilles	17	~58 yrs	N/A	PCD		47.1% ↑	p > 0.05
Couppé et al. (2015)	Human	Achilles	17	~60 yrs	N/A	WCD		47.1% ↑	p > 0.05
Volper et al. (2015)	Rat	Achilles	8	9	10	STZ (60)	20.7% ↑	0.44% ↓	p > 0.05
Volper et al. (2015)	Rat	Achilles	8	9	10	STZ (60) ⁱ	1.7% ↑	1.1% ↑	p > 0.05
Volper et al. (2015)	Rat	Achilles	9	18	1	STZ (60)	12.1% ↑	14.2% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (ventral)	9	18	1	STZ (60)	10.9% ↑	4.5% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (ventral)	8	9	10	STZ (60) ⁱ	21.5% ↑	10.2% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (ventral)	8	9	10	STZ (60)	28.5% ↑	25.1% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (dorsal)	8	9	10	STZ (60)	42.8% ↑	21.4% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (dorsal)	8	9	10	STZ (60) ⁱ	48.1% ↑	50.6% ↑	p > 0.05
Volper et al. (2015)	Rat	Tail (dorsal)	9	18	1	STZ (60)	1.1% ↑	22.1% ↑	p > 0.05

STZ = Streptozotocin (followed by dosage size in mg/kg), PCD = poorly-controlled diabetes, WCD = well-controlled diabetes, ZDSD = Zucker diabetic Sprague-Dawley, MGO = methylglyoxal (a producer of AGEs rather than a model of diabetes per se), Charcot = patients with diabetic neuropathic arthropathy, (ins.) = insertion, (mid.) = midsubstance, ⁱ = included insulin treatment, ^t = included regular moderate exercise

*Age and trial duration are both given in the format <weeks>.<days> unless specified otherwise

2.8. Diabetes

The need for research into diabetes is well established; £60 million is spent on it annually in the United Kingdom (Diabetes UK, 2017a). This is due to the great and very visible impact of the disease on society in general and sufferers in particular, details of which will be discussed in this section.

2.8.1. Pathophysiology

Diabetes is a disease related to the production and utilisation of the insulin hormone. Normally, insulin is produced by the pancreas and released into the bloodstream in proportion with the amount of glucose present there (Sonksen and Sonksen, 2000). It acts to accelerate the rate at which this glucose is absorbed by cells (although this absorption can still take place without insulin (Sonksen and Sonksen, 2000)) and also stimulates its conversion into glycogen (the form in which it can be stored) by the liver. In this way, it reduces the ‘blood sugar level’. Once reduced, the production of insulin likewise decreases and the liver begins converting the glycogen back into glucose, increasing this level again (Sonksen and Sonksen, 2000). A feedback loop thus exists, regulating glucose concentration to within tight limits. Some glucose perfuses out of the blood into tissues where ‘glycosylation’ reactions occur, mediated by enzymes.

However, in patients with diabetes mellitus (‘diabetes’), the rate at which blood glucose levels return to normal after a sharp increase (such as which occurs after eating) is reduced, creating a state of excess blood glucose (hyperglycaemia). The cause of this depends on the type of diabetes (World Health Organisation, 2013):

- Type 1: formerly ‘insulin-dependent’ or ‘childhood-onset’ diabetes; parts of the insulin-producing regions of the pancreas (the β -cells of the islets of Langerhans) are non-functional - possibly having been destroyed by an autoimmune reaction that is likely either genetically-caused or a response to an infection - creating a lack of insulin
- Type 2: formerly ‘non-insulin-dependent’ or ‘adult-onset’ diabetes; insulin is still

produced (although, over time, the rate may reduce) but cells are less able to use it to accelerate their absorption of glucose ('insulin resistance'). Comprising 90% of diabetes cases worldwide (World Health Organisation, 1999), it is primarily caused by obesity and physical inactivity.

- Gestational diabetes: during pregnancy (when demand for insulin is higher) production does not increase accordingly. The condition disappears after giving birth.
- Less common types include 'maturity onset diabetes of the young' (MODY), neonatal diabetes, Wolfram Syndrome, Alström Syndrome and cystic fibrosis-related diabetes (Diabetes UK, 2017b)

The increased level of blood glucose results in increased perfusion into tissues. This affects the glycosylation reactions, causing them to no longer be mediated by enzymes and thus be less controlled. This nonenzymatic process is termed 'glycation' and, notably, produces 'advanced glycation end-products' (AGEs) which bind, irreversibly, to proteins (Brownlee et al., 1989). It is the accumulation of these over a number of years that has been linked with diabetic complications in tissues (Brownlee et al., 1989; Grant et al., 1997; Brownlee, 1995). Note that AGE-levels also increase naturally in humans over time (Araki et al., 1992), meaning that age- and diabetes-related tissue changes are, generally, similar.

2.8.2. Prevalence and Impact

An estimated 415 million adults ($\sim 9.4\%$ of this age group) were living with diabetes worldwide in 2015 with a 55% increase expected by 2040 (Diabetes UK, 2015). In the United Kingdom, the estimated figures are 3.5 million diagnosed and 0.55 million undiagnosed (6.3% of the population altogether), up $\sim 150\%$ since 1996 and predicted to rise a further 25% by 2025 (Diabetes UK, 2015). The financial impact on UK sufferers is an estimated £23.7 billion per annum (expected to rise to £39.8 billion per annum by 2035/6) with the National Health Service spending approximately £1 million an hour - 10% of its budget - on diabetes (Hex et al., 2012).

2.8.3. Experimental Models for Diabetes

Extensive descriptions of these models, as well as of others, have been written by King (2012).

Streptozotocin: One of the most widely used methods involves the injection of streptozotocin (STZ), a chemical that is toxic to the insulin-producing β -cells of the pancreas (Szkudelski, 2001; Lenzen, 2008). This induces hyperglycaemia and models either Type 1 (if administered in multiple low doses) or Type 2 (if administered in a single large dose) diabetes (Reed et al., 2000; Zhang et al., 2008). According to Volper et al. (2015), a rat culled 10 weeks after STZ injection models 6-7 years of diabetes in humans.

Alloxan: Another chemical that targets pancreatic β -cells, although the mechanism via which this happens is different to that which occurs with STZ. However, it can have side effects on rats and the range of dosages over which it is effective without being non-lethal is small, leading to it being the less preferred method of the two (Mansford and Opie, 1968; Szkudelski, 2001; Lenzen, 2008).

Glucose Incubation: Alternatively, tendons may be isolated and soaked in a ribose- or glucose-rich liquid in order to mimic hyperglycaemia. However, the accuracy with which this model reproduces in vivo conditions is generally considered to be poor (Volper et al., 2015).

Other Considerations Related to Animal Models: In terms of species, Sprague-Dawley (Volper et al., 2015), Fischer 344 (Brennan, 1989) and Wistar rats (Kamalakkannan and Prince, 2006) have all been used in STZ models of diabetes. Body weight loss and poor growth is often reported in STZ- and alloxan-treated rats, with these potentially affecting tissue properties such as collagen solubility (Etherington and Bailey, 1982).

3. Investigating the Susceptibility of Tendon to Damage

3.1. Abstract

The accumulation of tendon damage is a debilitating and widely prevalent problem for which there is currently little mechanistic understanding. Tendinous structures play important physiological roles as energy-storing ‘springs’ during locomotion or as accurate positioning elements during fine motor control, depending on anatomical location. Their mechanical structures and properties are aligned with their functional roles, although diseases such as diabetes can disrupt this and lead to deterioration. These areas (functional role and diabetes) were thus investigated in order to gain greater insight into the process of tendon damage accumulation. Such knowledge will inform future research in the fields of tissue engineering, tendon damage prevention and diabetes diagnosis and monitoring. The mode of investigation was static fatigue testing on Achilles and tail tendons from a streptozotocin (STZ) murine model of diabetes. It was found that steady-state strain rate (a potential quantification of damage accumulation rate) significantly decreased in STZ-treated tendons vs controls (57.5 vs 826 μ strain/s in tail and 1.91 vs 34.4 μ strain/s in Achilles tendons). This is possibly due to the presence of advanced glycation end-products (AGEs) in the STZ-treated samples. This treatment, and thus diabetes, therefore potentially decreases the rate at which rat tendon is damaged.

3.2. Introduction

This introductory section will expand on the clinical motivation behind the investigation before briefly detailing the effects of both diabetes and functional role. Finally, the means of investigation that was used is discussed.

3.2.1. Problem Identification

The healthy functioning of tendon can become compromised as a result of diabetes, a disease associated with an increased risk of tendinopathy and tendon rupture (Abate et al., 2013) accompanied by debilitating pain, lengthy healing times and a loss of productivity. In 2015, over 415 million people suffered from diabetes worldwide and a 55% increase - to 12% of the world's population - was predicted over 35 years (Diabetes UK, 2015). The mechanisms behind tendon mechanical damage, and behind the increased susceptibility to tissue damage seen in diabetic patients, are not well understood. The effect of the mechanical differences seen between tendons with different functional roles (those used for fine motor skills are stiffer than those used for locomotion, for example (Shadwick, 1990)) are likewise yet to be fully investigated. Greater knowledge in this area is needed in order to enable advances in tendon treatment (both active and preventative) and tissue engineering.

3.2.2. The Effect of Diabetes

Another major risk factor for tissue damage is diabetes: in men younger than 44 years, the disease correlates significantly with Achilles tendinopathy (Holmes and Lin, 2006). It has also been linked to tendon deterioration, poor healing and structural abnormalities (Grant et al., 2005; Akturk et al., 2007; Batista et al., 2008; Abate et al., 2010; Fox et al., 2011; Ahmed et al., 2012), although the exact mechanisms by which these are caused are still largely unknown. Investigations into diabetic tendon can thus be especially beneficial: they have the potential to reveal more about these mechanisms whilst also allowing for comparisons with healthy tendon to be made. Such comparisons could enable additional information about damage in general to be gleaned. Greater insight in this area will also lead to clinicians being better informed as to patients' risks of developing tendon and musculoskeletal injuries and thus more able to offer advice (Fox et al., 2011).

3.2.3. The Effect of Functional Role

The structural properties of a tendon are influenced by its function: those involved in high load-bearing movements such as locomotion are stronger, more extensible and

more resilient than those involved in low load-bearing movements and fine motor control (Shadwick, 1990). This ensures that they are well-suited to their functional role. An example of this is the Achilles tendon (which bears up to 2-3 times the weight of the body when walking and up to 12.5 times when running) (Komi, 1990) having the ability to act as a spring: it absorbs and then releases mechanical energy - with an efficiency of 90-96% (Ker, 1981) - during a stride in order to aid the movement (Alexander, 1991). Conversely, ‘positional’ tendons such as those in human fingers or rat tails act as cords by transmitting muscle-generated force through them - without energy absorption - for precise controlling of bone movement around a joint (Birch et al., 2008). This requires them to be relatively inextensible (Birch et al., 2008). Controlling for functional role during experimentation can thus prove insightful as to the variation in damage accumulation properties that tendons with these different microstructures can show. Previous studies that have done this found energy-storage tendons to have superior fatigue resistance properties (Ker et al., 2000; Thorpe et al., 2015d). It also has clinical relevance: energy-storage tendons are considerably more prone to tendinopathy and rupture than those which are positional in nature (Ely et al., 2004; Birch, 2007). The particular energy-storage tendon that will be investigated here is the Achilles tendon due to its clinical importance. It is the most frequently ruptured tendon in humans (Maffulli et al., 1999) - constituting 20% of all large tendon injuries (Gillies and Chalmers, 1970) - and 6% of the population will experience Achilles tendinopathy at some point in their lifetime (Kujala et al., 2005). Additionally, the incidence rate of rupture has increased significantly over the past decades (Moller and Westlin, 1996; Nyssönen et al., 2008), rising by 900% in Finland (1979-2011) (Lantto et al., 2015), 48% in Denmark (1991-2002) (Sode et al., 2007) and 30% in Scotland (1980-1995) (Maffulli et al., 1999).

3.2.4. Mechanical Damage

While ‘damage’ is a pertinent topic for investigation, and is especially relevant when diabetes and functional role are also considered, there is no precise definition of this term or consensus as to how it should be measured in biological material. This was thus one of the knowledge gaps explored in the Literature Review of Chapter 2 where

it was noted that many previous studies have also expressed difficulty in defining it. However, a number of insights as to the markers of tendon deterioration (a state commonly induced via fatigue testing) that can be sought were found. These markers fall into two broad groups - phenomenological and mechanistic - with the former describing *how* tendon properties change and the latter answering *why*. More precisely, phenomenological models are those which fit mathematical formulas to experimental data while mechanistic ones consider the behaviour of constituent structures. Previously used phenomenological markers include permanent strain (Fung et al., 2009), loading modulus (stiffness/Young's modulus) (Volper et al., 2015) and strain energy (Alexander and Bennet-Clark, 1977). If the fatigue test used is a 'creep' test, markers related to time and strain can also be recorded as the stress applied to a sample is held constant. Surprisingly, despite it being known that strain rate influences a tendon's properties (Svensson et al., 2012), the characteristic creep rate (i.e. the minimum strain rate measured during a creep test) is seldom reported, with a few exceptions (Wang and Ker, 1995). Indeed, this steady-state strain rate (SSSR) represents a point of balance between the two creep regimes - an initial regime with decreasing rate and a final regime with increasing rate (leading to rupture). For this reason, it is often reported during material testing of metals (although in these instances the initial and final processes are work hardening and thermal softening, respectively, which are not relevant to biological material) (Sherby and Burke, 1968). Other descriptors of resistance to damage exist but often show high variability: the time at rupture, for example, is heavily biased by the weakest points in the material and occurs at the end of an unstable process (once the tendon starts to rupture it continues to do so at an exponentially increasing rate). The SSSR thus has the potential to be superior in this regard.

3.2.5. Static Fatigue Testing

The susceptibility of a biological material to the accumulation of mechanical damage may be quantified through static fatigue ('creep') testing, during which a constant stress is applied and the strain is then measured over time (Ker et al., 2000; Wren et al., 2003). The SSSR (i.e. the gradient of the strain-time curve at the point of inflection, see Figure

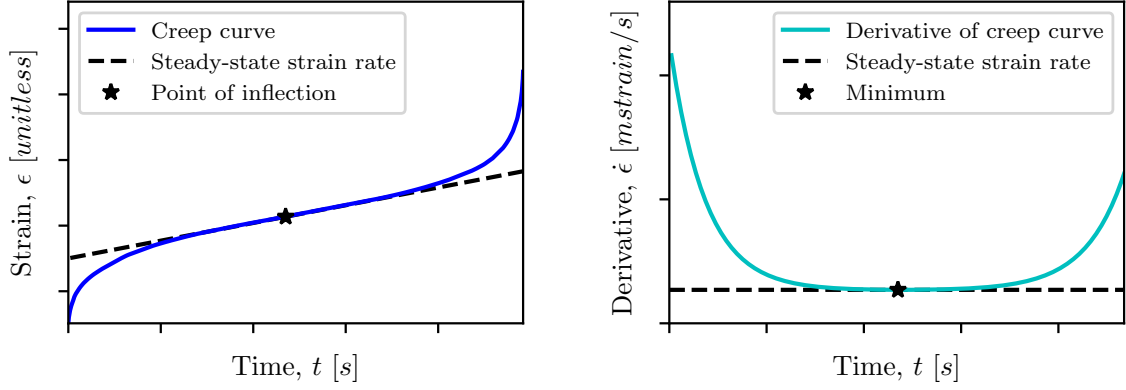


Figure 3.1: The creep response of a tendon under static fatigue (left) and its derivative (right). The minimum of the latter is equal to the gradient at the point of inflection (PoI) of the former, and this value is defined as the steady-state strain rate (SSSR).

3.1) is then calculated. Note that cyclic testing, which would involve an alternating load being applied to samples, is not being performed. Such a test would require the alternating load to always be positive (as a tendon cannot be loaded in compression axially) and thus the sample would effectively be experiencing a constant positive load and a zero-mean cyclic load at the same time. This would, concurrently, induce both static and dynamic fatigue, and it would be unclear as to what mechanical effects were being caused by which type of fatigue.

3.2.6. Murine Models

Laboratory experiments, including those concerning tendon, are often carried out using rat models for practical and ethical reasons. Diabetes can be investigated in these models through the use of streptozotocin (STZ), a chemical that is toxic to the insulin-producing β -cells of the pancreas (Szkudelski, 2001). Functional role can be investigated by testing both Achilles (energy-storage) and tail (positional) tendons (Fessel et al., 2014).

3.2.7. Aim

To compare susceptibility to mechanical damage between tendons of positional and energy-storing roles from an STZ model of diabetes with healthy controls. Further, the utility of the steady-state strain rate in capturing different damage behaviour is assessed.

This is achieved using static fatigue testing of Achilles and tail tendons from an STZ-treated murine model of diabetes and controls.

3.2.8. Hypotheses

The following hypotheses are tested:

- Samples from the diabetic model will show a higher susceptibility to mechanical damage than control tendons of the same type
- Tail tendons will show a higher susceptibility to mechanical damage than Achilles tendons from the same group
- A parameter exists that can be measured during fatigue tests and used to quantify these changes

where ‘type’ refers to the type of tendon (Achilles or tail) and ‘group’ refers to the experimental group (diabetic model or control).

3.3. Materials and Methods

The procedure followed during the experiment is detailed here.

3.3.1. Sample Collection

STZ-treated Achilles ($n = 7$) and tail ($n = 20$) tendons were obtained from adult male Wistar and Sprague-Dawley rats (Charles River Laboratories and Harlan/Envigo, UK) that had been culled for unrelated experiments (Wistar: Egom et al. (2016); Sprague-Dawley: Newton et al. (2017)). These experiments had not affected the tails or the limbs. In accordance with standard procedure (Like and Rossini, 1976), rats had been administered with STZ after reaching maturity which, due to the predictable relationship between rat age, maturity and weight (Taconic Biosciences, 2018), had been estimated from the animals’ masses (Jackson et al., 2017). Specifically, masses varied between 286 and 425 grams (approximately 9 to 12 weeks old). Culling was done after eight weeks of

treatment along with age- and weight-matched controls from which Achilles ($n = 18$) and tail ($n = 27$) tendons were similarly collected. Full details of the samples are listed in Table 3.1. Usually, two Achilles (one from each hind leg) and one tail tendon was obtained from each carcass, the exceptions being those that arrived with one leg already resected (as part of the aforementioned unrelated experiments) from which only the remaining Achilles could be used. Additionally, a number of extra tail tendons were tested (from rats 7, 8, 13 and 15) in order to increase the sample size. The data from rats 1 to 6 had initially been collected as part of a tail-only pilot study (Mansel et al., 2015) but was also included as the protocol was identical.

3.3.2. Dissection

A full video protocol of the dissection process was made with select still frames being included in Figure B.1 in Appendix B. With regards to Achilles tendons, care had to be taken to leave the tendinous aponeurosis intact at the proximal end in order to retain as much material as possible. This was to allow the sample to be gripped more easily during testing, and was achieved by scraping the red gastrocnemius muscle away with the blunt edge of a scalpel blade until only white material remained (Best et al., 1993; Probst et al., 2000). At the distal end, the calcaneus was left attached to the tendon. The tibial nerve and tendon of the plantaris muscle - structures easily mistaken for Achilles tendon fascicles - were purposefully removed. Additionally, the peritenon sheath which surrounds the tendon and requires considerable effort to remove without affecting the tendon proper was given particular focus. Contrastingly, tail tendons were collected with relative ease. Through-slices were made near the distal and proximal ends of the tail in order to access the mid-span, and tendon fascicles could be harvested from this with forceps. It was easier to harvest from the distal end as tendons visible here do not have attachments in the tail. Those visible from the proximal end may attach in the middle of the structure and thus be difficult to remove. Post-dissection, samples were immediately frozen in phosphate-buffered saline (PBS) at -18°C , which has been shown to preserve mechanical properties (Huang et al., 2011), and kept until needed (although not longer than five months (Lee and Elliott, 2017)).

Table 3.1: Details of the rats used and the samples obtained from each

Rat #	Strain	Group	Achilles	Tail	Mass [g]	Breeder
1	Sprague-Dawley	Control	0	5	350-375	Charles River
2	Sprague-Dawley	Control	0	5	350-375	Charles River
3	Sprague-Dawley	Control	0	5	350-375	Charles River
4	Sprague-Dawley	STZ	0	5	350-375	Charles River
5	Sprague-Dawley	STZ	0	5	350-375	Charles River
6	Sprague-Dawley	STZ	0	4	350-375	Charles River
7	Wistar	STZ	2	3	370	Charles River
8	Wistar	STZ	2	3	425	Charles River
9	Wistar	Control	2	1	420	Charles River
10	Wistar	Control	1	0	436	Charles River
11	Wistar	Control	2	1	412	Charles River
12	Sprague-Dawley	Control	2	1	300-325	Charles River
13	Sprague-Dawley	Control	2	3	300-325	Charles River
14	Sprague-Dawley	Control	2	1	300-325	Charles River
15	Sprague-Dawley	Control	2	3	300-325	Charles River
16	Sprague-Dawley	Control	2	1	300-325	Charles River
17	Sprague-Dawley	Control	1	0	300-325	Charles River
18	Wistar	Control	1	0	386	Harlan
19	Wistar	Control	1	1	387	Harlan
20	Wistar	STZ	1	0	286	Harlan
21	Wistar	STZ	1	0	304	Harlan
22	Wistar	STZ	1	0	300	Harlan
Totals	12 Sprague-Dawley	14 Control	25	47	17 Charles River	
	10 Wistar	8 Diabetic			5 Harlan	

3.3.3. Sample Preparation

Following 15 minutes of thawing, tendon diameters were measured at five points along the samples' lengths using an optical micrometer (Keyence, UK & Ireland) with two measurements 90° to each other being taken at every point. Cross-sectional areas were then calculated, assuming an ellipsoidal shape, at each of these points (de Oliveira et al., 2012). The smallest of these values was taken, representing the weakest section of the tendon (where damage was most likely to occur). Following measurement, marks were

made on the tendons' lengths using a fineliner pen. While excess PBS was removed from the samples before diameter measurement and again before marking, care was taken to prevent them from drying out completely (Ker, 2007). Re-hydration took place via submersion in PBS after each stage of the sample preparation process (Screen et al., 2004a).

3.3.4. Apparatus

A universal testing machine fitted with a calibrated 5 kN load cell was used for all experimental runs (Zwick/Roell UK), see Figure 3.2a. A camera was set up and connected to the testXpert II software that controlled the apparatus in order to record the runs and a manual timing device was placed in the background of the camera's field of view in front of a white backing (Figure 3.2c). Aluminium grippers and a Perspex PBS bath were custom-built in-house so as to fit onto the testing machine's existing clamps (which had been deemed inappropriate for this particular application). These grippers extended down into the PBS bath such that samples remained wholly submerged during runs.

3.3.5. Sample Loading

Gripping was achieved by placing one end of the sample between two pieces of rubber and clamping these together between the two aluminium plates of a gripper (screw-tightened). The other end of the sample was then either likewise clamped in the remaining gripper (Figure 3.2b) or, in the case of the calcaneus, was wedged into a V-shape on the gripper (Figure 3.3b) (Probst et al., 2000). Once the grippers had been loaded with a sample they could in turn be fastened onto the testing machine. This was achieved by hooking one into the machine's upper clamp and attaching the other to the Perspex bath via screws (Figure 3.2c). Care was taken to avoid applying longitudinal force to the tendon at any point during loading.

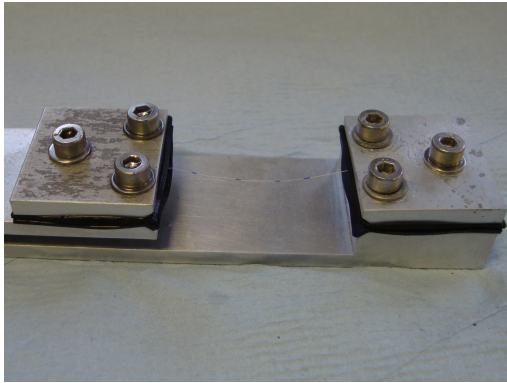
3.3.6. Static Fatigue Testing

An extension rate of 6 mm/min (Probst et al., 2000; Eliasson et al., 2007; de Oliveira et al., 2012; Ahmed et al., 2012; Volper et al., 2015), which is approximately physiologically

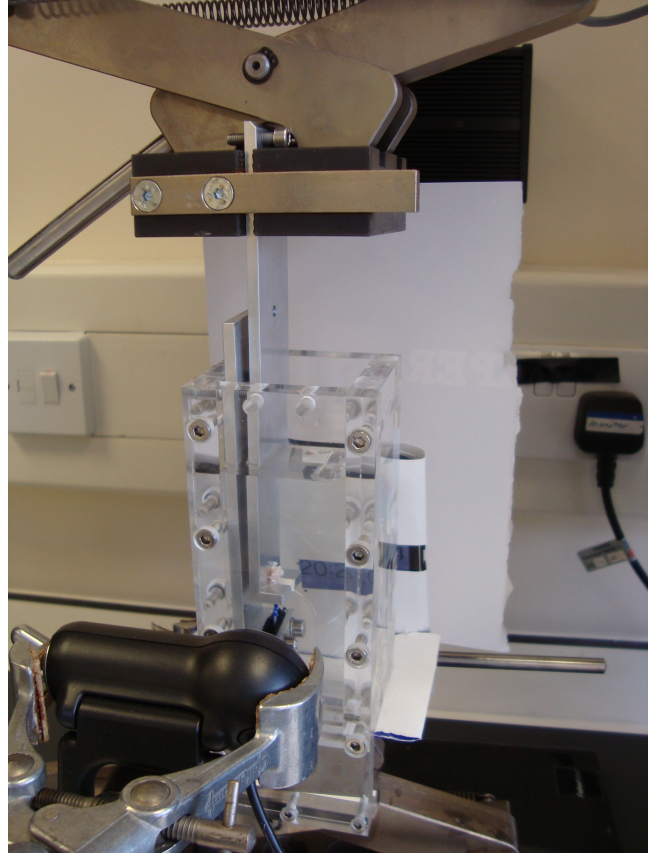
3. Investigating the Susceptibility of Tendon to Damage



(a) *Universal testing machine*



(b) *A tail tendon sample marked with four dots and loaded into the grippers*



(c) *The custom-built testing apparatus and camera during an experimental run*

Figure 3.2: *Apparatus used for the static fatigue testing*

relevant, was applied to the initially-slack sample until a force corresponding to either 10 MPa (Achilles tendons) or 30 MPa (tail tendons) was reached. These stresses were chosen as previous testing (data not shown) suggested that they would cause creep damage and eventual failure rather than fast failure (rupture within the first minute). Once achieved, these stresses were held constant until total rupture occurred. Note that preconditioning, which is often used in mechanical tests on tendon, was not performed in this instance: the protocol being described here was designed to investigate damage accumulation properties and therefore introducing damage before the tests began would have been counter-productive. Video capture was taken at a frame rate of 10 Hz and force, clamp-to-clamp separation and time data was collected at 11 Hz. The resolutions of these were 0.05 N, 10 μm and 0.1 s, respectively. Initial length of the sample was defined as that which was instantaneously measured when the load was equal to twice

the resolution limit of the load cell (Calve et al., 2004), i.e. 0.1 N. Once rupture had occurred, the sample was left submerged in the PBS bath for at least three minutes. This was to allow the collagen, now unloaded, to relax and assume its plastically-deformed length. Both fragments of the sample were then removed from the apparatus. The smaller of the two was disposed of while the larger was re-loaded and re-tested. The purpose of the re-test was merely to have the sample be loaded to its tare load of 0.1 N for a second time, this being necessary in order to measure initial length again and thereby calculate permanent strain (see Section 3.3.8).

3.3.7. Calibration

After the test, the force data was divided by the sample's cross-sectional area to obtain engineering stress. The start- and end-points of the test were then defined, these being the times at which the aforementioned tare load of 0.1 N was first reached and at which the target engineering stress (30 or 10 MPa) was last measured, respectively. Synchronisation between imaging and load cell data was achieved with an accuracy of one frame (~ 90 ms), see Figures 3.3a and 3.3b. A manual image analysis method (Figure 3.3c), corrected for foreshortening, was used to convert the 'clamp-to-clamp separation' as measured by the apparatus into 'sample length' (Figure 3.3b, accuracy ~ 150 μm) and subsequently into engineering strain (Figure 3.3d) using the aforementioned initial length. A flowchart of this process is shown in Figure 3.3e. Next, true stress and true strain were calculated using $\sigma_{true} = \sigma_{eng} (1 + \epsilon_{eng})$ and $\epsilon_{true} = \ln(1 + \epsilon_{eng})$. These formulas assume that the volume of the sample remains constant - i.e. the material has a Poisson's ratio of 0.5 - which is indeed what has been reported in the literature (to within experimental uncertainty) (Vergari et al., 2011). Finally, an adjustment factor was incorporated to correct for end-effects caused by the grips. This involved plotting compliance $\times$ cross-sectional area against initial length for each sample and finding the point at which the regression line crossed the x-axis (Ker, 1981; Legerlotz et al., 2010). This yielded an estimate of the magnitude of the end-effects, which was added to the samples' length data to give their effective lengths before re-running the analysis with the new values. While mathematically addressing the problem of end-effects is commonly done it should be

noted that this is not a comprehensive solution.

3.3.8. Data Analysis

Following the discussion in Section 2.3.1, a Standard Linear Solid Model was chosen for this experiment including (as discussed in Section 2.4.2) a modification that allows it to model static fatigue behaviour (Figure 3.4a). This model is a superposition of a viscoelastic, a sliding and a pullout response, as shown in Figure 2.11 where the mathematical descriptions of each of these responses is also shown. The governing equation of the overall model is a simple summation of each of these three constituent responses, and thus it reads as follows:

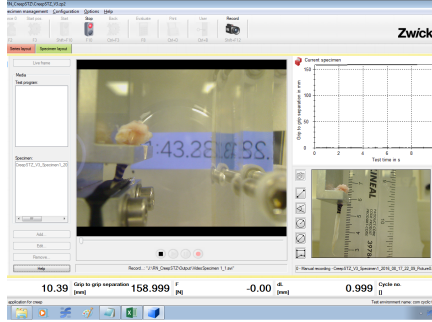
$$\epsilon = (\epsilon_0 - \epsilon_\infty) \times e^{\left(\frac{-t}{\tau_v}\right)} + \epsilon_\infty + m \times t + A \left(e^{\left(\frac{t}{\tau_d}\right)} - 1 \right) \quad (3.1)$$

See also Appendix A.3. For each sample, this equation was fitted to the strain vs time data (see Figure 3.4c; note that Figures 3.3d and 3.4c, d, e, and f come from the same specimen and so should be considered as a worked example of the data analysis process). This fitting procedure was performed in Python 3.6 using the `least_squares` function as provided by the `scipy.optimize` package¹. This involved estimates for Equation 3.1's constants being provided and then optimised for the given sample's strain, ϵ , and time, t , data. Usefully, these constants have physical meaning in the context of static fatigue tests, as shown in Figure 3.4b, and they are named as below:

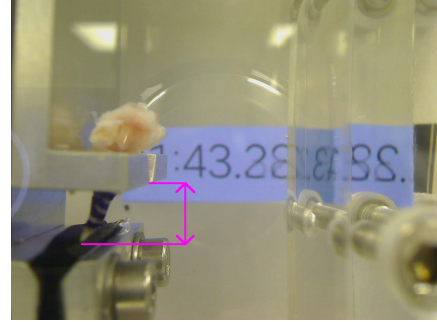
- Initial viscoelastic strain, ϵ_0
- Final viscoelastic strain, ϵ_∞
- Viscoelastic time constant, τ_v
- Sliding gradient, m
- Damage scale factor, A

¹This procedure is used to solve non-linear least-square problems that have bounds on the variables. The minimisation algorithm used was the Trust Region Reflective which the SciPy.org docs describe as “particularly suitable for large sparse problems with bounds. Generally robust method”.

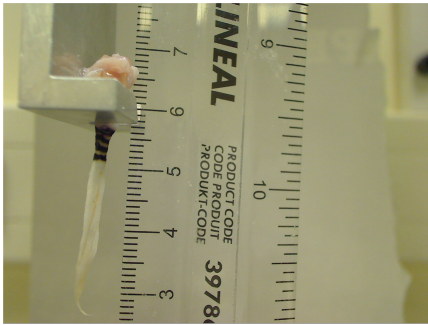
(a) Screenshot



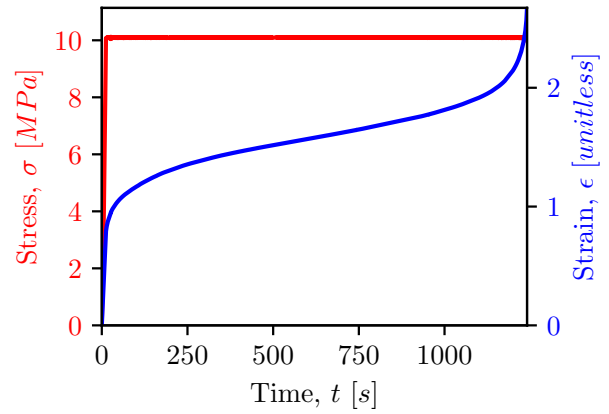
(b) Video Frame (Sample Length in Magenta)



(c) Ruler Photo



(d) Calibrated Stress, Strain and Time Data



(e) Flowchart of the Synchronisation Methodology

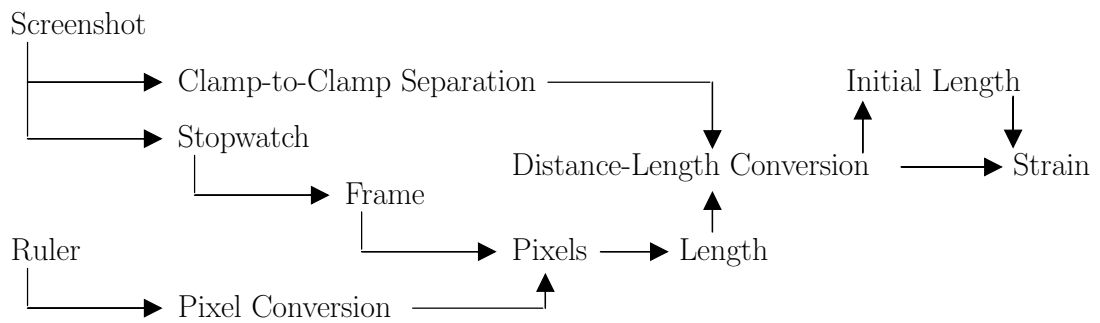


Figure 3.3: Summary of the calibration process for strain values

- Damage time constant, τ_d

The next step was to identify the graph's point of inflection (PoI). This was done by finding the minimum of the derivative of the fitted curve (Figure 3.4d). Explicitly, the derivative of Equation 3.1 is:

$$\dot{\epsilon} = \frac{-(\epsilon_0 - \epsilon_\infty)}{\tau_v} \times e^{\left(\frac{-t}{\tau_v}\right)} + m + \frac{A}{\tau_d} \times e^{\left(\frac{t}{\tau_d}\right)} \quad (3.2)$$

The minimum value of the derivative was defined as that sample's steady-state strain rate (SSSR) - the gradient at the PoI. The time and strain at the PoI were also calculated from this point.

A stress vs strain graph was then plotted and similarly fitted with a curve², see Figure 3.4e. This allowed the loading modulus (gradient of linear region) and strain energy at the PoI (area under the curve multiplied by the sample's volume, the latter being found by multiplying the cross-sectional area by the initial length) to be found. Next, the video frames that corresponded to the sample's initial length - i.e. from the instants when the applied load was 0.1 N - were obtained for both the primary run and the secondary (post-rupture) run, see Figure 3.4f. Comparing the number of pixels between the same two blue dots before and after rupture allowed permanent strain ϵ_p to be calculated via:

$$\epsilon_p = \frac{L_{0,D,S} - L_{0,D,P}}{L_{0,D,P}} \quad (3.3)$$

where $L_{0,D,P}$ is the initial distance (' L_0 ') between two of the four fineliner dots (' D ') during the primary (' P ') run and $L_{0,D,S}$ is the initial distance between these same two dots during the secondary (' S ') run.

3.3.9. Statistical Analysis

This took place in three parts:

²The toe and heel region was fitted with an exponential curve and the linear and constant regions were both fitted with straight lines.

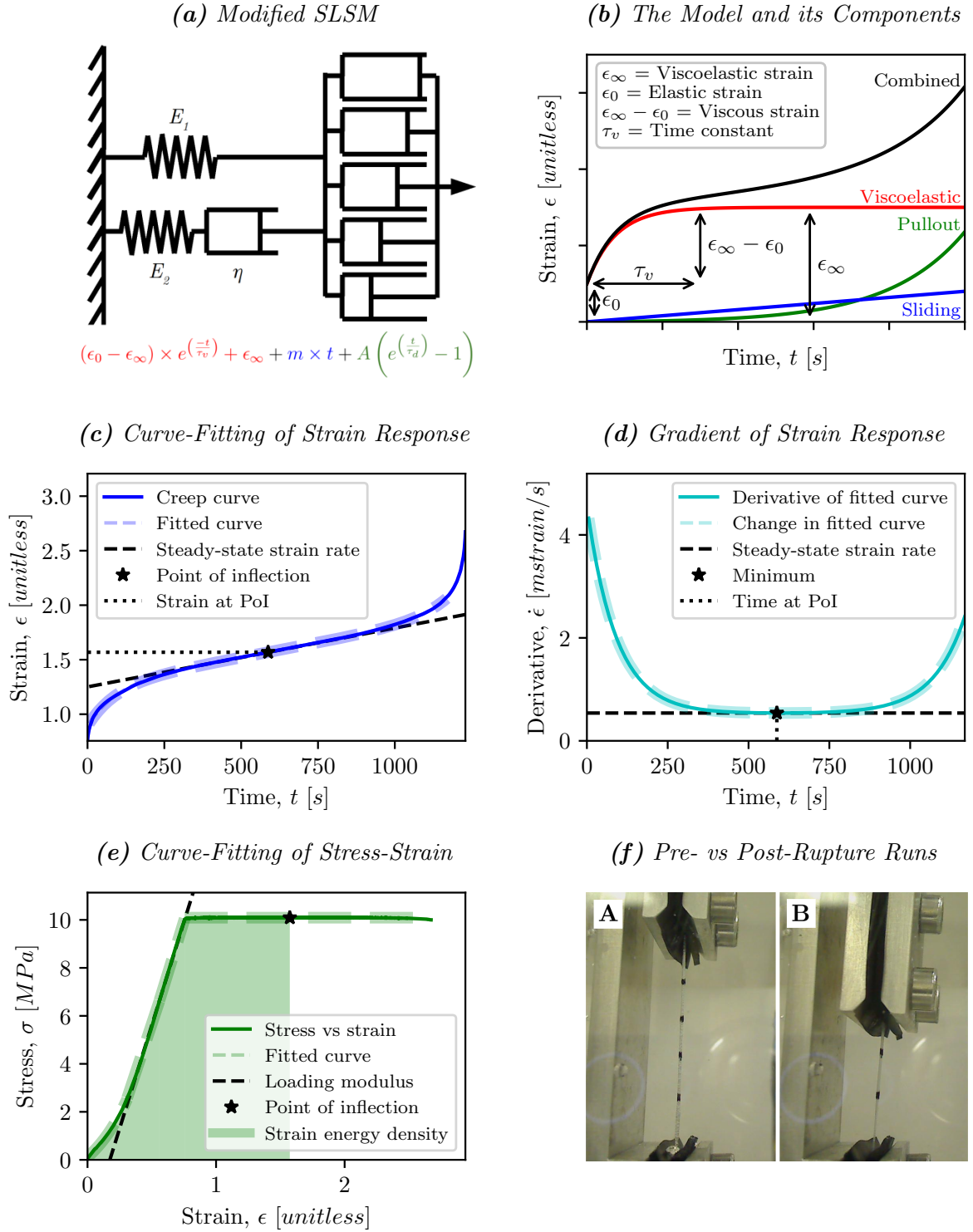


Figure 3.4: Summary of the data analysis process

Outlier Rejection: The standard deviation of each data point in each category (control Achilles, control tail, STZ Achilles and STZ tail) was calculated for each of the six reported results and the corresponding probability of a data point being found at that distance away from the mean was then derived. If this probability fell below a certain threshold - known as Chauvenet's criterion - based on the number of samples in the category then that data point was rejected as an anomaly. Only one instance of rejection occurred: the strain energy at PoI of an STZ-treated tail tendon.

Normality Testing: In order to ascertain if the data was Normally distributed, six tests for Normality were performed. These were the Lilliefors, D'Agostino, Jarque-Bera, Shapiro-Wilk and Anderson-Darling tests as well as qualitative quantile-quantile plot assessment. While a number of individual result categories were judged Normal by a majority of the tests, none had all categories deemed as such.

Significance Testing: The lack of Normality in general, together with the relatively small sample sizes and the need for consistency when handling the results, led to a decision to use the Mann-Whitney U test for statistical significance (Graziano and Raulin, 2014). This test is recommended when performing inferential statistics on two, independent groups of ordered data which are not necessarily Normally-distributed. As per the requirements for this test, the final results are presented as medians together with their corresponding inter-quartile ranges (IQRs). $p \leq 0.05$, two-tailed, was taken as significant.

3.3.10. Uncertainty Analysis

The six results being reported are, fundamentally, based upon measurements of stress, strain and time. Such measurements need, in turn, to be derived from the force, distance and time readings made by the apparatus (which have uncertainties equal to their resolutions: 0.1 N, 10 μm and 0.1 s, respectively) through calibration that is performed using micrometer measurements (uncertainty of 10 μm) and by counting pixels in various video frames (uncertainty of 2 pixels). By propagating these errors, the uncertainties associated with stress (0.39%), strain (0.283%) and time (0.0167%) could be calculated for representative data points. From this, the uncertainty of each individual result could

be estimated. These final uncertainties ranged from 0.017% (time at the PoI) to 1.35% (loading modulus).

3.4. Results

The static fatigue test protocol was performed on all samples and the creep (strain vs time) plots are shown in Figure 3.5. Notably, all control tail tendons ruptured before 2 000 seconds whilst their STZ-treated counterparts all failed after this point. A similar pattern can be found in the Achilles tendon data - all control samples ruptured before 6 000 seconds and all STZ samples after 10 000 seconds - although four anomalies exist (one STZ sample that ruptured quickly, one control sample that ruptured after 14 000 seconds and a further two control samples that did not rupture at all for a run length of 5 hours). Note that all samples reached creep load well before $10\tau_v$ - the accepted criterion for an inconsequential fast loading rate (see description of Figure 2.4) - although this data is not shown except for one sample in Figure 3.3d. See Appendix E for τ_v values.

3.4.1. STZ Model vs Healthy Controls

Significance due to STZ treatment was found in the values of steady-state strain rate and also in the times at which these values were measured (i.e. the time at the point of inflection). In both cases, this significance held for both tendon types and the differences were greater than an order of magnitude (close to 20-fold increases and decreases in the measured values, respectively). As mentioned, data from a pilot study that measured the steady-state strain rates and loading moduli of tail tendons, according to the same protocol, has been included. This is reflected in the larger sample sizes of these particular results in Figures 3.6a and 3.6c ($n = 27$ for control tail tendons - although two samples did not have measurable steady-state strain rates - and $n = 20$ for STZ-treated tails tendons).

3.4.2. Energy-Storage vs Positional Functional Roles

As shown in Table 3.2 and Figure 3.6, significant differences were found due to functional role in all of the six markers apart from permanent strain (which showed no significance between any groups) regardless of tendon type. In steady-state strain rate and loading modulus, these differences were increases (in tail tendons compared to Achilles) whilst in the other markers measured at the point of inflection they were decreases.

3.4.3. Mechanistic Markers

Additional results are reported in Appendix E.

3.4.4. Wistar vs Sprague-Dawley

As two different strains of rat were used in this experiment the possibility existed that this would influence the result. However, no significant differences were found when these two variables were separated out (see Table F.1 and Figure F.1 in Appendix F). These two rat strains thus appear to have no relevant differences in the mechanical properties of their tendons, justifying the grouping of their results together.

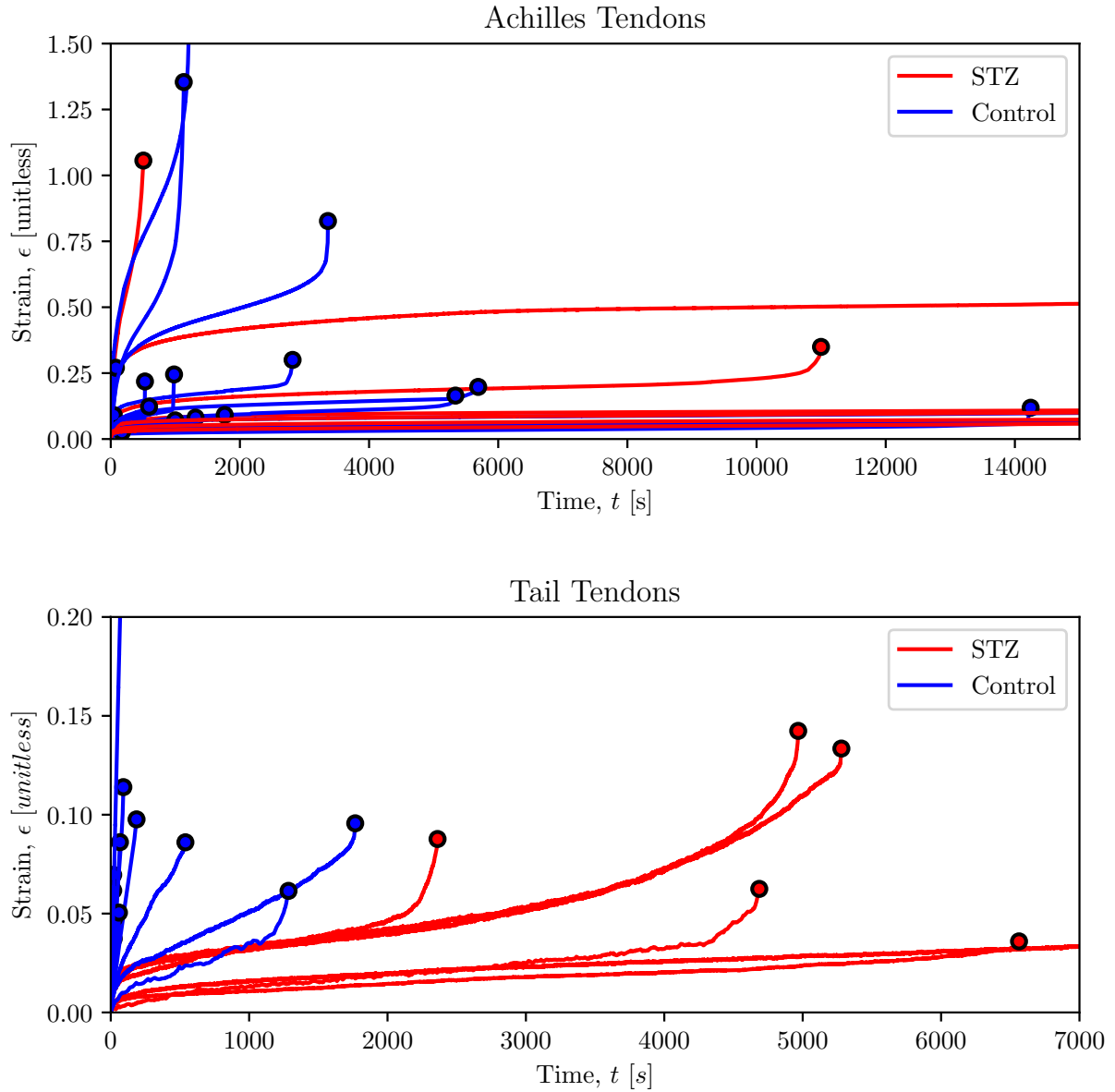


Figure 3.5: Creep plots of all samples - Achilles tendons (top) and tail tendons (bottom) - showing that most control samples ruptured before 6 000 seconds and 2 000 seconds (Achilles and tail tendons, respectively). The STZ-treated samples mostly ruptured after these time points.

3. Investigating the Susceptibility of Tendon to Damage

Table 3.2: Data is reported as median $\pm$ IQR and p -values (Mann-Whitney U test) are shown in italics alongside the experimental groups they refer to. Significance ($p \leq 0.05$) is denoted with a star ($\star$) when it refers to STZ and with a circle ($\circ$) when it refers to functional role. For sample sizes, see Figure 3.6

<i>Steady-State Strain Rate, $\dot{\epsilon}_{PoI}$ [$\mu\text{strain/s}$]</i>			
	STZ	Control	<i>Significance</i>
Tail	57.5 $\pm$ 84.9	826 $\pm$ 1010	$p=0.000001\star$
Achilles	1.91 $\pm$ 3.23	34.4 $\pm$ 66.2	$p=0.04\star$
<i>Significance</i>	$p=0.01\circ$	$p=0.0001\circ$	
<i>Loading Modulus, E [MPa]</i>			
	STZ	Control	<i>Significance</i>
Tail	227 $\pm$ 436	186 $\pm$ 654	$p=0.40$
Achilles	38.7 $\pm$ 29.2	63.4 $\pm$ 62.0	$p=0.11$
<i>Significance</i>	$p=0.0001\circ$	$p=0.0000002\circ$	
<i>Permanent Strain, ϵ_P [%]</i>			
	STZ	Control	<i>Significance</i>
Tail	6.09 $\pm$ 7.21	2.30 $\pm$ 3.25	$p=0.16$
Achilles	5.17 $\pm$ 6.44	2.46 $\pm$ 4.46	$p=0.93$
<i>Significance</i>	$p=0.43$	$p=0.34$	
<i>Strain Energy at the PoI, U_{PoI} [J]</i>			
	STZ	Control	<i>Significance</i>
Tail	0.797 $\pm$ 0.175	1.01 $\pm$ 0.498	$p=0.12$
Achilles	30.8 $\pm$ 5.72	47.0 $\pm$ 32.0	$p=0.11$
<i>Significance</i>	$p=0.003\circ$	$p=0.00001\circ$	
<i>Time at the PoI, t_{PoI} [min]</i>			
	STZ	Control	<i>Significance</i>
Tail	25.0 $\pm$ 35.8	1.07 $\pm$ 2.97	$p=0.0009\star$
Achilles	257 $\pm$ 147	11.1 $\pm$ 41.2	$p=0.008\star$
<i>Significance</i>	$p=0.04\circ$	$p=0.0003\circ$	
<i>Strain at the PoI, ϵ_{PoI} [%]</i>			
	STZ	Control	<i>Significance</i>
Tail	6.12 $\pm$ 3.49	6.78 $\pm$ 5.21	$p=0.28$
Achilles	84.6 $\pm$ 82.8	41.8 $\pm$ 46.3	$p=0.14$
<i>Significance</i>	$p=0.003\circ$	$p=0.00001\circ$	

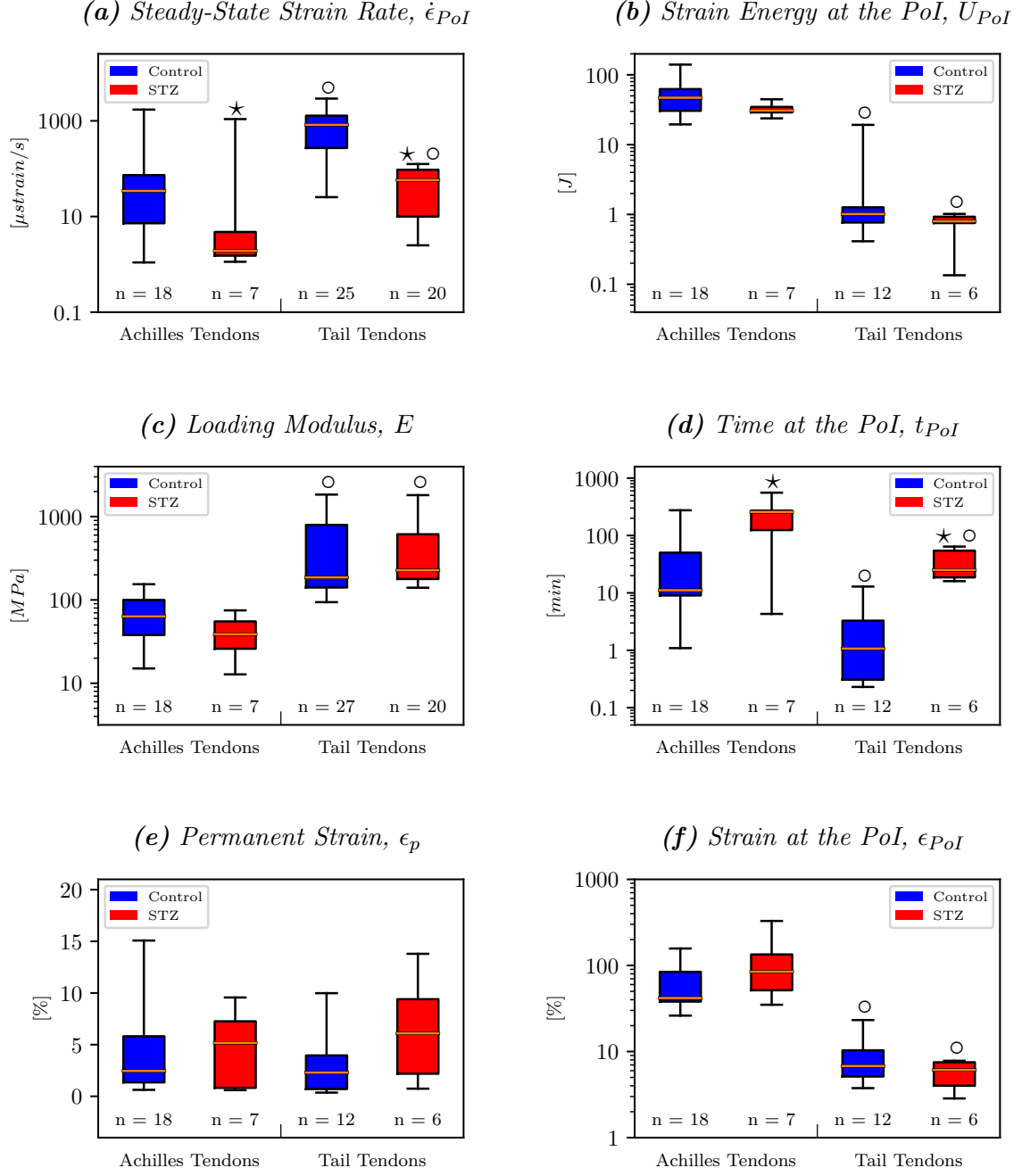


Figure 3.6: Box-and-whisker plots of the phenomenological markers showing significant differences ($p \leq 0.05$, Mann-Whitney) in steady-state strain rate (a) and time at the point of inflection (d) in STZ-treated samples. Significance due to functional role is seen in all results except permanent strain (e).

3.5. Discussion

The results show that STZ and functional role have significant effects on certain markers of tendon damage, which will be discussed here. Note that the estimated uncertainties of the results - which ranged from 0.017% (time at the PoI) to 1.35% (loading modulus) - were deemed too small to be relevant. In particular, this is due to the large scatter in the data (i.e. the large relative sizes of the inter-quartile ranges compared to the medians). Note also that one of the anomalies that was seen in the Achilles creep curves (Figure 3.5) might be explained by the weight of the rat in question: one of the control samples that took longer than expected to fail came from rat 10 which happened to be particularly large (see Table 3.1). However, the other two anomalous control samples and the STZ sample that failed quickly all came from rats of average weight (rats 13, 18 and 7), and rats 7 and 13 both had contralateral Achilles tendons tested without notable results. There is thus little evidence to suggest that animals that are over- or under-weight (while still being within the bounds of expected natural variation) lead to anomalies in the results. The considerable spread seen in the weights of the animals (despite mass - as opposed to age - having been used to determine the point of cull) can thus be ignored.

3.5.1. STZ Model vs Healthy Controls

The importance of the point of inflection of a strain response has already been mentioned: it represents the transition from a net recoverable response to a net damaging response and is relatively stable (and thus more likely to be an intrinsic property of the material) as opposed to stochastic. Accordingly, four measurements were taken at the PoI: the strain, the strain energy, the gradient (the steady-state strain rate) and the time. The latter two results were significant, suggesting that STZ causes tendon to strain more slowly and thus delay the onset of the net damage response. The lack of significance in strain and strain energy at the PoI suggests that net damage is a function of one (or both) of these: tendon damage is caused by a tendon being strained to a certain amount as opposed to it being subjected to a certain load or being loaded for a certain length of time. This evidence is not conclusive, however, and testing at different stresses would

be needed to further separate out the effects of stress, strain and strain energy. Previous literature to propose a strain-based model of damage such as this includes Fung et al. (2009) and Wren et al. (2001), whereas a stress-based model is supported by Schechtman and Bader (2002) and Wang and Ker (1995). No reports could be found of strain energy-based models. In terms of loading modulus, the measured differences not only lacked significance but also consistency: Achilles tendons saw a decrease with STZ while tail tendons saw a slight increase. This is in contrast with the majority of the literature which found a marginal increase with STZ in both tendons, see Figure 2.18. When compared directly with previously reported values for loading modulus as measured by tests that also used a strain rate of 6 mm/min, the control and STZ Achilles results both agree with those of de Oliveira et al. (2012) and Ahmed et al. (2012) to within the given standard deviations. However, control Achilles' results disagree with Eliasson et al. (2007) and all four groups disagree with Volper et al. (2015). It is important to only compare loading moduli with other experiments if they used the same strain rate. This is because a tendon's mechanical properties depend on the rate at which it was loaded (Cohen et al., 1976). Note, however, that Volper et al. (2015) tested Achilles tendons that had been sectioned (which likely introduced damage) and used a clamping method that was found to be unsuccessful when it was attempted during this experiment (namely, gluing the dry ends of the samples down). Similarly, Eliasson et al. (2007) used sandpaper to grip the samples and moist gauze for hydration as opposed to a PBS bath. The influence of the gripping and hydration methods should not be disregarded; they are considerable challenges that many previous studies cite as difficulties (Ker, 2007). Finally, the permanent strain results showed no significance between any groups. This is a measurement that is likely related to the elastic parts of a tendon's structure (because ruptured samples were allowed to relax until the viscoelastic effects had dissipated before measurement took place) as is loading modulus (which is measured over too short a period of time for viscoelasticity to be influential). Thus, the fact that both of these results show no significance with STZ suggests that this particular model of diabetes affects the viscous components of tendon more than it does the elastic ones. Additionally, no significant differences in permanent strain again supports a strain-based damage model:

the tendons appear to have failed when a certain level of permanent strain was reached which thus might mean that ‘permanent strain at failure’ is an intrinsic material property.

3.5.2. Energy-Storage vs Positional Functional Roles

Five out of the six phenomenological markers showed significant differences due to functional role in both tendon types. Broadly, this supports the idea that a tendon’s resistance to fatigue damage is related to its functional role. Indeed, Ker et al. (2000) argued that it is related to the level of stress experienced by the tendon in life. Moreover, in all cases, the results suggest that a tendon’s properties aid it in performing its function. For example, lower SSSR values were found in the highly-stressed Achilles tendons which would allow them to strain more slowly, delaying the onset of damage to a degree that less-stressed tail tendons would not need. Likewise, Achilles tendons are subjected to larger strains in vivo and thus would benefit if their net damage responses only started occurring when they were more stretched, which is indeed what is seen in the strain-at-the-PoI results. They are also loaded for longer periods of time in vivo, thus being suited to the higher times-at-the-PoI which were measured. Their ability to act as springs in order to aid in locomotion is reflected in their larger ability to absorb strain energy before the inflection point occurs, whilst the ability of tail tendons to effectively transmit force from muscle to bone is reflected in their larger loading moduli. Finally, it might be the case that permanent strain, the only marker to not show significance, affects the tendon greatest on a molecular level (i.e. permanent strain might be mostly due to permanent molecular sliding). As the two tendon types are most similar when viewed on the molecular level, a property that was related to molecular behaviour would be less likely to show a difference.

3.5.3. Implications

The results of both sets of markers (the phenomenological markers reported here and the mechanistic ones reported in Appendix E) suggest that STZ causes the viscoelastic process to lengthen without affecting its magnitude. Moreover, this is achieved due to changes in the viscous deformation mechanisms (i.e. those related to the loading and movement of

water molecules, to the sliding of collagenous structures and to the untwisting of collagen molecules) rather than elastic ones. In tail tendons, it is specifically the viscosity of these viscous mechanisms that is affected (Figure E.2b) whilst in Achilles tendons it is the initial loading of the water (Figure E.2f). As it is known that diabetes (and STZ, although this was not confirmed in this experiment) increases the abundance of hydrophilic advanced glycation end-products (AGEs; thought to increase the strength and stiffness of intermolecular bonds) in tendon, this is thus the most likely underlying explanation for these effects (Fessel et al., 2014). However, in tail tendons, the intermolecular bonds are likely already very stiff; the structure is taut and non-energy-absorbing. As such, increasing the AGE levels probably has little effect on these bonds and, rather, it may be the hydrophilic properties that are more influential. This would explain the increased viscosity in tail tendons (Figure E.2b): additional hydrophilic AGEs resist the movement of water molecules. Conversely, in Achilles tendons, the more compliant structure would be more affected by the addition of stiff AGEs and, as a result, relatively more energy would be absorbed by intermolecular bonds during initial loading. This, in turn, would explain the decreased initial loading of the water (Figure E.2f): the Achilles tendon is less reliant on it to absorb energy. In addition to AGEs, another known effect of diabetes is to increase the susceptibility of a patient to tendon disease. Initially, the current results appear to contradict this: net damage is delayed as a result of STZ. However, it is also known that there exists a healthy level of tendon over-loading and fatigue; a small amount of ‘damage’ triggers a mechanobiological reaction that ensures the maintenance of homeostasis. It is thus possible that by delaying net damage diabetes might allow a living structure to deteriorate over time. As for the concept of ‘damage’, the results suggest that this is best quantified by strain (although it may be that *permanent* strain and/or strain *energy* is a more complete answer - additional experimentation would be needed to explore these markers in isolation). Notably, loading modulus was not found to be significant, which contradicts some of the reports listed in Table 2.1. With regards to the effects of functional role in general, the results clearly suggest that mechanical properties are affected by and adapted to suit the tendon’s purpose and the in-life forces it experiences (Ker et al., 2000). Exceptions to this can occur when the marker in question is dependent

solely on the collagen molecules (e.g. permanent strain) or when STZ is present. In the case of the former, the hierarchical level on which the mechanism is relevant is small enough that the functional role is irrelevant: collagen is effectively identical on a molecular level in both tails and Achilles. In the case of the latter, the diabetic model appears to be able to mask the effects of functional role in a minority of cases.

3.5.4. Limitations and Future Research

As with any animal model, the degree to which the findings can be applied to humans is open to debate. In particular, while the rats in this experiment had been culled eight weeks after receiving STZ - an established model of chronic Type 2 diabetes (Mansor et al., 2013) - it is known that the effects of diabetes in humans can take many years to manifest. It is thus possible that time has a considerable influence, or that there are secondary effects of diabetes that need to build up before becoming noticeable. Secondly, the exact mechanisms of the effects of AGEs are still not fully understood. The role that they played in producing these results would require further investigation. A technique such as X-ray diffraction could be considered if greater understanding of the microstructural changes was desired. Additionally, the number of AGEs that were present in the tendons was not quantified. Ideally, the magnitude of effect due to STZ would need to be compared against the level of AGEs measured inside a tendon (and against the quantity of STZ that was administered). Another question that has not been fully answered is which of the markers represent ‘damage’ as discussed in Section 2.5. While steady-state strain rate has shown significant results and appears to be insightful, it would need to be compared with other indicators of tendon deterioration. With regards to the experiment itself it was noted that, while many of the problems related to sample gripping were overcome, the majority of Achilles tendons failed at either the calcaneus or the aponeurosis (all tail tendons failed mid-span). Although this is to be expected - these regions are naturally weaker than the mid-span due to their higher levels of fibrocartilaginous material - it is also known that end-effects caused by sample gripping can influence measurements of tendon mechanical properties (Legerlotz et al., 2010). While it is true that these effects were corrected for, the process used to perform this correction is merely an approximation. As such, the

values for the properties reported during this experiment should be taken as minimums. A possible improvement would be the use of video extensometry; using computer vision to track the strain of the mid-section only. This would provide greater accuracy than using grip-to-grip distance.

Time to Failure: Previous work (Ker et al., 2000) has found that, when creep tested with a common load, the time taken for a tendon to rupture correlates with ‘stress-in-life’ (the amount of load that the tendon in question would experience *in vivo*). When creep tested with a load relevant to their stress-in-life, however, the time to failure of all tendons was found to be similar. Note that these experiments were performed on wallaby tendons and so may not be relevant to rat tendons; indeed, neither of these findings manifested in this experiment. Here, while the tail tendons were loaded to 30 MPa, attempts to load Achilles tendons to this stress always resulted in premature rupture (data not shown) and hence a load of 10 MPa was used instead. It is thus unlikely that loading the tail and Achilles tendons to the same stress would have shown a greater time to failure in the Achilles. Similarly, whilst the stress-in-life of the Achilles tendon is ~ 4.5 time greater than that of the tail tendon (in wallabies) (Ker et al., 2000) it is unlikely that loading the (rat) tendons from this experiment in this proportion would have yielded consistent measurements. A likely explanation for this discrepancy is stress concentrations: the previous experiment used a different gripping mechanism which may have induced end-effects in the material to a greater or lesser degree than the current experiment. Also, the natural differences between rat and wallaby material likely influences this behaviour and may account for the relative lack in strength of the rat Achilles.

3.6. Conclusions

The effect of an STZ model of diabetes and of functional role on rat tendons’ susceptibility to mechanical damage has been measured. More specifically:

- Samples from the diabetic model showed a reduced susceptibility to mechanical damage than control tendons of the same type

3. Investigating the Susceptibility of Tendon to Damage

- STZ has the effect of lengthening the viscoelastic response - which delays the onset of net damage - although its magnitude (and that of the elastic mechanisms) is not changed
- It is likely that AGEs cause these effects by increasing the difficulty with which adjacent fibres and water molecules can move (tail tendons) and by reducing the amount of stress initially taken up by the ground substance (Achilles tendons)
- Tail tendons showed a higher susceptibility to mechanical damage than Achilles tendons from the same group
- Energy-storage tendons are less stiff and thus their response to (and their absorption of) load in the short-term is greater
- Steady-state strain rate is a parameter that shows clear differences between STZ-treated and control groups and between energy-storage and positional functional role groups
- Tendon damage is most likely strain-based

4. Steady-State Strain Rate as a Measure of Damage Accumulation

4.1. Abstract

A previous investigation into the effect of a streptozotocin (STZ) rat model of diabetes on the damage accumulation properties of tendon has found the gradient of the strain vs time curve at its point of inflection (the ‘steady-state strain rate’ or SSSR) to differ significantly with the treatment. This has raised the possibility of the measurement having utility as a descriptor of a material’s susceptibility to fatigue damage. Such a finding would have implications for research both into tendon damage and into the mechanisms behind the effect of diabetes on collagenous tissue. An experiment was performed on tail tendons from rats from the same STZ model of diabetes, together with controls, using a pseudo-static fatigue test that allowed for the measurement of the change in various material properties as the test progressed. Results indicate that SSSR is strongly correlated with the rate of increase of elastic strain ($p = 0.01$ for both STZ and control samples) and with the rate of decrease of loading modulus in control samples ($p = 0.02$) but not STZ samples ($p = 0.24$). This data confirms the relationship between SSSR and tendons’ damage susceptibility that was proposed in Chapter 3. Additionally, in control samples, the strain response’s point of inflection occurs sooner when SSSR is larger ($p = 0.04$) while, in STZ samples, it occurs only after a larger amount of strain energy has been absorbed when SSSR is larger ($p = 0.03$). These findings complement both the inverse relationship between SSSR and STZ treatment that was reported in Chapter 3 and the established relationship between damage susceptibility and diabetes (which STZ is a model of) from the literature (Lui, 2017). These effects are possibly due to the presence of advance glycation end-products (AGEs) in STZ-treated samples. SSSR can thus serve as a marker of fatigue damage susceptibility and be used to differentiate between materials with different propensities to damage.

4.2. Introduction

In this section, the purpose behind an investigation into steady-state strain rate will be outlined and the concepts surrounding the measurement will be discussed.

4.2.1. Problem Identification

In Chapter 3, one of the parameters measured as part of the static fatigue testing - steady-state strain rate (SSSR) - showed highly significant results across experimental groups (STZ-treated samples vs controls) for both tendon types: $p = 0.000001$ for tails and $p = 0.04$ for Achilles. As SSSR is a relatively unexplored concept in biological material, no literature exists as to its mechanical importance or mechanistic implications. However, as a link between it and the STZ diabetic rat model has now been found - and the clinical importance of diabetes already established - motivation thus exists to investigate it further. In particular, it should be investigated with regards to its relationship with tendon damage, a phenomenon that has clinical relevance of its own (Mazzone and McCue, 2002) in addition to being a possible route into investigating diabetes (due to it being influenced by the disease (Abate et al., 2013)). Greater understanding of the microstructural processes behind tendon damage is needed in order to inform research into tendon behaviour and, specifically, into the prevention, mitigation and treatment of tendinopathies (Szczesny and Elliott, 2014a).

4.2.2. Steady-State Strain Rate

SSSR is an important engineering measurement in, for example, metals (where it is usually referred to as the ‘secondary creep rate’ or just ‘creep rate’) when it is used in fatigue considerations and when designing-for-use (Hearn, 1997). In Chapter 3, SSSR was defined for a tendon as a measure of the rates of strain at the end of the recoverable (viscoelastic) response, at the start of the damage response and of the constant response. It is thus proportional to the rate at which these combined processes happen. As these processes are governed by activity on the macro- and nanoscale (see Section 2.5) and strain is often considered to be the basis of tendon damage (Wren et al., 2001; Fung et al., 2009), SSSR

has the potential to elucidate details about these behaviours. It would thus represent the ease with which damage occurs and the potential rate at which it occurs. Note also that, as the experiment is performed at a constant stress, SSSR also represents the rate of absorption of strain energy. This may or may not be a more correct definition of damage accumulation.

4.2.3. Material Properties

A material property is that which is the same for all samples of a given type, independent of measurement technique. The value is a function of the material itself, not of the test. Material properties are useful in characterising, comparing and understanding material behaviour. In particular, if such a property is measured in a sample both before and after a certain procedure is performed, or treatment is applied, then any differences between the two measurements can, with a fair degree of certainty, be attributed to the treatment. This premise can be used to form the basis of an experimental design. While SSSR has not yet been established as a tendon material property, the coefficients of variation calculated using data from Chapter 3 suggest that it might be. A coefficient of variation is a measure of variability in a dataset, equal to the standard deviation divided by the mean (Abdi, 2010) and the SSSR values of the STZ tail tendons, control tail tendons and STZ Achilles tendons from Table 3.2 yielded relatively small values (0.74, 0.83 and 0.69, respectively) after adjusting for outliers. Only control Achilles tendons returned a large value (1.97). This is, however, only an estimate of material property status: the values in question are not Normally distributed and as such taking their standard deviations can, at best, merely approximate their dispersions. If it were indeed the case that SSSR was a material property it would be immediately relevant to future research: a portion of the samples in a batch could be used for SSSR measurement (which is a necessarily destructive process) and these results then generalised to represent the entire batch. A treatment could then be performed on the remaining samples and their SSSRs measured after this is completed. Any difference between the average of these post-treatment SSSRs and the average of the pre-treatment SSSRs would then be attributable to the protocol. Such information would add insight and aid investigations into the mechanisms behind tendon damage.

4.2.4. Phenomenological Markers of Tendon Damage

Various material properties have been used, directly or indirectly, as phenomenological markers¹ of the current damage level (i.e. measurements relating to the phenomena arising from damage rather than to the damage mechanisms themselves). Indirectly, changes in tendon strain-at-failure, stress-at-failure (UTS) and loading modulus, as measured during ramp-to-failure and dynamic fatigue tests, have been used by Volper et al. (2015) and Shepherd et al. (2014b) to investigate the effects of a diabetic model and the effects of functional role, respectively. While these authors did not claim that their phenomenological results implied that diabetic and energy-storing tendons damage more easily, both of these groups have shown a higher propensity to damage in vivo (Deshmukh and Akarte, 2017; Jozsa et al., 1989). Thus, a correlation might exist between changes in these measurements and damage. Other such potential markers of damage from past studies include decreased time-to-failure (Ker et al. (2000), static fatigue) and increased permanent strain (Abrahams (1967) and Veres et al. (2013b), dynamic fatigue). On the other hand, a change in loading modulus is the only property that has been used as a marker of damage *directly*: Lemaitre (1984) introduced a ‘damage variable’ which effectively accounts for damage by decreasing the mathematical value of the modulus in calculations. Similarly, Wang and Ker (1995) (static fatigue) introduced a ‘damage hypothesis’ which was quantitatively defined as a decrease in this measurement. Thus, a change in loading modulus is currently the best phenomenological estimate for the amount of ‘damage’ in a tendon (as is, equivalently, the rate of change of modulus the best estimate for the rate of damage). Strain- and stress-at-failure are also accepted when investigating elastic properties (i.e. when performing ramp-to-failure tests) while a change in permanent strain may also be relevant to fatigue damage. However, time-to-failure as measured over a fatigue test is probably too stochastic to be reliable; the fast-failure response of a tendon immediately prior to failure is governed by random factors such as the exact moment when fibrils pull out from one another and/or the

¹The terms “marker” and “indicator” can be used interchangeably in this context. “Marker” implies that correlation (but not necessarily causation) has been established whilst “indicator” implies that the relationship is based on cross-sectional evidence as opposed to longitudinal studies (Burt, 2001). Both are thus applicable here.

statistical distribution of defects and the weakest interfibrillar bonds. They therefore cannot be predicted accurately. Note that no example has been found of a *predictive* phenomenological marker: a measurement that could predict how much damage a certain tendon will accumulate during a certain loading regime.

4.2.5. Fatigue Testing

This experimental methodology was introduced in Chapter 3. While static fatigue testing enables the measurement of a variety of markers, including the SSSR, a disadvantage that exists when compared to cyclic fatigue testing, for example, is that each measurement can only be performed once (as opposed to every cycle). Thus, the change in the value of a marker cannot be tracked. This inhibits investigations into tendon damage as two of the aforementioned indicators of damage - the change in loading modulus and the change in permanent strain - cannot be quantified. However, in order to perform a comparison between SSSR and recognised markers of damage it will be necessary to measure all of these values on the same sample during the same experimental run and, as such, an alteration must be made. Therefore, a new testing procedure has been defined - an ‘interrupted creep test’ - which consists of a standard creep test during which load is periodically removed from the sample (in order to allow for the repeated measurement of permanent strain) and then re-applied (in order to allow for the repeated measurement of loading modulus). Upon test completion, a ‘pseudo-SSSR’ can be measured by considering only those portions of the test during which the sample was under load and finding the minimum of the derivative of the strain vs time graph formed by concatenating this data. This protocol thus combines features of both static and cyclic fatigue testing. It must be noted that any measurements taken during such a test will only be relevant in relation to other measurements from the same test; the non-standard nature of the procedure makes comparisons with standard values (e.g. of loading modulus) measured during other tests meaningless. One of the factors introduced by the unloading of samples that is not seen in standard static fatigue tests is the formation of fibril kinks, a phenomenon which is equivalent to introducing additional damage (Herod and Veres, 2018).

4.2.6. Streptozotocin Treatment

The investigation into steady-state strain rate was performed using the same model of diabetes which had been found, in Chapter 3, to significantly decrease this measurement's value. The model has already been discussed at length there, as well as in Chapter 1.

4.2.7. Aim

To establish the utility of steady-state strain rate as a descriptor of tendon susceptibility to fatigue damage. This was achieved using 'interrupted' static fatigue testing of rat tail tendons from an STZ model of diabetes and from healthy controls at a range of stress levels. Damage was defined both as a decrease in loading modulus and as an increase in permanent strain.

4.2.8. Hypothesis

Steady-state strain rate can be used to differentiate between materials with different susceptibilities to fatigue damage. Secondly, the following hypotheses were also tested:

- Steady-state strain rate will be correlated with changes in previously-used markers of damage
- Steady-state strain rate is a material property and as such will be independent of the applied stress
- Loading modulus will decrease and permanent strain will increase over the course of an interrupted static fatigue test
- These changes will be greater in magnitude in samples with larger steady-state strain rates
- SSSR will show a decreased rate of damage in STZ-treated tendons compared to controls

In summary, SSSR is thought to be a material property that predicts the response to load over time, and thus predicts how quickly the tendon will damage. This is opposed to

changes in loading modulus and permanent strain which represent how much damage has already occurred, independent of time. Comparing the SSSRs of two groups of tendons - one treated in some way and the other untreated - could allow an inference about the effect of that treatment on the tendons' resistance to damage to be made.

4.3. Materials and Methods

While similar to that used in Chapter 3, the procedure used in this experiment differed from a traditional static fatigue test in a number of ways.

4.3.1. Sample Collection

Tail tendons from male Wistar rats (Harlan/Envigo, UK) were used with all rats having been culled as part of an unrelated experiment (on diabetic cardiomyopathy: Le Page et al. (2015)). These rats fell into two experimental groups - diabetic and control - with the model of (Type 2) diabetes in question having involved a high fat diet and a 25 mg/kg injection of streptozocin (Mansor et al., 2013) at 8 weeks of age. Ages at cull ranged from 10 to 14 weeks, implying that all had reached maturity (Provenzano and Vanderby, 2006) and thus ensuring that the tendons and the properties thereof were fully developed. The tails were stored at -70°C until being fully thawed (~ 30 min) when needed. 16 tails, all of which were blind-labelled with regards to their experimental group (STZ-treated or control), were dissected with five tendons being harvested from each. These were placed in phosphate-buffered saline (PBS) to prevent dehydration and one tested immediately whilst the others were re-frozen for later use (at which point they were re-thawed for ~ 15 min). No tendon was frozen for longer than five months in total (which would have affected the mechanical properties (Lee and Elliott, 2017)).

4.3.2. Sample Preparation

As in the previous experiment, sample cross-sectional area A was calculated by taking two measurement of the diameter - d_1 and d_2 - at 90° to each other using an optical micrometer (Keyence, UK & Ireland) and assuming an ellipsoidal shape (i.e. $A = \pi \times \frac{d_1}{2} \times \frac{d_2}{2}$). This was

repeated for five points along the samples' lengths and the smallest of these (representing the weakest section of each tendon, where damage would most likely occur) was used. Although excess liquid was removed from the samples before diameter measurement took place they were never allowed to dry fully, being returned to PBS as soon as possible.

4.3.3. Apparatus and Sample Loading

The identical apparatus and loading method was used for this experiment as in the previous. The following elements were involved:

- Universal material tester with 5 kN load cell (Zwick/Roell UK)
- testXpert II software (Zwick/Roell UK)
- Webcamera (Logitech QuickCam Pro 9000)
- Manual timing device with white backing
- Custom-made aluminium grippers and Perspex PBS bath

A video protocol was made for the apparatus setup and sample loading, frames of which are included in Appendix G. Samples were loaded so as to be initially slack.

4.3.4. Interrupted Static Fatigue Testing

Each of the five samples harvested from each tail was assigned a 'creep stress' - either 30, 28, 26, 24 or 22 MPa. This was the stress at which static fatigue testing would be performed. From this, the required 'creep force' was calculated by multiplying by cross-sectional area. A 'relaxation force' of 0.1 N was also defined for all samples (this being twice the resolution limit of the load cell which is used as the 'no load' force, see Section 3.3.6). The testing protocol was then started, beginning with the 'loading phase': the material tester's clamps moving apart at a rate of 6 mm/min (the same strain rate as in Chapter 3) until a load equal to the creep force was measured over the sample. At this point the force was held constant for 60 seconds ('creep phase') after which it was removed (the clamps moved together at 6 mm/min) until 'no load' was registered ('unloading phase'). This force was held constant for 600 seconds ('relaxation phase'),

sufficient time for viscoelastic relaxation effects to dissipate (Sverdlik and Lanir, 2002; Screen et al., 2013). These four phases were then repeated until rupture occurred. As before, force, clamp-to-clamp separation and time data was collected at 11 Hz.

4.3.5. Calibration

Following test completion the raw data was transferred and imported into a Python script for calibration. The overall start and end of the test was defined as the point at which no load (0.1 N) was first registered and at which the applied force dropped below 99% of the creep force for the last time, respectively. The starts and ends of each of the testing protocol's phases were then found (Figure 4.1a). Clamp-to-clamp separation was converted into sample strain in the same way as described previously: a manual image analysis method (accuracy of ~ 90 ms) using a graduated ruler as a reference found the sample length at 'no load' (accuracy of ~ 150 μm) and the difference was subtracted from the raw data to obtain sample length at all time points. Dividing through by this initial length yielded engineering strain which was then converted into true strain. Force data was divided by cross-sectional area to obtain engineering stress which was likewise converted into true values. These conversions were performed by assuming incompressibility of the tissue (i.e. a Poisson's ratio of 0.5, which is indeed what has been reported in the literature to within experimental uncertainty (Vergari et al., 2011)) and thus using the following equations: $\sigma_{true} = \sigma_{eng} (1 + \epsilon_{eng})$ and $\epsilon_{true} = \ln(1 + \epsilon_{eng})$. An adjustment to account for end-effects at the grips was also made (Ker, 1981; Legerlotz et al., 2010).

4.3.6. Data Analysis

The strain at the end of each relaxation phase was defined as the permanent strain after each cycle. The rate of change in permanent strain ($\dot{\epsilon}_p$) was then calculated as the gradient of a straight line fitted to these points, as shown in Figure 4.1b. A curve-fitting routine was then applied to each of the cycles, identifying a combined viscoelastic and sliding response in every creep phase (Figure 4.1c). A number of values could then be calculated from each of these, as defined in Section 3.3.8, and a linear fit was then again used to

4. Steady-State Strain Rate as a Measure of Damage Accumulation

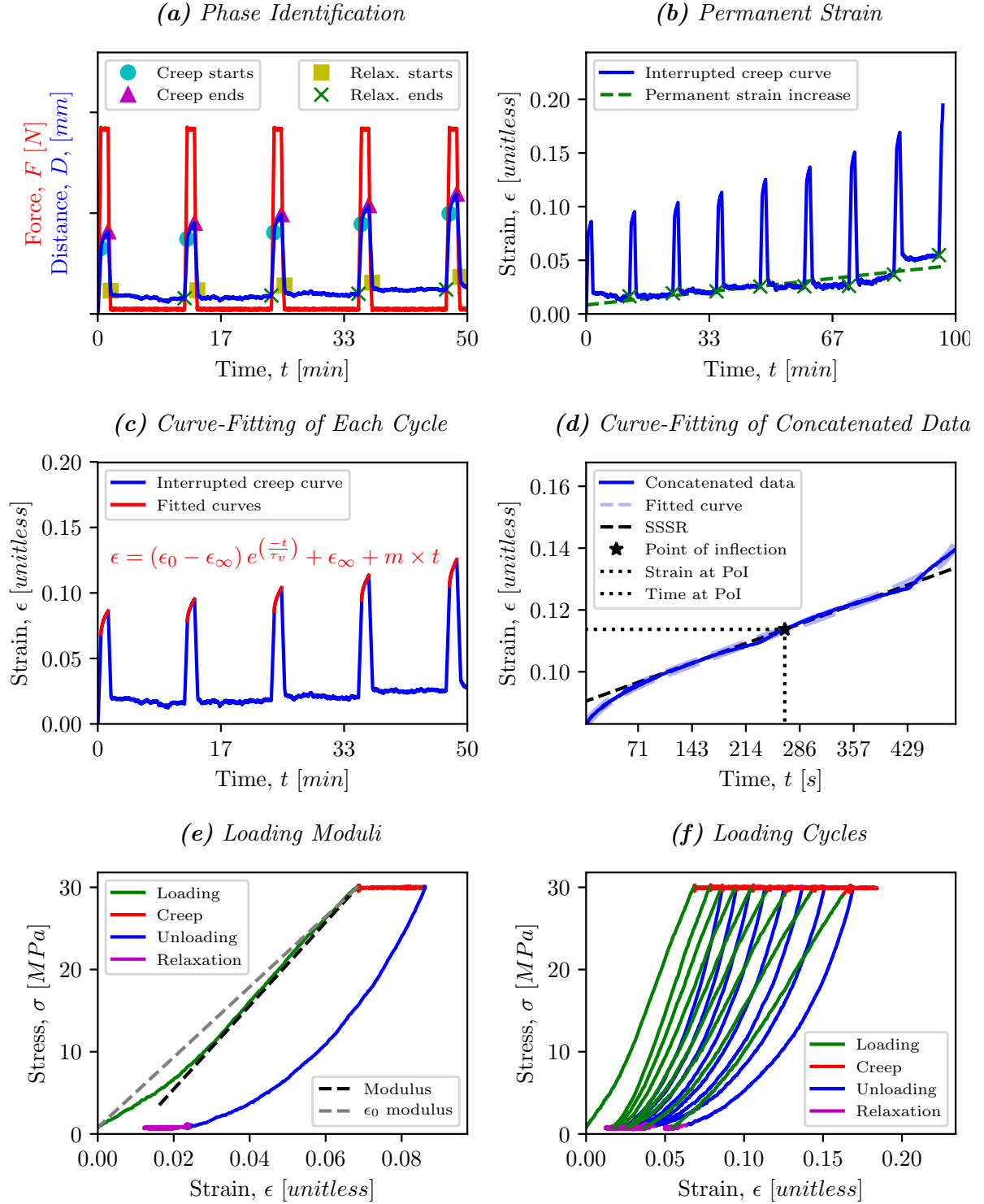


Figure 4.1: Summary of the data analysis process. Note that the data in Figures (a), (c) and (e) has been truncated for clarity.

quantify their rates of change. For consistency, data points associated with the first and last cycles were omitted. The results obtained in this fashion are as follows:

- Rate of change of viscoelastic time constant ($\dot{\tau}_v$)
- Rate of change of elastic strain ($\dot{\epsilon}_0$)
- Rate of change of viscous strain ($\dot{\epsilon}_{\infty-0}$)
- Rate of change of viscosity ($\dot{\eta}$)
- Rate of change of Kelvin-Voigt stiffness ($\dot{E}_1$)
- Rate of change of Maxwell stiffness ($\dot{E}_2$)

with the first three having being derived from the previously discussed model (the modified SLSM, see Figure 2.11 and Figure 3.4b) and the latter three having been derived from its associated equation (see Figure 3.4a, Appendix E.1 and Equations A.21 to A.23 in Appendix A.3). See also Figure 4.2 for explanations of the various strain-based measurements.

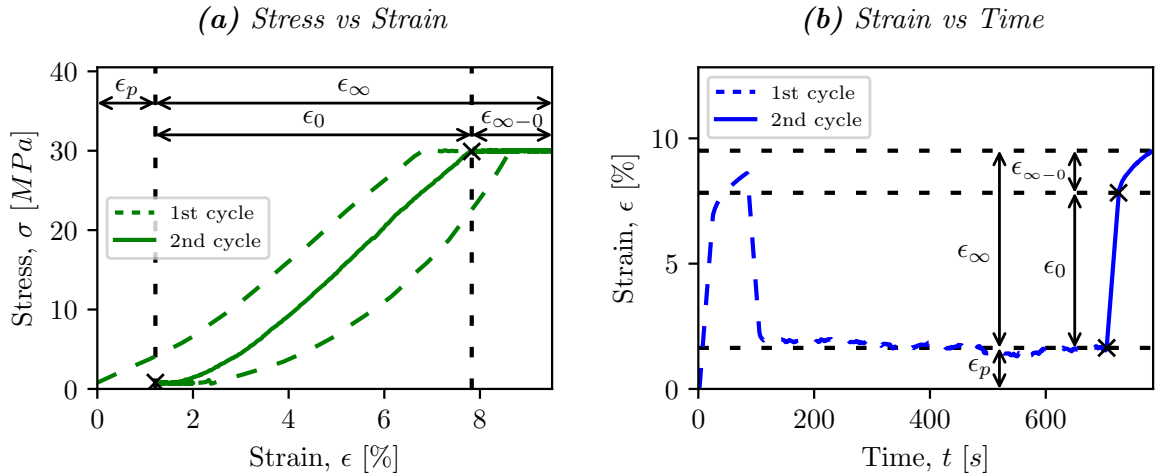


Figure 4.2: Two plots both showing the first one-and-a-half cycles of a particular sample's test. Permanent strain (ϵ_p) and non-permanent ('recoverable' or 'viscoelastic') strain (ϵ_∞) are both shown, the latter being divided up further into elastic (ϵ_0) and viscous ($\epsilon_{\infty-0}$) strains.

An algorithm was used to automatically concatenate the creep phases (Figure 4.1c) into a single pseudo-static fatigue test or 'interrupted creep test' (Figure 4.1d). This was then

treated in the same manner as the data from Chapter 3: a curve was fitted and the minimum of the derivative thereof taken as the (pseudo-)steady-state strain rate (SSSR; $\dot{\epsilon}_{PoI}$). This was also used to define the point of inflection (PoI), allowing strain, time and strain energy at the PoI to be found (Figure 4.1d). The latter required stress vs strain to be plotted for the concatenated curve, and this was also done for each cycle individually (Figure 4.1e) as well as for the entire run (Figure 4.1f). These allowed the loading modulus (E) - the gradient of the linear portion of the relevant loading phase - to be calculated for each cycle:

$$E = \frac{\Delta\sigma_{linear}}{\Delta\epsilon_{linear}} \quad (4.1)$$

where ‘linear’ refers to a straight section of the loading phase of a stress vs strain plot. Additionally, an ‘elastic strain modulus’ (E_{ϵ_0}) was calculated as the gradient of the straight line connecting the start of the loading phase to the point at which elastic strain ϵ_0 was reached:

$$E_{\epsilon_0} = \frac{\sigma_{creep} - \sigma_{relax}}{\epsilon_0} \quad (4.2)$$

for creep stress σ_{creep} and relaxation stress σ_{relax} (the latter being close to zero). As such, this parameter represents the behaviour of the material in a way that includes the viscoelastic toe and heel regions. Both of these moduli are represented in Figure 4.1e and both sets of readings were fitted with straight lines from which their rates of change were reported (as before).

4.3.7. Statistical and Uncertainty Analyses

These were performed identically to Section 3.3; the Mann-Whitney U test was used (Graziano and Raulin, 2014) and $p \leq 0.05$ taken as significant. It was confirmed that no outliers existed in the data, that a majority of the datasets were not Normally distributed and that the data’s uncertainties were relatively small.

4.4. Results

Following the completion of all testing, and of initial analysis, the experimental blind was removed to reveal that nine of the 16 tails had come from the diabetic model with the remaining seven having been healthy controls. While five tendons had been tested from each tail (80 samples total), 21 of these subsequently had to be excluded (leaving 59 viable for analysis): 6 of these were due to the tendon having gone slack during one or more of the relaxation phases (visible as a drop-off in the strain vs time plots and falsely resulting in a decrease of measured permanent strain) and 15 of these were due to rupture having occurred before the completion of three full cycles (rendering a calculation of the change in measurement from the middle cycles impossible). As such, the sample sizes for each group were as follows:

Table 4.1: Sample sizes for the analysis of the interrupted static fatigue test data

	Creep Stress [MPa]					Total
	22	24	26	28	30	
STZ	6	7	5	7	5	$n = 30$
Control	7	6	4	6	6	$n = 29$

Steady-state strain rate (the rate of strain at the point of inflection; $\dot{\epsilon}_{PoI}$) was calculated for each of the 59 samples, as shown in Figure 4.3. The value of this measurement increases with the amount of creep load applied for both control and STZ-treated samples, and the latter correlation is statistically significant (Figure 4.3). It was also lower, significantly, in STZ samples (Figure 4.4) which replicates the results found in Chapter 3.

4.4.1. Phenomenological Markers

In addition to SSSR, a number of phenomenological results were calculated for each sample. For each of the ten categories shown in Table 4.1 (five creep stresses times two experimental groups) these were averaged and plotted against SSSR (Figure 4.5). All results (i.e. not as averages) were then compared across experimental groups (Figure

4.7).

Correlations with SSSR: Statistically significant correlations were found between samples' SSSRs and their rates of change of loading modulus ($\dot{E}$) for control samples only (Figure 4.5a) with the same being true for their rates of change of elastic strain modulus ($\dot{E}_{\epsilon_0}$; Figure 4.5c). The correlation between SSSR and the time at the PoI (Figure 4.5d) was likewise significant for control tendons only while between SSSR and strain energy at the PoI it was significant for STZ samples only (Figure 4.5b). However, in the latter two cases the correlation was negative in control samples but positive in the STZ samples, and the same juxtaposition is seen in strain at the PoI (Figure 4.5f). The rate of change of permanent strain is positively correlated with SSSR in both groups, but not significantly (Figure 4.5e).

STZ Model vs Healthy Controls: the rates of change of loading modulus, of elastic strain modulus and of time at the PoI are significantly larger in STZ samples (Figures 4.7a, 4.7c and 4.7d, respectively) while the rates of change of strain energy at the PoI, of strain at the PoI and of permanent strain remain unchanged (Figures 4.7b, 4.7f and 4.7e, respectively). These results mirror those reported in Chapter 3 from tail tendons, except for loading modulus which was originally unchanged with STZ.

Previously-Used Markers of Damage: The moduli (both loading modulus and elastic strain modulus) decreased over the course of the interrupted creep tests. The rates at which these changes in modulus occurred increased as SSSR increased (significant in control samples only; Figures 4.5a and 4.5c) but were significantly smaller in STZ-treated samples than in controls (Figures 4.7a and 4.7c). Permanent strain increased over the course of the tests with the rate of increase itself increasing if SSSR was larger (Figure 4.5e). This rate was no different in STZ samples compared to controls (Figure 4.7e).

4.4.2. Mechanistic Markers

These six results represent the rates of change of mechanistic markers of fatigue response as derived from the applied model (namely the modified standard linear solid model (Frisén et al., 1969; Fung, 2004) as was used in Chapter 3, detailed in Figure 2.11 and

derived in Appendices A.3 and A.5). They were likewise averaged and plotted against SSSR (Figure 4.6) and, separately, compared across experimental groups (Figure 4.8).

Correlations with SSSR: The rates of change of markers from the SLSM (viscosity, Kelvin-Voigt stiffness and Maxwell stiffness) were all negatively correlated with SSSR, with these correlations being significant for viscosity in STZ-treated samples only (Figure 4.6b), for Kelvin-Voigt stiffness in control samples only (Figure 4.6d) and for Maxwell stiffness in both groups (Figure 4.6f) with the largest coefficients of determination (r^2 values) also being found here (0.93 and 0.98). Regarding the rates of change of markers from the equation (viscoelastic time constant, elastic strain and viscous strain) all the correlations with SSSR were positive, although in STZ-treated samples the viscoelastic time constant and viscous strain correlations were notably flat (Figures 4.6a and 4.6e, respectively). The correlation in control samples, however, was significant for viscous strain. The rate of change of elastic strain was correlated with SSSR in both groups (Figure 4.6c), and these coefficients of determination were the second largest out of all the results (0.93 and 0.92).

STZ Model vs Healthy Controls: Rates of change of each of the SLSM markers increased with STZ (Figures 4.8b, 4.8d and 4.8f) while rate of change of elastic strain decreased with STZ (Figure 4.8c). The rates of change of viscoelastic time constant and of viscous strain remained unchanged (Figures 4.8a and 4.8e, respectively). These results mirror those from Chapter 3 for viscosity and viscous strain, while viscoelastic time constant has lost significance and elastic strain, Kelvin-Voigt stiffness and Maxwell stiffness have gained it.

4. Steady-State Strain Rate as a Measure of Damage Accumulation

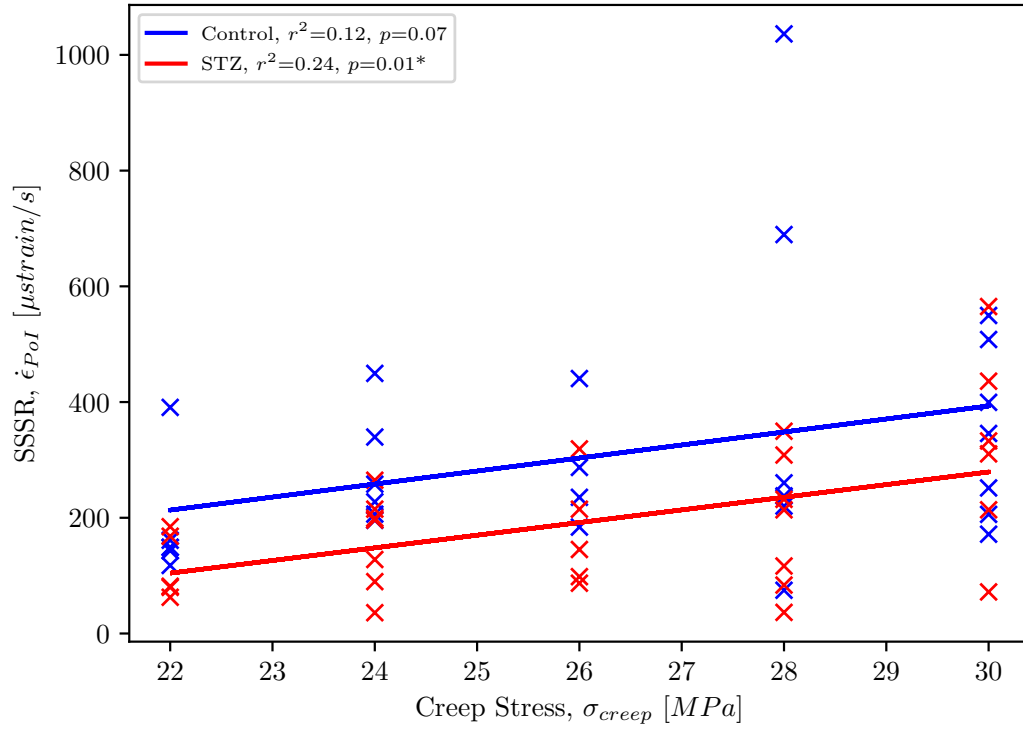


Figure 4.3: Dispersion (given by the coefficients of determination, r^2) and correlation (given by the significance levels, p) of the steady-state strain rate data at the various creep stresses for both experimental groups. Significance is determined by a two-sided hypothesis test the null hypothesis, H_0 , of which states that the gradient of the line of best fit is zero. H_0 is rejected if $p \leq 0.05$, as indicated by an asterisk*.

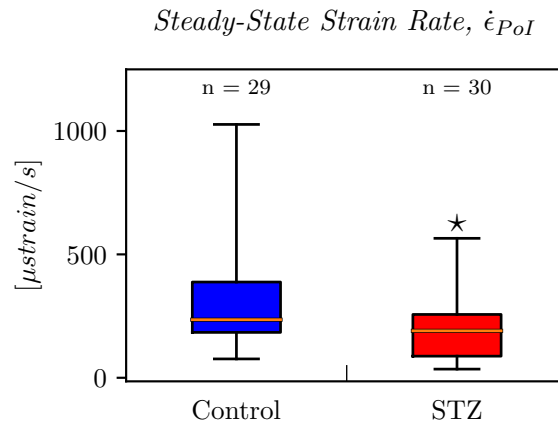


Figure 4.4: Box-and-whisker plots of the SSSR values show a significant difference associated with STZ treatment ($p \leq 0.05$, Mann-Whitney)

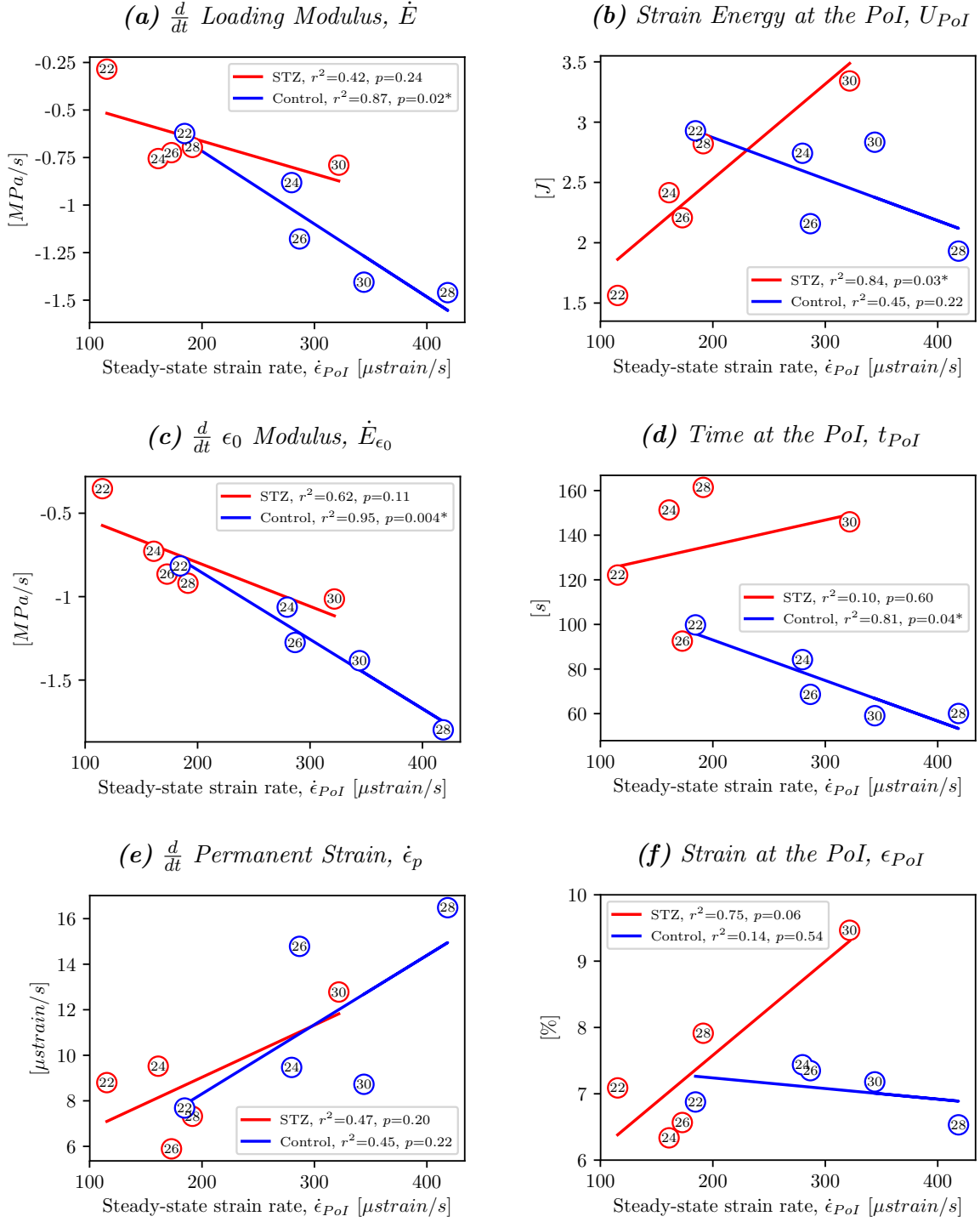


Figure 4.5: Correlations between SSSR and the phenomenological markers (b, d, and f) or their cycle-on-cycle changes (a, c, e). Results at each creep stress (22, 24, 26, 28 and 30 MPa, as indicated) have been averaged. The coefficients of determination (r^2) have been included in the legends along with the significance levels of a two-sided hypothesis test the null hypothesis, H_0 , of which is that the associated gradient is zero ($p \leq 0.05$ is indicated by an asterisk*). Axes do not start at the origin; results are for comparison only and are not valid as standalone values.

4. Steady-State Strain Rate as a Measure of Damage Accumulation

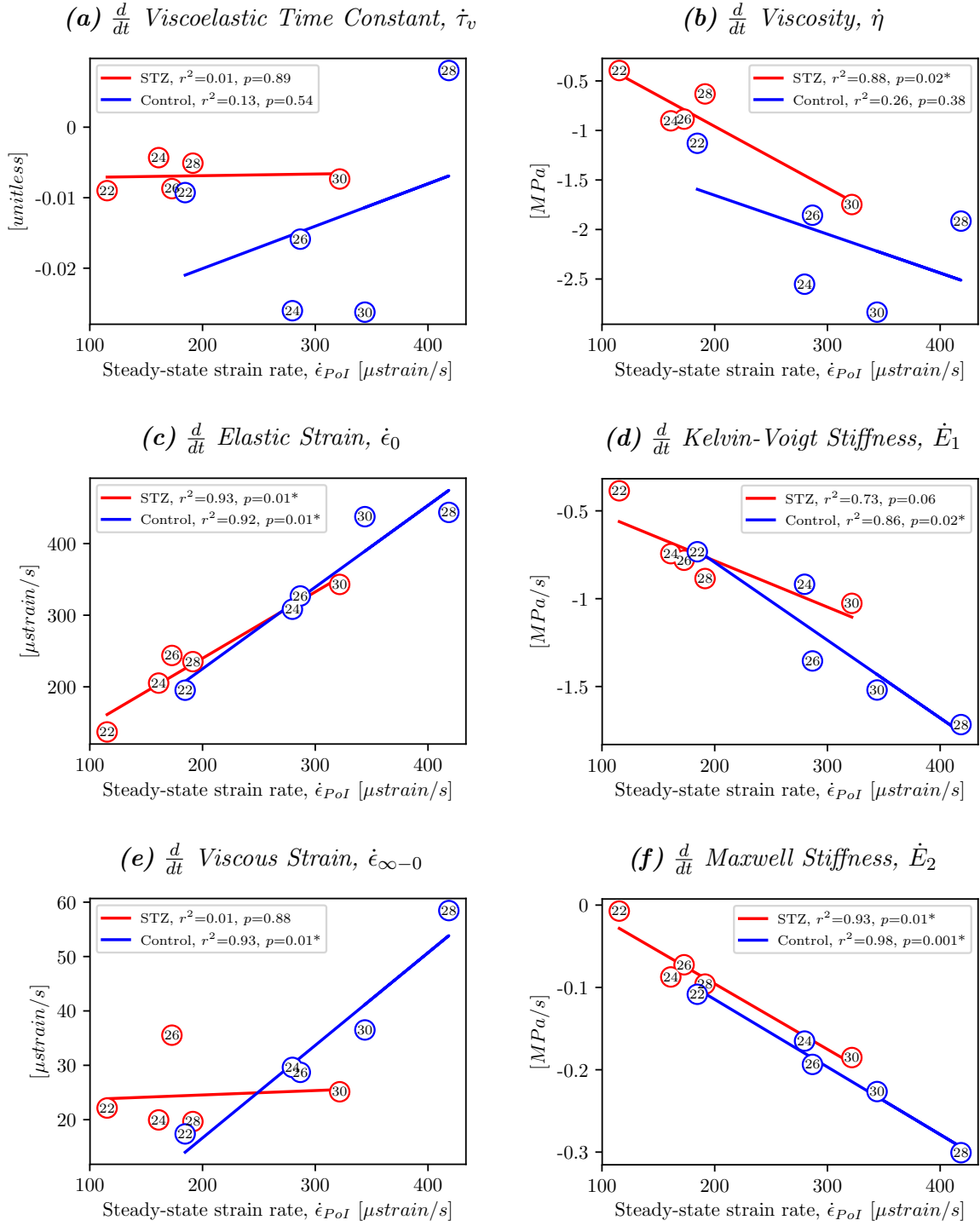


Figure 4.6: Correlations between SSSR and cycle-on-cycle changes in the characteristics of the applied model. Results at each creep stress (22, 24, 26, 28 and 30 MPa, as indicated) have been averaged. The coefficients of determination (r^2) have been included in the legends along with the significance levels of a two-sided hypothesis test the null hypothesis, H_0 , of which is that the associated gradient is zero ($p \leq 0.05$ is indicated by an asterisk*). Axes do not start at the origin; results are for comparison only and are not valid as standalone values.

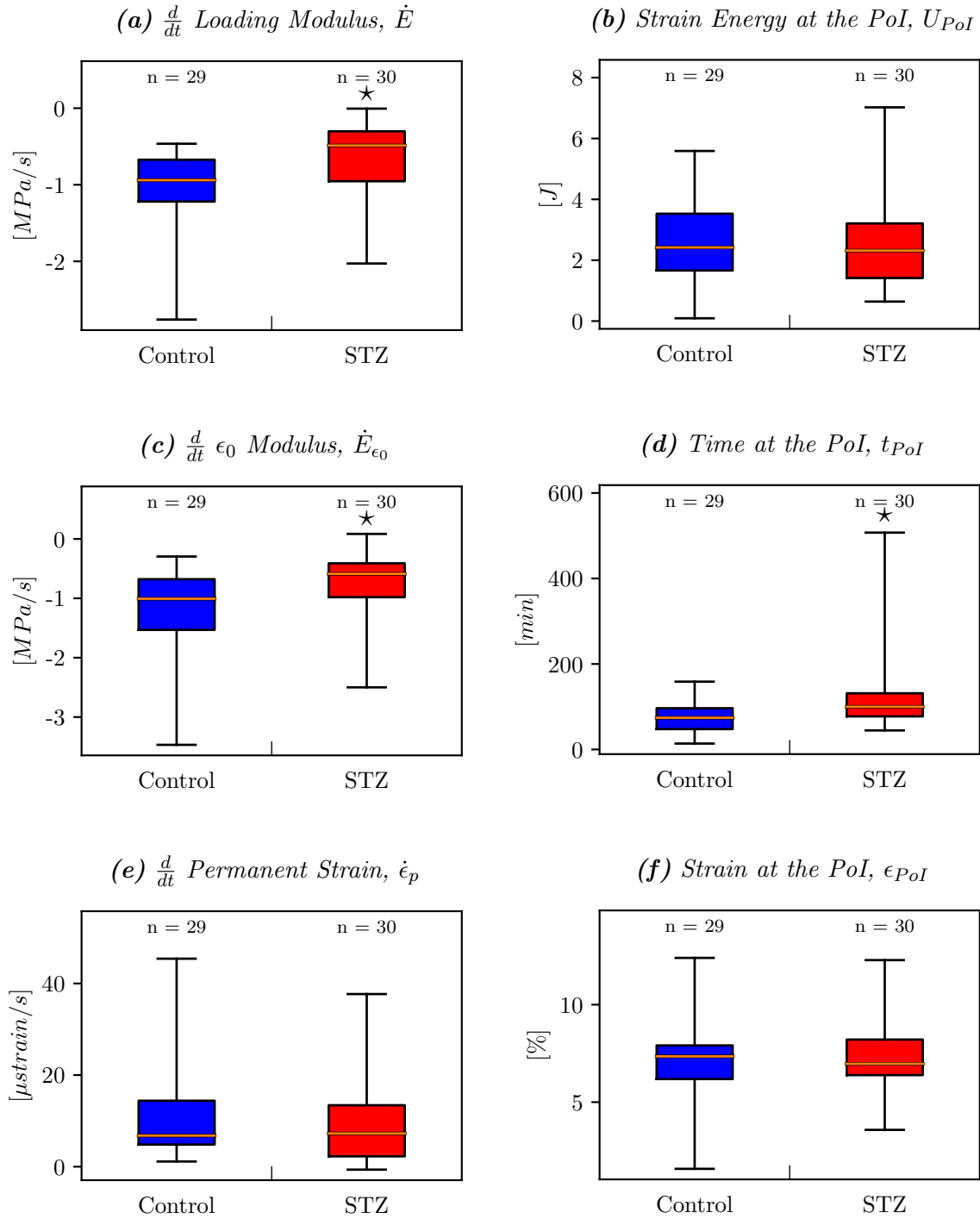


Figure 4.7: Box-and-whisker plots of the phenomenological markers showing significant differences associated with STZ treatment ($p \leq 0.05$, Mann-Whitney) in rate of change of loading modulus (a), rate of change of elastic strain modulus (c) and time at the point of inflection (d).

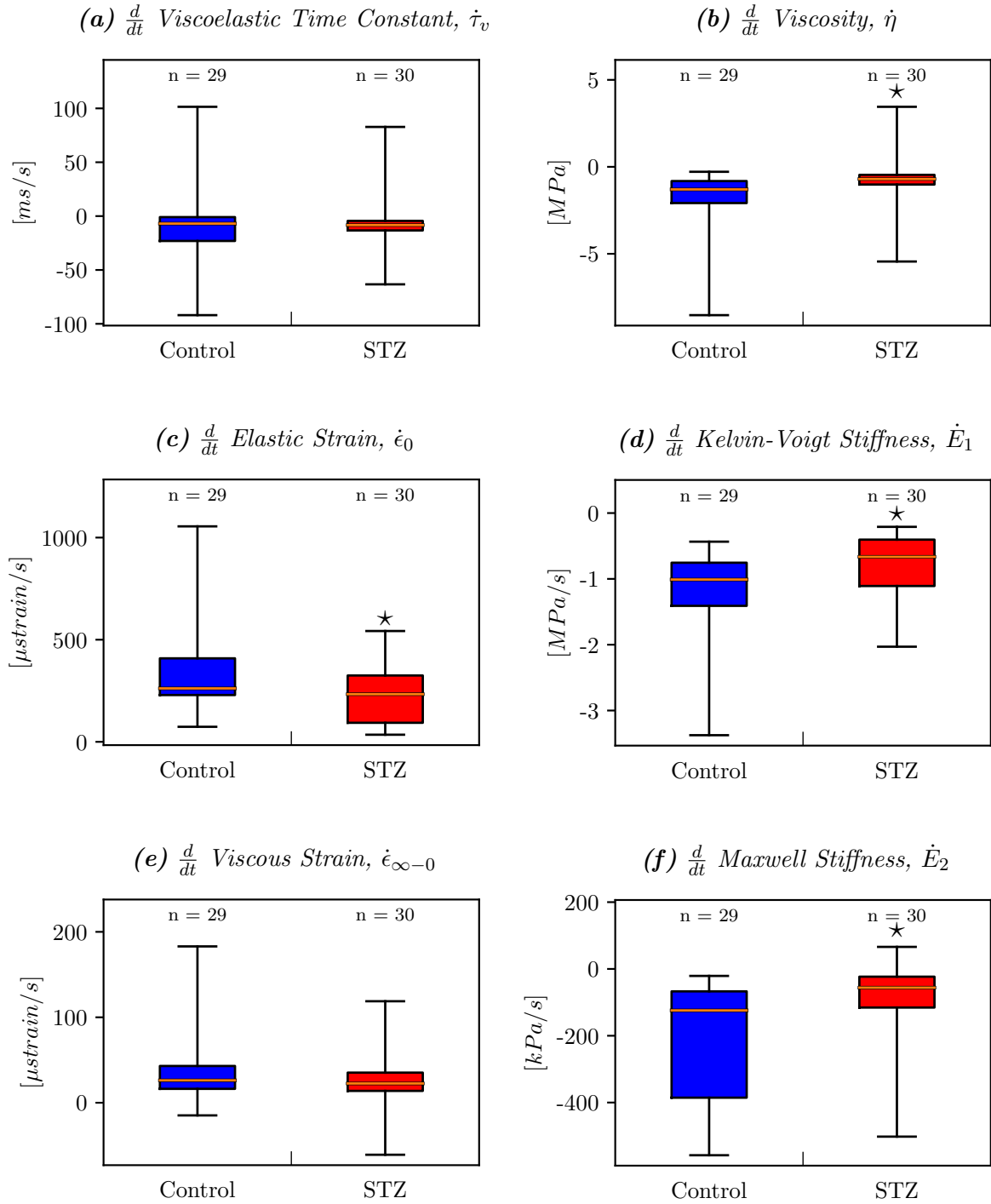


Figure 4.8: Box-and-whisker plots of the characteristics of the applied model, representative of mechanistic markers. Significant differences ($p \leq 0.05$, Mann-Whitney) associated with STZ treatment are seen in the rates of change of viscosity (b), elastic strain (c), Kelvin-Voigt stiffness (d) and Maxwell stiffness (f).

4.5. Discussion

Interrupted static fatigue tests were performed on rat tail tendons from an STZ model of diabetes as well as from healthy animals. The data from the creep phases of these tests was extracted and analysed as pseudo-static fatigue tests using a modified form of the Standard Linear Solid Model. The results indicate that steady-state strain rate is correlated with a number of properties and characteristics of tendon under static fatigue loading, with these being influenced by the STZ model. Many of these results were measured at the point of inflection (PoI) of the relevant concatenated strain response. The PoI is significant as it can be considered the ‘tipping point’ of tendon damage; it marks the balance between the material being able to contain the load being placed upon it (represented by the viscoelastic response) and it stretching exponentially more quickly until failure (represented by the pullout response). Thus, measurements taken here - such as the SSSR - may be able to act as descriptors of the material’s ability to resist strain and mechanical damage.

4.5.1. Steady-State Strain Rate as a Material Property

The plot of SSSR vs creep stress in Figure 4.3 indicates that the two variables are positively correlated, with this correlation being significant for STZ samples ($p = 0.01$) and near-significant for control samples ($p = 0.07$). As such, SSSR is not independent of loading conditions and thus does not appear to be a material property. At the same time, the high levels of dispersion in the data for both groups (the r^2 values are relatively low; 0.24 and 0.11 for STZ and control respectively) suggest that it is not possible to accurately predict what a sample’s SSSR will be for a given creep stress. Thus, SSSR is unlikely to be an innate property of the material but rather a function of other factors, some of which may be stochastic characteristics particular to the specific sample.

4.5.2. Phenomenological Markers

A sample’s SSSR influences its stress- and strain-response to load over time.

Correlations with SSSR: A larger SSSR is correlated with a quicker decrease in loading modulus which suggests that it is an indicator of a sample's increased susceptibility to mechanical damage. Moreover, it is correlated with a quicker decrease in 'elastic strain modulus' (E_{ϵ_0}), which is similar to loading modulus (E) but is explicitly related to creep stress (σ_{creep}) and elastic strain (ϵ_0) as opposed to being approximately related to them (see Figure 4.1e and Equations 4.1 and 4.2). Indeed, Figures 4.3 and 4.6c confirm that SSSR is correlated with, respectively, the creep stress and the rate of change of elastic strain. This is especially the case with the latter which has both low dispersion and large significance, suggesting that SSSR and the rate of change of elastic strain likely both measure the same aspect of damage. Possibly, this could also be true for other strain-based measures.

In addition to a larger SSSR being associated with a quicker decrease in loading modulus it is also associated with permanent strain increasing more quickly (Figure 4.5e), albeit not significantly and with moderate dispersion. This is another indication that SSSR is related to damage susceptibility. It is also associated with the PoI occurring sooner (Figure 4.5d) but at approximately the same level of strain (Figure 4.5f) - i.e. at a lower level of strain energy (given that stress is constant) - in control samples but not in STZ samples. Indeed, the PoI occurs at approximately the same time but at a larger strain in STZ samples, implying that a larger level of strain energy has been absorbed. This is corroborated by the strain energy at the PoI results (Figure 4.5b). As discussed in Section 4.2.2, the PoI may represent the transition point between the recoverable and the damage responses of tendon to load. Thus, in effect, it appears as if STZ treatment causes the samples to damage more easily if the load (and SSSR) is low but less easily if the load (and SSSR) is large.

A possible explanation for this may be found by considering the mechanistic markers' results from Chapter 3, which have been included in Appendix E. In particular, in tail tendons, it was found that the viscosity (η ; the resistance to the flow of water molecules from the inside to the outside of the fibrils) increased significantly with STZ treatment while Kelvin-Voigt stiffness (E_1 ; approximately the stiffness of the collagenous

matrix) and Maxwell stiffness (E_2 ; approximately the stiffness of the non-collagenous matrix) remained unchanged. Thus, in STZ tendons, water remains within the fibrils for longer. While the presence of these molecules can contribute to strength during the early stages of a static fatigue test (acting as a ‘shock absorber’, although shock is not the loading protocol in this instance) they do not add structural integrity per se; once collagen molecules start to pull out due to fatigue the water will simply flow away. This would explain the aforementioned reduced fatigue resistance in STZ samples at low loads: more water being present in the collagenous matrix as a result of it not being able to flow out into the ground substance as easily would reduce its fatigue strength. At high loads, however, the deformation of the structure becomes relevant: a tendon would decrease in cross-sectional area (due to the Poisson effect) less if it contained many water molecules, artificially reducing the stress it experienced. Due to the square-linear relationship between length and radius for a constant-volume material such as tendon (the governing equation is $V = \pi r^2 L$ for a fibril of volume V , radius r and length L) this effect would only be noticeable at high strains. Hence, this would explain the aforementioned increased fatigue resistance in STZ samples at high loads.

STZ Model vs Healthy Controls: Similar to what was found in Chapter 3, STZ treatment lead to the PoI occurring later and, when it did occur, occurring with a slower rate of strain. However, the act of unloading and loading the samples, as happened during the interrupted creep tests, caused STZ to also increase stiffness. It may be that the mechanisms behind STZ’s effects on tendon - e.g. increased crosslink strength due to AGE presence - become more pronounced under repeated loading.

Previously-Used Markers of Damage: SSSR is indeed correlated with previously-used markers of damage - decreasing loading modulus and increasing permanent strain. Additionally, it is correlated with decreasing elastic strain modulus, a measurement related to loading modulus but altered in order to take advantage of the large correlation that exists between SSSR and the rate of change of elastic strain. In all cases, the results suggest that STZ-treated samples damaged more slowly than control samples.

4.5.3. Mechanistic Markers

Steady-state strain rate was also correlated with a number of the rates of change of model-derived, mechanistic markers.

Correlations with SSSR: A larger SSSR has no correlation with the rate of decrease of the time constant of the viscoelastic processes within the tendon. It also has no effect on the rate of increase of viscous strain for STZ-treated samples, but is highly correlated with it in control samples. Conversely, it increases the rate of decrease of viscosity in STZ samples but has no effect in control samples. In other words, if AGEs are not present, a higher rate of strain causes more strain to occur due to water molecules having flowed out of the collagenous matrix/due to non-collagenous matrix deformation. If they are indeed present, the increased intermolecular bond strength prevents this increase in strain from occurring, and instead the decrease in the resistance to the flow of water molecules/to non-collagenous deformation happens more quickly (although, possibly, less of it is happening due to less viscous strain occurring). Finally, SSSR is highly correlated with an increase in the rate of increase of elastic strain, an increase in the rate of decrease of Kelvin-Voigt stiffness and an increase in the rate of decrease of Maxwell stiffness. This supports the phenomenological results discussed previously, as all three of these phenomena are related to loading modulus decreasing more quickly (which is something that has already been established as occurring).

STZ Model vs Healthy Controls: These results (Figure 4.8) differ considerably from their equivalents in Chapter 3. The process of removing and then re-applying load during a fatigue test appears to have the effect of masking the increase STZ causes in the viscoelastic time constant whilst galvanising a decrease in elastic strain, an increase in Kelvin-Voigt stiffness and an increase in Maxwell stiffness. Notably, however, these latter three are all related to an increase in loading modulus which, as mentioned earlier, occurs with STZ treatment only under a repeated loading regime. There is no change to viscosity being increased or to viscous strain being unaffected by STZ.

4.5.4. Implications

These results suggest that SSSR is proportional to the rate of decrease of loading modulus and, thus, to the rate of damage (as modulus has been taken to be a marker of this in the past). Therefore, the results of Chapter 3 do indeed suggest that STZ decreases the susceptibility of a tendon to damage, and thus that diabetes does the same. It appears as if the mechanism behind this effect is increased crosslink strength and stiffness affecting the viscous behaviour. Also, damage may be better understood by considering the accumulation of strain in a sample than by its change in modulus. Additionally, the SSSR is not a material property; its value depends on the loading conditions of the tendon during the static fatigue test in which it is measured. However, as long as the loading parameters are kept consistent, it can still be used in the design of an experiment: comparing the average SSSR of the samples in a control group to the average SSSR of the samples in an experimental group will elucidate the effect of the relevant treatment on damage susceptibility. Indeed, it is a superior measurement that the oft-used change in loading modulus when fatigue damage is being investigated as it incorporates the time element of fatigue. Loading modulus, on the other hand, is an elastic property measured over only a short period of time; it is limited in its relevance to fatigue behaviour. With regards to the loading regime used in this investigation it indeed seems to be the case that repeated loading and unloading affects the tendon (and thus it can only be considered to be a *pseudo*-static fatigue test). Specifically, this may manifest as an exacerbation of the effects of STZ, making them more pronounced. This means that, as STZ has been associated with a decrease in SSSR (Chapter 3) which now has been associated with a decrease in damage susceptibility, repeated loading effectively increases damage. This agrees with the literature which identifies the unloading of tendon as the cause of kink formation and kinks, in turn, as having a damaging effect on tendon (Herod and Veres, 2018).

4.5.5. Data Analysis

A number of the aspects of the data analysis process as introduced in Section 4.3.6 will be justified and expanded upon here:

- The ten-minute relaxation phases that occurred before permanent strain measurements ensured that all viscoelastic behaviour was allowed to dissipate; 600 seconds was considerably longer than the viscoelastic time constants calculated for tail tendons in Appendix E.
- As mentioned, each cycle's creep phase was fitted with a combined viscoelastic and sliding response. The pullout response, which is included in the full model, was omitted. The equation used for the fitted routine reflected this - it did not contain an exponentially increasing term - and it is shown in Figure 4.1c. The reason for using a reduced model is that each creep phase was only 60 seconds in length; this was deemed too short for a pullout response to be influential.
- All of the 'rate of change' parameters were calculated by measuring them individually in each cycle (except for the first and the last cycles), plotting them against concatenated time and taking the gradient of the straight line approximation. A linear fit was chosen as it was deemed sufficient for this purpose: estimating a variable's overall increase or decrease over time with the exact relationship being unimportant.
- The reason for omitting the readings from the first and last cycles when calculating rates of change (as mentioned in Section 4.3.6) was that these were too long and too short for consistent data to be obtained, respectively. This was determined after manual assessment of the data points for a number of sets of readings. The first cycle is long because it involves stretching the tendon for the first time; the material has not been 'broken in' and so has additional internal resistance relative to the subsequent loading phases. Practically, this is likely due to the kink formation that occurs during unloading phases (Herod et al., 2016) and thus which has not yet happened when the first creep phase is performed. On the other hand, the final loading cycle is always truncated because this is when rupture takes place, and so it can never be completed. These complications had noticeable effects on the data; it was often observed that the middle data points were highly linear while the first and last stood out as obvious outliers. A representative example of this - a calculation

of a sample's rate of change of viscous strain - is shown in Figure 4.9a: the middle points follow a linear trend while the first and last do not.

- The permanent strain values were an exception to the above and so these rates of change were indeed calculated from all cycles' readings.
- The concatenation step that created the interrupted creep tests involved finding the 'strain resumption' points, these being when the sample strain returned to the same levels as were recorded at the end of the previous creep phases, see Figure 4.9b. These mark when the 'interrupted' static fatigue test 'resumed' creep loading. Once found, it became possible to remove the data from the interrupted sections and concatenate the remaining data.

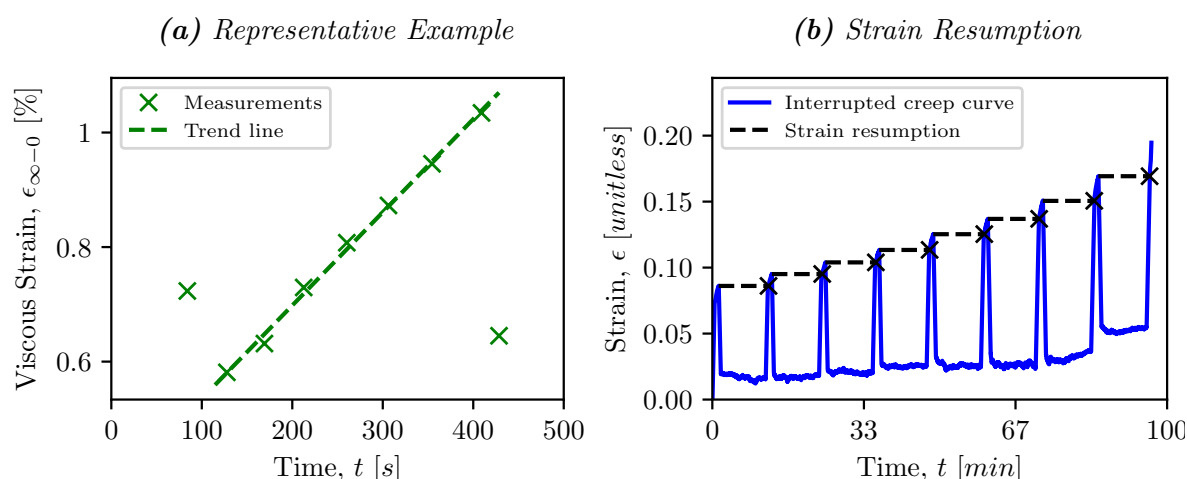


Figure 4.9: (a) The viscous strain ($\epsilon_{\infty-0}$) as measured during each loading phase for a particular sample. Note the strong linear trend that can be fitted when the first and last points are removed - a common observation. (b) Illustration of the algorithm used to locate the points where strain resumed its pre-unloading-phase level. Following this step the algorithm could remove the intermediate data and concatenate that which remained into a pseudo-creep curve.

4.5.6. Limitations and Future Research

It is not clear if the trends seen here are applicable to tendons that serve other functional roles, such as energy-absorbing Achilles tendons. Indeed, the significant influence that functional role has on mechanical properties, which was seen in the previous experiment, shows that not all tendons can be assumed similar. Further testing is thus needed, using

the same protocol but with rat Achilles tendons and stresses in the region of 10 MPa (this being approximately equivalent, in terms of damage, to 30 MPa in a tail tendon - see Chapter 3). Additionally, the concept of energy absorption could be considered: it may be that a tendon will fail once it has absorbed a certain amount of strain energy. This would require the stress vs strain plots (Figure 4.2a) to be re-analysed but it would not be trivial: some energy is lost as dissipation due to internal friction (hysteresis) and this would need to be separated out from that which is being absorbed due to strain. The proportion of strain energy absorption that manifests as permanent strain (as opposed to the proportion that manifests as non-permanent strain and thus which can be recovered) might also be relevant. Also, future testing might consider augmenting the loading regime in order to not fully unload the samples; doing so is known to induce kinks which is damaging in and of itself (Herod and Veres, 2018). A regime that avoided this would more closely represent a true creep test. Finally, the spring-and-dashpot viscoelastic model that has been used to derive the mechanistic markers is relatively simplistic. Caution should be taken when using it to directly map macro-scale measurements onto micro-scale mechanisms, and future experiments should consider using more intricate models.

4.6. Conclusions

Steady-state strain rate can indeed be used to differentiate between materials with different susceptibilities to fatigue damage. This is due to it being correlated with a increase in the rate of decrease of loading modulus - a marker accepted as being indicative of such - with both this rate and SSSR being smaller in samples treated with STZ (which is known to affect damage susceptibility). This conclusion is also supported by SSSR being correlated with an increase in the rate of increase of permanent strain, another previously-used marker. Specifically, SSSR is correlated with the rate of change of elastic strain, a property which influences loading modulus to a large degree. The mechanisms behind this correlation with elastic strain are correlations with the rates of change of Kelvin-Voigt stiffness and of Maxwell stiffness. These represent, roughly, the material's resistance to elastic strain (Kelvin-Voigt stiffness) and viscoelastic strain (both together).

5. Quantification of Advanced Glycation End-Products

5.1. Abstract

Previous data from investigations into a streptozotocin (STZ) rat model of diabetes has shown a significant decrease in the susceptibility to mechanical damage with treatment (Chapters 3 and 4). While it has been speculated that this change is due to the presence of advanced glycation end-products (AGEs) in the structure, this has not been verified. As such, AGEs were quantified in tail tendons from STZ-treated rats and from healthy controls using spectrofluorimetry to measure the concentration of fluorescing collagen in the tissue. The results showed no significant difference between the groups ($p = 0.15$). A concentration standard curve was also generated, which serves as evidence of the validity of the assay. Following this experiment, the exact mechanisms behind the effects of STZ and diabetes remain unknown and in need of elucidation if a greater understanding of tendon damage - and the prevention and treatment thereof - is to be developed.

5.2. Introduction

Background information relevant to the experiment and the means of investigation will be discussed here.

5.2.1. Problem Identification

In two previous experiments (Chapters 3 and 4) it was found that steady-state strain rate (SSSR) - a property of a material that quantifies its susceptibility to fatigue damage - decreases significantly in rat tendon from a streptozotocin model of diabetes. This has relevance for research into tendon damage and the effect that diabetes has on it. However, it has been taken for granted that the samples had been affected by STZ in the same way that tendons are altered by diabetes in vivo. Specifically, it has been assumed

that advanced glycation end-products were present, and that the mechanical changes seen so far can be attributed to these. In order to gain a greater understanding of the exact microstructural processes that cause increased susceptibility to fatigue damage, both in vivo and in tendons from this particular model, this AGE presence will need to be confirmed. If significant levels are indeed present it will support the theory that these crosslinks caused the measured changes. If not, a different potential explanation should be sought.

5.2.2. The Effect of STZ and of Diabetes

The exact mechanisms that cause the change in SSSR seen in STZ samples have not yet been established. It has been theorised that AGEs, the presence of which increases in diabetic tendons (as well as in those of advanced age), cause these effects by creating stronger intermolecular crosslinks (Thorpe et al., 2010; Eriksen et al., 2014). These reduce the amount of sliding that takes place between molecules (Fessel et al., 2014) and increase failure stress and strain (Reddy, 2004; Thorpe et al., 2015b). This, in turn, disrupts the natural mechanobiological processes related to healthy collagen turnover (Kjær et al., 2005; Abate et al., 2009) and thus increases the incidence of tendinopathies (Lui, 2017). However, various other processes may also affect the diabetic tendon and/or the STZ-treated tendon, and the questions surrounding cause-and-effect have not yet been definitively answered. A greater understanding of the role AGEs and other processes play in affecting the tendons of diabetic patients can have considerable real-world impact: already, clinically-approved non-invasive diagnostic and monitoring devices exist that use skin autofluorescence to measure patients' AGE levels (Meerwaldt et al., 2004). An improved ability to interpret this data will help inform clinicians about a patient's status and their risk of cardiovascular disease, atherosclerosis, kidney disease and diabetes-related illness (Lutgers et al., 2009).

5.2.3. The Streptozotocin Rat Model of Diabetes

The model in question was developed by Mansor et al. (2013). It uses rats that have been fed with a high fat diet for three weeks and injected with STZ (25 mg/kg) after the second

week. This protocol replicates Type 2 diabetes by inducing symptoms and co-morbidities associated with that disease: increased blood glucose levels, increased cholesterol (Mansor et al., 2013), impaired insulin secretion from the β -cells of the pancreas (Schmedl et al., 1994; Reed et al., 2000), obesity (following the high fat diet) and, possibly, weight loss (following STZ injection). It is thus an attractive model for use in studies of diabetes although, as with all animal research, its ability to be generalised to humans is limited. This drawback must be weighed up against the advantages of using a rat model: cost-effectiveness, high turnover (rat maturity is reached within a few months), ease of sample harvesting, large quantities of samples (both in terms of rats and tendons) and a relatively low barrier to ethical approval.

5.2.4. Spectrofluorimetry

If electromagnetic radiation of a certain wavelength is directed towards a material with fluorescent properties, such as tendon, it will absorb and then emit a certain amount of that energy. This phenomenon occurs in a predictable way and, as such, can be measured and used to reveal information about the microstructure (Nahata, 2011). Of most importance to this investigation are collagen AGEs, the excitation and emission spectra of which are 370 and 440 nm respectively (Reiser, 1994). This knowledge can be used to measure their presence in prepared quantities of tendon.

5.2.5. Aim

To quantify the presence of advanced glycation end-products in tail tendons from a streptozotocin rat model of diabetes. This was performed using spectrofluorimetry. Such evidence is needed in order to help verify that the effects induced by this rat model are the same as those induced in humans by diabetes, and thus confirm its usefulness as a model of diabetes in tendon.

5.2.6. Hypothesis

The concentration of AGEs will be higher in the STZ-treated tendons than in the control samples.

5.3. Materials and Methods

In addition to the Written Protocol and Video Protocol which are included in Appendices H and I, respectively, the procedure followed during this investigation is briefly detailed here. This method is based upon that used by Li et al. (2013).

5.3.1. Sample Collection

Tails from STZ-treated adult (10-14 wks) male Sprague-Dawley rats (Harlan/Envigo, UK) that had been culled for unrelated experiments (on diabetic neuropathy: Newton et al. (2017)) were obtained, along with age-matched healthy controls. These were stored at -70°C for a maximum of five months (Lee and Elliott, 2017) until needed and were labelled in a way that kept the experimenter blind as to the group of each. A sample size calculation was performed (Chow et al., 2008) using the results of a previous experiment that had used the same method (Li et al., 2013). This indicated that $n = 5$ for each group (i.e. ten samples in total) would provide sufficient power for statistical significance. However, as the experiment would be run blind, it was decided to test 20 samples in order to increase the probability that at least five from each group would be tested. Additionally, two samples - one from each group - were tested separately in order to create a concentration standard curve (as detailed in Section 5.3.6).

5.3.2. Apparatus and Materials

Before testing began, the following items were obtained and/or prepared:

- Hydrochloric acid (HCl), 6M concentration
- Phosphate buffered saline (PBS), 10X and 1X concentrations
- Liquid nitrogen supply
- Safe-lock Eppendorf tubes (Sigma-Aldrich), each of known mass
- Hot block for use with Eppendorf tubes (Techne)
- Centrifuge (Spectrafuge, Labnet)

- Microplate spectrometer (FLUOstar Omega, BMG Labtech)

Training in order to handle the liquid nitrogen and use the microplate spectrometer was obtained.

5.3.3. Day One

Following the defrosting of five tails (~30 min), five tendons were harvested from each and stored in PBS until needed. Each group of five tendons was placed into a mortar and flash frozen with liquid nitrogen before being ground with a pestle. The material was then transferred into an Eppendorf tube after which drops of HCl were added via pipette (sufficient to completely cover the material). The tubes were then closed and placed into the hot block for 24 hours at 110°C.

5.3.4. Day Two

The hot block was turned down to 35°C and the lids of the tubes opened. Another 24 hours were then allowed to pass.

5.3.5. Day Three

With all moisture now evaporated from the tubes, each was weighed in order to obtain the sample mass (current mass of tube, including sample, minus original mass). For each tube, 1 ml of 1X PBS was added for every 14 mg of sample it contained in order to create suspensions. These were then centrifuged for 10 min at 1500 g. Three aliquots of each supernatant were created in a 96 well plate along with H₂O and PBS wells for reference. These were then placed into the microplate reader and assayed for AGE fluorescence (excitation wavelength of 370 nm, emission wavelength of 440 nm (Li et al., 2013)). The entire process was then repeated an additional three times after which all 20 samples had been tested.

5.3.6. Concentration Standard Curve

The amount of HCl that needed to be added to each sample was not specified and it was not known if this quantity would affect the result. As such, ten additional groups

of tendons from two tails (five from an STZ-treated tail and five from a control tail) were also tested, each having either 5, 10, 15, 20 or 25 ml of HCl added per g of tendon. These results formed a concentration standard curve to elucidate the effect of HCl on the measured value of fluorescence.

5.3.7. Analysis

The fluorescence values measured by the spectrometer were not calibrated and as such are reported as ‘arbitrary fluorescence units’ per mass of sample (aFU/mg). The fluorescence readings for each of the three aliquots were summed before dividing by sample mass. The primary results (i.e. not those from the concentration standard curve) were tested for Normality and the STZ-treated samples failed, implying that the results in general could not be considered Normal. As such, a Mann-Whitney U test (which does not assume a distribution) was used to test significance (Graziano and Raulin, 2014). $p < 0.05$ was taken as significant. Outlier identification was performed using Chauvenet’s criterion and no outliers were found. The greatest source of experimental uncertainty related to the use of the pipette: 0.05 ml.

5.4. Results

Following all testing the results were collated and are shown in Table 5.1. Upon removal of the experimental blind it was revealed that 12 samples had been from the STZ-treated group with the remaining 8 having been controls. Uncertainty due to the use of the pipette amounted to between 2.3% and 7% of the results, depending on the mass of the sample in question.

Table 5.1: Data reported as median $\pm$ IQR with significance (Mann-Whitney U test)

Fluorescence [aFU/mg]			
	STZ	Control	Significance
Tail	429 $\pm$ 94.1	464 $\pm$ 104	$p=0.15$

The results are also shown in Box-and-Whisker form in Figure 5.1a. The difference between the groups was not significant. The concentration gradient is shown in Figure 5.1b. Neither of the groups show a significant relationship with the quantity of acid used, and this lack of correlation becomes more evident when both groups are considered together.

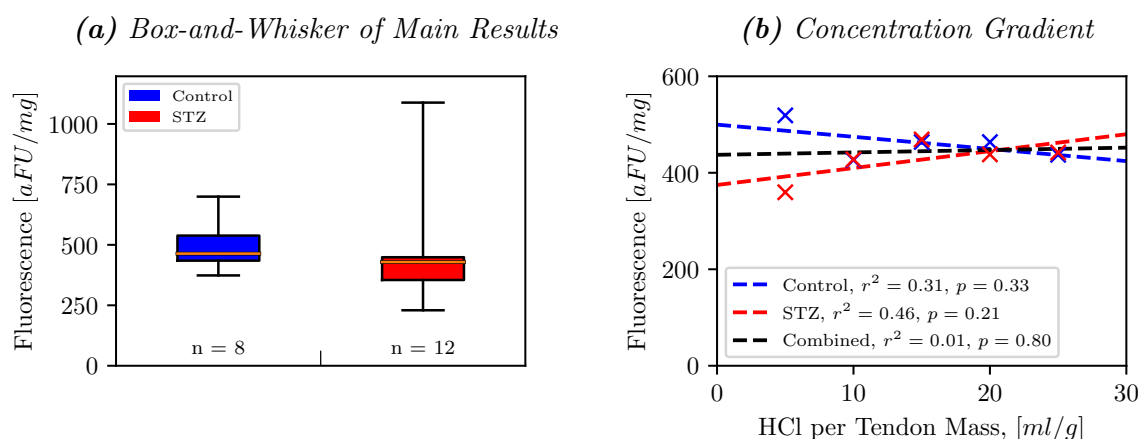


Figure 5.1: The fluorescence (in ‘arbitrary fluorescence units’) per unit mass due to AGE presence in STZ-treated and control tail tendons. The results from 20 tendons are shown in (a) and those from two tendons, divided into five samples each and treated with various quantities of HCl, are shown in (b). In the latter, trend lines have been added for both sets of data as well as an additional (black) line fitted to all points.

5.5. Discussion

The results showed no significant difference in fluorescence between STZ-treated and control tendons, and no relationship between the amount of HCl and fluorescence. As the level of uncertainty (2.3% to 7%) is small relative to the interquartile ranges (22% for both groups) it can be dismissed as inconsequential.

5.5.1. Quantification of AGEs

The lack of significance implies that there is no difference in the amount of AGEs present in the two types of tendon. This contradicts the hypothesis of this experiment. It also contradicts the assumptions that were made about what mechanistic effect STZ has on tendon in this particular model of diabetes.

5.5.2. Concentration of Hydrochloric Acid

As the abovementioned results are unexpected it is useful to seek confirmation that the assay used in this experiment is indeed accurate. For this reason, a concentration gradient was set up, shown in Figure 5.1b. This involved testing five groups of tendons from the same tail, each using a different quantity of hydrochloric acid (HCl) in the relevant step of the protocol, for both an STZ-treated and a control tail. In order to quantify the existence of a relationship between HCl and fluorescence a statistical test was performed on the results which, if significant, would have indicated such. However, the opposite was seen: both STZ and control samples returned low significance levels ($p = 0.21$ and $p = 0.33$, respectively) and, indeed, all data points combined returned a particularly low significance ($p = 0.80$) together with a low coefficient of determination ($r^2 = 0.01$) which actively suggests a lack of a correlation. This supports the accuracy of the assay. Additionally, the STZ and control results within this concentration gradient did not differ considerably, reinforcing the main results. An alternative method of confirming the reliability of the assay would have been to repeat the experiment with a commercially-available standard assay and/or to have performed it on samples with a known quantity of AGEs. The various sets of results could then have been compared. However, as it was felt that the protocol had been set up and carried out to a satisfactory level of accuracy, it was deemed that such measures were not necessary.

5.5.3. Implications

It is now not clear what mechanisms have caused the results seen in the previous two experiments to occur. One possible explanation is that the viscous components of the tendon (e.g. the ground substance) are affected differently by the hyperglycaemia in vivo than the collagenous components are. A major difference between the rat model of diabetes being considered here and diabetes in a human patient is the length of time over which the disease acts - a few weeks in the rats vs years in a human (notwithstanding the possibility that a few weeks for a rat might be equivalent to many years in a human (Sengupta, 2013)). It may be the case that the increased levels of glucose affect the ground substance - interacting with the glycosaminoglycans (GAGs) present in it - more

quickly that it affects the collagen. This could be due to it having the ability to diffuse through the liquid more easily than through the solid. Alternatively, it may be that these GAGs are simply more reactive to glucose (or more plentiful) and are thus affected first. Given more time, it may indeed be the case that AGE levels in the collagen increase. The idea that the viscous components are especially affected is supported by the results of Chapters 3 and 4: the tail tendons showed significant changes in viscosity but not in the Kelvin-Voigt or Maxwell stiffnesses due to STZ in Chapter 3 (Figure E.2). Similarly, while changes in all three of these measurements were significant in Chapter 4 (Figure 4.8) there was a considerable increase in significance of the rate of change of viscosity in STZ samples compared to controls while the significance levels of the two stiffnesses did not change as drastically (Figure 4.6). The Achilles tendons in Chapter 3 were, conversely, not affected in this way and as such they likely respond differently to STZ. It may be, for example, that the larger size of the Achilles increases the diffusion distance from blood vessels to deep fibrils to an influential degree. It is not known if performing spectrofluorimetry on these would produce an AGE difference due to STZ or not.

Alternatively, it is possible that direct chemical effects of glucose on structural matrix proteins are not the mechanism behind the measured effects at all. One theory suggests that disruptions to the mechanobiological and homeostatic metabolic processes have significant effects (Birch, 2007; Magnusson et al., 2010). The increased glucose levels in the tendon may inhibit or mask the mechanical or cellular behaviour in such a way as to cause one or both of these disruptions, with no (or merely an incidental) change in AGEs. Alternatively, molecular denaturation (with or without associated kink formation as would occur in dynamic and static fatigue, respectively) and/or biological damage from cell cleavage (marked by the presence of matrix metalloproteinases - MMPs) might be the most influential mechanism(s). If this is indeed the case it may be that damage ‘operates’ on a hierarchical level below that of AGEs. Regardless, it is unlikely that the unexpected results of this experiment are due to the model of diabetes being poor; it is well established that STZ injection coupled with a high fat diet induces an accurate diabetes-like state (modelling either Type 1 or Type 2 depending on whether the STZ dose is high or low) (Mansor et al., 2013). Certainly, it is not the STZ per se that is

causing the measured effects, but rather the induced hyperglycaemia (without ketosis) and normoinsulinaemia following compromise of the pancreatic β -cells.

5.5.4. Future Research

The same protocol can be repeated on Achilles tendons from the same two groups to confirm the results in functionally distinct tendons. In terms of seeking an alternative explanation to AGEs, X-ray diffraction can be used to investigate the micro- and nanostructures, the deformation mechanisms and the mechanical properties of STZ and control tendons and their responses to damage (fatigue and otherwise) (Bianchi et al., 2016). Additionally, the amount of GAGs present in the tendon and/or their reactivity with glucose could be quantified. Finally, given the aforementioned possibility that the time over which AGEs were allowed to develop might be relevant, the rate constant of the Maillard reaction should be investigated in rat models and compared to that measured in humans. This reaction is what creates AGEs (Monnier et al., 1992) and, if the time constants are significantly different, it may explain the difference in their presence.

5.6. Conclusions

The presence of advance glycation end-products has been quantified, using spectrofluorimetry, and found to not be significantly different in tail tendons from an STZ rat model of diabetes compared to controls ($p = 0.15$). The previously-measured effects of this model on this material might thus not be due to these crosslinks. Alternatively, they may indeed be due to the AGEs but via a different mechanism. The accuracy of the assay was confirmed via a concentration gradient which showed no relationship between HCl quantity and fluorescence.

6. Discussion and Conclusions

Following the literature review and the three investigations that have been performed, this chapter summarises the outcomes and conclusions that can be drawn.

6.1. Steady-State Strain Rate

This has been shown to be lower in tendons treated with STZ (Figure 3.6a), in tendons with an energy-storage-related functional role (Figure 3.6a) and in tail tendons with a lower absolute rate of change of loading modulus (significant in control tail tendons only; Figure 4.5a). These results suggest that SSSR is an indicator of susceptibility to damage in tendon. However, it appears as if STZ has a masking effect on damage properties, causing tail tendons' properties to become more similar to those of Achilles tendons and also causing them to be less closely related to the rate of strain compared to healthy samples. With regards to the first of these - the effect of a difference in functional role being masked - it is possible that STZ simply affects one type of tendon more than the other (and the results suggest that this would be positional tendons). With regards to the latter - the relationship between damage (defined as the rate of decrease of loading modulus) and steady-state strain rate being masked - an explanation can be found in the fact that the rate of change of elastic (initial) strain remains highly significant in STZ samples (Figure 4.6c). This is because damage is probably more closely related to strain than it is to stress - something which has already been proposed in the literature (Wren et al., 2001; Fung et al., 2009) - and it may also be that STZ affects the viscoelastic properties more than it does the elastic ones. This latter assertion is supported by STZ having affected viscosity more than Kelvin-Voigt or Maxwell stiffness in tail tendons during the static fatigue tests (Figures E.2b, d and f). This explanation for the loss of correlation between SSSR and rate of change of loading modulus with STZ is presented more clearly in the next section, together with Figure 6.1.

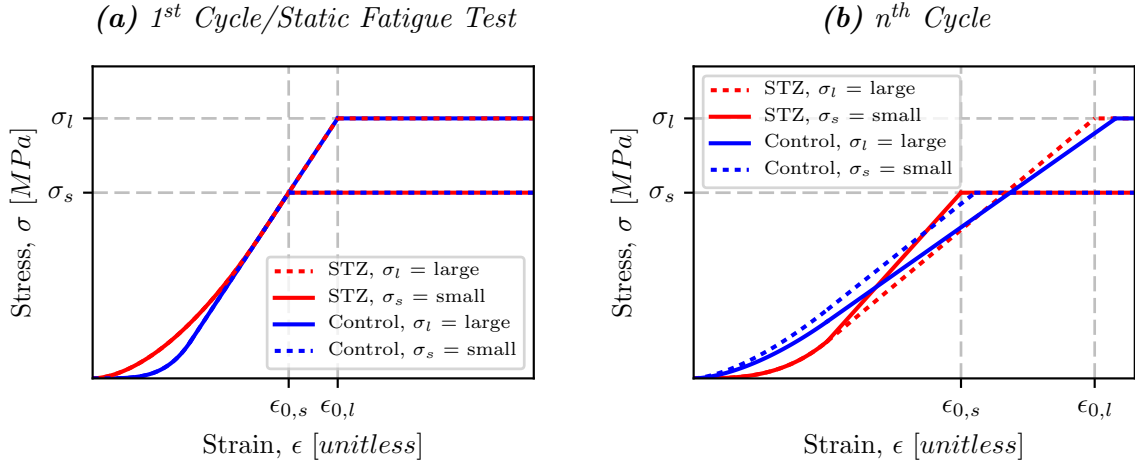


Figure 6.1: Illustration of the ‘masking’ effect of STZ treatment

6.1.1. Correlation Between SSSR and $\frac{d}{dt}$ Loading Modulus

Consider four hypothetical tail tendons - two STZ-treated and two controls - each loaded over a short period of time to either a large or a small creep stress (σ_l or σ_s where $l = large$ and $s = small$), see Figure 6.1. In the first loading cycle of an interrupted fatigue test (or equivalently, in the only loading cycle of a traditional static fatigue test), shown in 6.1a, all four have the same loading modulus (as per Figure 3.6c) but the STZ samples have a larger toe-heel region due to their larger viscoelastic effects and larger viscoelastic time constants (which imply that the initial responses will be stiffer than those of the control samples) (Figures E.2a and b). Both heavily loaded samples have the same elastic strain ($\epsilon_{0,l}$) as do both lightly loaded samples ($\epsilon_{0,s}$) with the latter being smaller than the former (again, $l = large$ and $s = small$). After an arbitrary number of cycles, n , as shown in 6.1b, all four samples have larger elastic strains and lower moduli although these changes are greater in the more heavily loaded samples (as per Figures 4.6c and 4.5a). Additionally, the increases in elastic strain are proportional to the amount of damage each sample has sustained (as rate of change of elastic strain is proportional to SSSR - Figure 4.6c - which represents susceptibility to fatigue damage - Section 4.6 - and which is higher in more heavily load samples - Figure 4.3) which is why these are slightly larger in the control samples. For the control samples, the decreases in loading moduli are also proportional to damage (Figure 4.5a) while the changes in the toe-heel regions are arbitrary: the elastic strains and the loading moduli are the driving forces behind the shapes of the graphs at

this point. This is because they are both determined by the amount of damage that has been sustained while the toe-heel region (i.e. the short-term viscoelastic effects) can be whatever it needs to be in order for this shape of graph to occur. For the STZ samples, however, the treatment has affected the viscous properties (the manifestations of this can be seen in the long-term viscoelastic effects shown in Figures 4.6a and e - which are both strongly independent of SSSR - Figure 4.6b and Figures E.2a and b) and thus these are now a driving force of the graphs' shapes. Elastic strain is also, still, an influential factor in graph shape (because damage is probably strain-based, as discussed in Section 4.5) which means that loading modulus (which is both stress- and strain-based, hence why it is less closely associated with damage than elastic strain is) has only enough influence for its rate of change to merely be approximately proportional to damage (and thus to SSSR). It has been 'masked' by STZ.

6.2. The Effect of STZ

The previous section discussed how STZ masks some of the phenomena seen in control tendons. The mechanism behind this masking effect might be a strengthening of the covalent bonds between components of the ground substance such as proteoglycans and GAGs - similar to what, in Chapter 5, was hypothesised to be occurring in the collagenous matrix due to AGEs. Indeed, an increase in the stiffness of the interfibrillar matrix due to STZ has already been reported (de Oliveira et al., 2017). As the ground substance is unlikely to be affected by fatigue damage relative to the collagen (mechanical damage is not usually associated with a liquid) this would explain why the rates of change of the viscoelastic time constant and of viscous strain are independent of damage (Figures 4.6a and e) while the rate of change of viscosity (which represents the transfer of load from the ground substance to the gradually more damaged collagen; Figure 4.6b) is. STZ affecting the ground substance more than the collagen would also explain the different toe-heel regions but similar loading moduli in Figure 6.1a. Note that the 'effect of STZ on tendon' is actually an indirect effect: STZ causes the pancreas to produce less insulin which, coupled with a high-fat diet, induces hyperglycaemia in the rats. The increased

blood sugar then perfuses into the tendons and affects them from there - it is not the STZ that affects them per se.

6.3. Tendon Damage

While it has been established that SSSR reveals information about susceptibility to fatigue damage, this is not the only concept potentially related to damage out of those discussed. Permanent strain, for example, has been discussed in Section 4.5 and is loosely correlated with SSSR; it is probably associated with damage but not as strongly as loading modulus. The point of inflection, on the other hand, has been claimed to be the ‘point of balance’ between the net effect of a load being damaging or recoverable. In STZ-treated samples the PoI occurs significantly later (Figure 3.6d) but at the same levels of strain and strain energy (Figures 3.6f and b). If it were the case that strain and strain energy were related to damage - which, following what has been discussed in each chapter, is indeed likely - then this would suggest that a tendon becomes ‘net damaged’ once it has absorbed a certain amount of strain energy/strained to a certain point (given that stress is constant in a static fatigue test, these two are equivalent). The decrease in susceptibility to damage caused by STZ treatment would thus be by virtue of it delaying the PoI, with SSSR then decreasing as a knock-on effect of this delay. However, the rates of change of strain energy, time and strain at the PoI do not correlate consistently with SSSR: indeed, STZ causes them to reverse their correlations from negative to positive (Figures 4.5b, d and f). This is possibly, again, due to STZ affecting the ground substance. In healthy samples, high loads/larger SSSRs/larger susceptibilities to damage (these three concepts being roughly equivalent) are significantly correlated with a decrease in the time at the PoI and not correlated with strain or strain energy at the PoI (although there is a marginal negative correlation with both), which is as expected. The marginal negative correlations may be due to the kinking that likely occurs during unloading: this has the effect of introducing extra damage without an increase in strain or strain energy (thus keeping these values artificially low) and it is known that higher loads introduce kinks more readily (Veres et al., 2013a) (which keeps these values especially low). After STZ treatment, however,

the affected ground substance now causes the rates of change of the viscoelastic properties (viscoelastic time constant and viscous strain) to be independent of load (Figures 4.6a and e) and thus the viscoelastic response is independent of load. Thus, while the time at the PoI is still delayed compared to healthy samples, it also becomes independent of load. This means that net damage is being reached after the same amount of time regardless of load, and thus the correlation seen in healthy samples in Figure 4.5d disappears. Also, as viscous strain is not independent of load, the strain at the PoI is largely determined by the initial elastic strain which, understandably, would be higher for higher loads. Hence the positive correlation in Figure 4.5f. The combined effect of time and strain at the PoI being independent of and positively correlated with load, respectively, is necessarily that strain energy at the PoI is positively correlated with load, which is indeed seen in Figure 4.5b.

6.4. Limitations and Future Research

While it has effectively been found that STZ decreases a tendon's susceptibility to damage, this is at odds with the clinical observation that diabetic patients are more prone to tendon injuries (Abate et al., 2013). One possibility is that tendon damage is actually healthy in regular, small amounts as it catalyses collagen turnover and cell activity (Kjær et al., 2005; Abate et al., 2009), and thus decreasing susceptibility to it decreases the mechanobiological healing response. An experiment might thus be designed to investigate this using a bioreactor: as opposed to dealing with the tipping point between the recoverable response and the damage response of a tendon under creep load (i.e. the point of inflection) the tipping point between the healthy and damaging processes that occur over time in a living material due to load could be looked at. The repair process can also be investigated by performing in vivo experiments, using factors such as diabetes but also exercise (which is also known to influence the repair process (Langberg et al., 2002)) as variables.

Additional sources of uncertainty associated with the experiments include their use of an animal model - as discussed elsewhere (Section 2.8.3) - and their use of the fairly simple

standard linear solid model (SLSM) which may have hidden some of the complexities of the tendons' responses. The decision to use a model of viscoelasticity, which the SLSM is, may also not have been the most accurate (although it is commonly done (Fung, 2004)) when, for example, a poroelastic model may possibly have been better. Such a model would be built on the fact that tendon is comprised mostly of a collagenous matrix (which behaves elastically) and water (which is virtually incompressible) (Reese and Weiss, 2013). When tension is applied to this model the matrix starts to deform and the water starts to flow out of it (similar to a towel being wrung), but viscosity slows this flow down and absorbs energy in doing so. As such, there is a large resistance to this deformation in the short term. This is a 'shock absorber' mechanism; a sudden change in load will be 'carried' partly by water, protecting the matrix from potential damage. However, as time goes on, the pressurised water will continue to flow out of the structure whereafter this effect is no longer seen. This time-dependent response is the viscoelasticity - a viscous substance acting as a damper to an otherwise elastic material.

Another aspect that could be addressed in future work is the testing of additional collagenous material. It is not necessarily the case that the results generated here will be seen in all sample types, and other potential materials include bladder (relevant to urodynamics), horse flexor and extensor tendon (frequently damaged and commercially relevant) and human material (including patellar, rotator cuff and bicep tendon, all of which are frequently injured).

Additionally, the apparatus that was used did not allow for true values of stress to be applied during the test. Instead, engineering values of stress were used which likely caused the samples to fail artificially early. Although a conversion was made from engineering to true values during the data analysis processes this was post-hoc and the samples had already been affected. Data more representative of the materials' mechanical structures might thus be obtainable if live measurement of the samples' diameters was performed (e.g. using computer vision) and used in a feedback loop to control the applied force. It may be that samples would not fail at all under such a protocol; the long time-dependant viscoelastic mechanisms (fibril sliding and the movement of water) might

naturally dissipate if true stress was constant. Options for this type of more advanced programming of the control software should be explored; specifically, an upgrade should be sought that will allow for scripts written in the ZIMT (Zwick Interpreter for Materials Testing) language to be utilised.

Finally, various other methods of investigation have the potential to be useful when exploring the tendon properties discussed here, including x-ray diffraction and multi-photo microscopy (imaging methods used to characterise the multi-scale properties of the structure) coupled with a traditional dynamic fatigue loading regime. Such an investigation may have the potential to elucidate the exact mechanisms of STZ's effect of tendon and the influence of previously-discussed complications such as kinking, fibril disorder, ground substance behaviour and differing functional roles (both with and without STZ treatment). Similarly, such methods may help reveal more information about the nature of cell-matrix interactions; this is an area of prime importance if new strategies for tendon healing and repair are to be developed (Screen et al., 2015).

6.5. Conclusions

Insight into the mechanisms behind, and the markers of, tendon damage and its causes has been gained. This was done by considering a rat model of diabetes and its effects on the properties of tail and Achilles tendons. Additionally, the following conclusions have been drawn:

- Steady-state strain rate is a property of a tendon that quantifies its susceptibility to fatigue damage
- The streptozotocin rat model of diabetes decreases the susceptibility of a tendon to fatigue damage
- The mechanisms of the effects of STZ treatment are not associated with any change in measurable AGE concentration. This does not, however, imply that the same is true for the mechanisms of the effects of diabetes in humans.

- An energy-storing functional role decreases the susceptibility to fatigue damage
- The standard linear solid model is adequate when greater insight into the material behaviour is desired
- Following the literature review, it is concluded that ‘tendon damage’ has no categorical definition although phenomenological markers (e.g. a decrease in loading modulus) are often used to represent it. Ex vivo, strain can be recoverable (viscoelastic) with the point of inflection representing the balance between this response and the damage (permanent) response. In vivo, a healing process exists which leads to ‘healthy’ and ‘unhealthy’ loading regimes both being possible.

6.6. Implications

Following the outcomes of this research a number of suggestions can be made, in various areas of tendon work:

- Basic science: SSSR is a new tool/experimental parameter that allows experiments that interrogate susceptibility to clinically-important damage and the functional differences between tendons to be designed (by including comparisons of the SSSRs of different samples)
- Solid mechanics: the rate at which strain occurs represents susceptibility to fatigue damage and should be quantified. This has relevance for material science and can be used when developing new composites and biomaterials.
- Ex vivo experimentation: fatigue damage is vital to the understanding of tendon damage; investigations into damage cannot be comprehensive unless fatigue is considered
- Diabetic models: the short time over which STZ acts in this particular rat model might limit its relevance to diabetes. The time constant of the Maillard reaction should be investigated in this regard.

- Experimental design: research into how tendon injuries are treated, and how their risk factors are addressed, should use strain rate as one of their measures of success
- Medical trials: treatments for tendinopathy could be assessed using changes in tendon strain rate
- Tissue engineering (bioreactors): bioreactors should be calibrated so as to develop tendons with similar strain rate properties as those found in healthy samples
- Tissue engineering (scaffolds): it may be that scaffolds used in tendon regenerative medicine could benefit from having the same steady-strain rate and/or susceptibility to damage as healthy tendons. Specifically, it may help with the seeding of stem cells onto the scaffolds - this is a process that involves cyclic mechanical stimulation (Butler et al., 2010) - and thereby improve the mechanical properties of the tissue produced. Scaffolds' SSSRs might be controllable by varying the degree to which their molecules are crosslinked.
- Cell biology: using the mechanical properties of extracellular matrix as a way of modulating cell behaviour for the purpose of targeting diseases is already being considered (Lampi and Reinhart-King, 2018)
- Biochemistry: the susceptibility of patients, especially diabetics and the elderly, to tendon damage could be reduced by treatments that target AGE formation, AGEs themselves, the glycation process or a combination of these
- Clinical work: SSSR should be measured in patients - using ultrasound (Hsiao et al., 2015) or magnetic resonance elastography (Cortes et al., 2015) - in order to aid the diagnosis and monitoring of tendinopathies. 'Healthy' values of SSSR should be established ('healthy rates of damage accumulation') (Magnusson et al., 2010) as it is known that both beneficial and detrimental rates of strain can exist (see 'Disrupted Mechanobiology' in Section 2.4.3)

Bibliography

- Abate, M., Gravare-Silbernagel, K., Siljeholm, C., Iorio, A. D., Amicis, D. D., Salini, V., Werner, S., and Paganelli, R. (2009). Pathogenesis of tendinopathies: inflammation or degeneration? Arthritis Research and Therapy, 11(3):235.
- Abate, M., Salini, V., Antinolfi, P., and Schiavone, C. (2014). Ultrasound morphology of the Achilles in asymptomatic patients with and without diabetes. Foot & ankle international, 35(1):44–9.
- Abate, M., Salini, V., and Schiavone, C. (2016). Achilles tendinopathy in elderly subjects with type II diabetes: the role of sport activities. Aging Clinical and Experimental Research, 28(2):355–358.
- Abate, M., Schiavone, C., and Salini, V. (2010). Sonographic evaluation of the shoulder in asymptomatic elderly subjects with diabetes. BMC Musculoskeletal Disorders, 11(1):278–284.
- Abate, M., Schiavone, C., and Salini, V. (2012). Neoangiogenesis is reduced in chronic tendinopathies of type 2 diabetic patients. International Journal of Immunopathology and Pharmacology, 25(3):757–761.
- Abate, M., Schiavone, C., Salini, V., and Andia, I. (2013). Occurrence of tendon pathologies in metabolic disorders. Rheumatology, 52(4):599–608.
- Abdi, H. (2010). Coefficient of Variation. In Salkind, N., editor, Encyclopedia of Research Design. Sage, Thousand Oaks, CA.
- Abrahams, M. (1967). Mechanical behaviour of tendon In vitro - A preliminary report. Medical & Biological Engineering, 5(5):433–443.
- Abramowitch, S. D., Clineff, T. D., Withrow, J. D., Papageorgiou, C. D., and Woo, S. L.-y. (1999). The Quasi-Linear Viscoelastic Properties of the Healing Goat Medial Collateral Ligament: An Experimental & Analytical Approach. In 23rd Annual Meeting of the American Society of Biomechanics, Pittsburgh, PA.
- Ackerman, J. E., Geary, M. B., Orner, C. A., Bawany, F., and Loiselle, A. E. (2017). Obesity/Type II diabetes alters macrophage polarization resulting in a fibrotic tendon healing response. PLoS ONE, 12(7).
- Ackermann, P. W., Li, J., Finn, A., Ahmed, M., and Kreicbergs, A. (2001). Autonomic innervation of tendons, ligaments and joint capsules. A morphologic and quantitative study in the rat. Journal of Orthopaedic Research, 19(3):372–378.
- Ahmed, A. S., Schizas, N., Li, J., Ahmed, M., Östenson, C.-G., Salo, P., Hewitt, C., Hart, D. A., and Ackermann, P. W. (2012). Type 2 diabetes impairs tendon repair after injury in a rat model. Journal of Applied Physiology, 113(11):1784–91.
- Akturk, M., Ozdemir, A., Maral, I., Yetkin, I., and Arslan, M. (2007). Evaluation of Achilles tendon thickening in type 2 diabetes mellitus. Experimental and Clinical Endocrinology & Diabetes, 115(2):92–6.
- Alexander, R. M. (1991). Energy-saving mechanisms in walking and running. The Journal of Experimental Biology, 160(1):55–69.
- Alexander, R. M. and Bennet-Clark, H. C. (1977). Storage of elastic strain energy in muscle and other tissues. Nature, 265(5590):114–117.
- Allenmark, C. (1992). Partial Achilles tendon tears. Clinics in Sports Medicine, 11(4):759–769.
- Amiel, D., Frank, C. B., Harwood, F., Fronek, J., and Akeson, W. H. (1984). Tendons and ligaments: a morphological and biochemical comparison. Journal of Orthopaedic Research, 1(3):257–265.
- Andreassen, T. T., Seyer-Hansen, K., and Bailey, A. J. (1981). Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. Biochimica et Biophysica Acta (BBA) - General Subjects, 677(2):313–7.
- Aparecida de Aro, A., de Campos Vidal, B., and Pimentel, E. R. (2012). Biochemical and anisotropical properties of tendons. Micron, 43(2):205–214.
- Araki, N., Ueno, N., Chakrabarti, B., Morino, Y., and Horiuchi, S. (1992). Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. The Journal of biological chemistry, 267(15):10211–4.

Bibliography

- Archambault, J. (2003). Tendon micromechanics and research methods in tendinopathy. Journal of Musculoskeletal Neuronal Interactions, 3(4):326–328.
- Arnoczky, S. P., Lavagnino, M., and Egerbacher, M. (2007). The mechanobiological aetiopathogenesis of tendinopathy: is it the over-stimulation or the under-stimulation of tendon cells? International Journal of Clinical and Experimental Pathology, 88:217–26.
- Arnoczky, S. P., Tian, T., Lavagnino, M., and Gardner, K. (2004). Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism. Journal of Orthopaedic Research, 22:328–333.
- Avery, N. C. and Bailey, A. J. (2005). Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. Scandinavian Journal of Medicine & Science in Sports, 15(4):231–240.
- Avery, N. C. and Bailey, A. J. (2006). The effects of the Maillard reaction on the physical properties and cell interactions of collagen. Pathologie Biologie, 54(7):387–395.
- Baer, E., Hiltner, A., and Keith, H. D. (1987). Hierarchical Structure in Polymeric Materials. American Association for the Advancement of Science, 235(4792):1015–1022.
- Bai, P., Phua, K., Hardt, T., Cernadas, M., and Brodsky, B. (1992). Glycation alters collagen fibril organization. Connective Tissue Research, 28(1):1–12.
- Bailey, A. J., Robins, S. P., and Balian, G. (1974). Biological significance of the intermolecular crosslinks of collagen. Nature, 251:105–109.
- Baldock, C., Oberhauser, A. F., Ma, L., Lammie, D., Siegler, V., Mithieux, S. M., Tu, Y., Chow, J. Y. H., Suleman, F., Malfois, M., Rogers, S., Guo, L., Irving, T. C., Wess, T. J., and Weiss, A. S. (2011). Shape of tropoelastin, the highly extensible protein that controls human tissue elasticity. Proceedings of the National Academy of Sciences, 108(11):4322–4327.
- Baravarian, B. and Redkar, A. (2016). Emerging Innovations In Minimally Invasive Percutaneous Achilles Tendon Repair. Podiatry Today, 29(5).
- Barnard, K., Light, N. D., Sims, T. J., and Bailey, A. J. (1987). Chemistry of the collagen cross-links. Origin and partial characterization of a putative mature cross-link of collagen. The Biochemical Journal, 244(2):303–9.
- Batista, F., Nery, C., Pinzur, M., Monteiro, A. C., de Souza, E. F., Felipe, F. H. Z., Alcântara, M. C., and Campos, R. S. (2008). Achilles tendinopathy in diabetes mellitus. Foot & Ankle International, 29(5):498–501.
- Batson, E. L., Paramour, R. J., Smith, T. J., Birch, H. L., Patterson-Kane, J. C., and Goodship, A. E. (2003). Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? Equine Veterinary Journal, 35(3):314–318.
- Beaumont, P. W. R. (1989). The failure of fibre composites: An overview. The Journal of Strain Analysis for Engineering Design, 24(4):189–205.
- Bedi, A., Fox, A. J. S., Harris, P. E., Deng, X.-H., Ying, L., Warren, R. F., and Rodeo, S. A. (2010). Diabetes mellitus impairs tendon-bone healing after rotator cuff repair. Journal of Shoulder and Elbow Surgery, 19(7):978–988.
- Benjamin, M., Kaiser, E., and Milz, S. (2008). Structure-function relationships in tendons: a review. Journal of Anatomy, 212:211–228.
- Bennett, M. B., Ker, R. F., Imery, N. J., and Alexander, R. M. (1986). Mechanical properties of various mammalian tendons. Journal of Zoology, 209(4):537–548.
- Best, T. M., Collins, A., Lilly, E. G., Seaber, A. V., Goldner, R., and Murrell, G. A. (1993). Achilles tendon healing: a correlation between functional and mechanical performance in the rat. Journal of Orthopaedic Research, 11(6):897–906.
- Bianchi, F., Hofmann, F., Smith, A. J., and Thompson, M. S. (2016). Probing multi-scale mechanical damage in connective tissues using X-ray diffraction. Acta Biomaterialia, 45(August):321–327.
- Birch, H. L. (2007). Tendon matrix composition and turnover in relation to functional requirements. International Journal of Experimental Pathology, 88(4):241–8.
- Birch, H. L., Worboys, S., Eissa, S., Jackson, B., Strassburg, S., and Clegg, P. D. (2008). Matrix metabolism rate differs in functionally distinct tendons. Matrix Biology, 27(3):182–189.

- Boivin, G. P., Elenes, E. Y., Schultze, A. K., Chodavarapu, H., Hunter, S. A., and Elased, K. M. (2014). Biomechanical properties and histology of db/db diabetic mouse Achilles tendon. Muscles, Ligaments and Tendons Journal, 4(3):280.
- Bojsen-Møller, J. and Magnusson, S. P. (2015). Heterogeneous loading of the human Achilles tendon in vivo. Exercise and Sport Sciences Reviews, 43:190–197.
- Bonifasi-Lista, C., Lake, S. P., Small, M. S., and Weiss, J. A. (2005). Viscoelastic properties of the human medial collateral ligament under longitudinal, transverse and shear loading. Journal of Orthopaedic Research, 23:67–76.
- Bourne, J. W., Lippell, J. M., and Torzilli, P. A. (2014). Glycation cross-linking induced mechanical - enzymatic cleavage of microscale tendon fibers. Matrix Biology, 34:179–184.
- Bozec, L., van der Heijden, G., and Horton, M. (2007). Collagen fibrils: nanoscale ropes. Biophysical Journal, 92(1):70–75.
- Brennan, M. (1989). Changes in solubility, non-enzymatic glycation, and fluorescence of collagen in tail tendons from diabetic rats. Journal of Biological Chemistry, 264(35):20947–52.
- Brownlee, M. (1995). Advanced Protein Glycosylation in Diabetes and Aging. Annual Review of Medicine, 46(1):223–234.
- Brownlee, M., Cerami, A., and Vlassara, H. (1989). Advanced Glycation End Products in Tissue and the Biochemical Basis of Diabetic Complications. Journal of Medicine, 320(21):1362 – 1367.
- Brožek, J. (1966). Body composition: Models and estimation equations. American Journal of Physical Anthropology, 24(2):239–246.
- Bruehlmann, S. B., Matyas, J. R., and Duncan, N. A. (2004). ISSLS Prize Winner : Collagen Fibril Sliding Governs Cell Mechanics in the Anulus Fibrosus. Spine, 29(23):2612–2620.
- Bucci, L. R. (1994). Nutrition applied to injury rehabilitation and sports medicine. CRC Press, 4th editio edition.
- Burt, B. A. (2001). Definitions of Risk. Journal of Dental Education, 65(October):1007–1008.
- Butler, D. L., Gooch, C., Kinneberg, K., Boivin, G. P., Galloway, M. T., Nirmalanandhan, V. S., Shearn, J. T., Dymment, N. A., and Juncosa-Melvin, N. (2010). The use of mesenchymal stem cells in collagen-based scaffolds for tissue-engineered repair of tendons. Nature Protocols, 5(5):849–863.
- Calleja, M. and Connell, D. A. (2010). The Achilles tendon. Seminars in musculoskeletal radiology, 14(3):307–322.
- Calve, S., Dennis, R. G., Kosnik, P. E., Baar, K., Grosh, K., and Arruda, E. M. (2004). Engineering of functional tendon. Tissue Engineering, 10(5-6):755–61.
- Carr, A. J. and Norris, S. H. (1989). The Blood Supply of the Calcaneal Tendon. British Editorial Society of Bone and Joint Surgery, 71(B):100–101.
- Cheng, V. W. T. and Screen, H. R. C. (2007). The micro-structural strain response of tendon. Journal of Materials Science, 42(21):8957–8965.
- Chow, S., Shao, J., and Wang, H. (2008). Sample Size Calculations in Clinical Research. CRC Biostatistics Series, 2nd edition.
- Ciccone, W. J., Bratton, D. R., Weinstein, D. M., and Elias, J. J. (2006). Viscoelasticity and temperature variations decrease tension and stiffness of hamstring tendon grafts following anterior cruciate ligament reconstruction. The Journal of Bone and Joint Surgery - American Volume, 88(5):1071–8.
- Cohen, R. E., Hooley, C. J., and McCrum, N. G. (1976). Viscoelastic creep of collagenous tissue. Journal of Biomechanics, 9:175–184.
- Connizzo, B. K., Bhatt, P. R., Liechty, K. W., and Soslowky, L. J. (2014). Diabetes alters mechanical properties and collagen fiber re-alignment in multiple mouse tendons. Annals of Biomedical Engineering, 42(9):1880–8.
- Cook, J. L., Feller, J. A., Bonar, S. F., and Khan, K. M. (2004). Abnormal tenocyte morphology is more prevalent than collagen disruption in asymptomatic athletes’ patellar tendons. Journal of Orthopaedic Research, 22(2):334–338.
- Cooper, R. R., Misol, S., and Stimmel, P. (1970). Tendon and ligament insertion. A light and electron microscopic study. The Journal of Bone and Joint Surgery, 52(1):1–20.
- Cortes, D. H., Suydam, S. M., Silbernagel, K. G., Buchanan, T. S., and Elliott, D. M. (2015). Continuous Shear Wave Elastography: a New Method to Measure in-vivo Viscoelastic Properties of Tendons. Ultrasound in Medicine & Biology, 41(6):1518–1529.

Bibliography

- Couppé, C., Svensson, R. B., Kongsgaard, M., Kovanen, V., Grosset, J.-F., Snorgaard, O., Bencke, J., Larsen, J. O., Bandholm, T., Christensen, T. M., Boesen, A. P., Helmark, I. C., Aagaard, P., Kjær, M., and Magnusson, S. P. (2015). Human Achilles tendon glycation and function in diabetes. *Journal of Applied Physiology*, 9(23):130–7.
- Cowan, P. M., North, A. C. T., and Randall, J. T. (1955). X-ray diffraction studies of collagen fibres. *Symposia of the Society for Experimental Biology*, 9:115–126.
- Cronin, N. J., Peltonen, J., Ishikawa, M., Komi, P. V., Avela, J., Sinkjaer, T., and Voigt, M. (2010). Achilles tendon length changes during walking in long-term diabetes patients. *Clinical Biomechanics*, 25(5):476–482.
- Danielson, K. G., Baribault, H., Holmes, D. F., Graham, H., Kadler, K. E., and Iozzo, R. V. (1997). Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *The Journal of cell biology*, 136(3):729–43.
- de Oliveira, R. R., Bezerra, M. A., de Lira, K. D. S., Novaes, K. A., Teixeira, M. F. H. B. I., Chaves, C. d. C., and de Moraes, S. R. A. (2012). Aerobic physical training restores biomechanical properties of Achilles tendon in rats chemically induced to diabetes mellitus. *Journal of Diabetes and its Complications*, 26(3):163–8.
- de Oliveira, R. R., de Lira, K. D. S., Silveira, P. V. d. C., Coutinho, M. P. G., Medeiros, M. N., Teixeira, M. F. H. B. I., and de Moraes, S. R. A. (2011). Mechanical properties of achilles tendon in rats induced to experimental diabetes. *Annals of Biomedical Engineering*, 39(5):1528–1534.
- de Oliveira, R. R., Martins, C. S., Rocha, Y. R., Braga, A. B. R., Mattos, R. M., Hecht, F., Brito, G. A. C., and Nasciutti, L. E. (2013). Experimental diabetes induces structural, inflammatory and vascular changes of Achilles tendons. *PLoS ONE*, 8(10):e74942.
- de Oliveira, R. R., Medina de Mattos, R., Magalhães Rebelo, L., Guimarães Meireles Ferreira, F., Tovar-Moll, F., Eurico Nasciutti, L., and de Castro Brito, G. A. (2017). Experimental diabetes alters the morphology and nano-structure of the Achilles tendon. *Plos One*, 12(1):e0169513.
- Deshmukh, D. P. and Akarte, A. G. (2017). Musculoskeletal manifestations in type 2 diabetes mellitus. *International Journal of Research in Medical Sciences*, 5(2):398–402.
- Detournay, E. and Cheng, A. H.-D. (1993). Fundamentals of Poroelasticity. In Fairhurst, C., editor, *Comprehensive Rock Engineering: Principles, Practice and Projects*, volume IIA, chapter Chapter 5, pages 113–171. Pergamon Press, edition 3 edition.
- Di Lullo, G. A., Sweeney, S. M., Körkkö, J., Ala-Kokko, L., and San Antonio, J. D. (2002). Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *Journal of Biological Chemistry*, 277(6):4223–4231.
- Diabetes UK (2015). Facts and Stats. Technical report, Diabetes UK.
- Diabetes UK (2017a). Diabetes UK calls for more funding for diabetes research.
- Diabetes UK (2017b). Other types of diabetes.
- Diamant, J., Keller, A., Baer, E., Litt, M., and Arridge, R. G. C. (1972). Collagen; Ultrastructure and Its Relation to Mechanical Properties as a Function of Ageing. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 180(1060):293–315.
- Docheva, D., Müller, S. A., Majewski, M., and Evans, C. H. (2015). Biologics for tendon repair. *Advanced Drug Delivery Reviews*, 84:222–239.
- Drake, R. L., Vogl, A. W., and Mitchell, A. W. M. (2015). *Gray’s Anatomy for Students*. Churchill Livingstone, Philadelphia, PA, 3rd edition.
- Duncan, N. A., Bruehlmann, S. B., Hunter, C. J., Shao, X., and Kelly, E. J. (2014). In situ cell-matrix mechanics in tendon fascicles and seeded collagen gels: implications for the multiscale design of biomaterials. *Computer Methods in Biomechanics and Biomedical Engineering*, 17(1):39–47.
- Dunkman, A. A., Buckley, M. R., Mienaltowski, M. J., Adams, S. M., Thomas, S. J., Satchell, L., Kumar, A., Pathmanathan, L., Beason, D. P., Iozzoc, R. V., Birk, D. E., and Soslowsky, L. J. (2013). Decorin expression is important for age-related changes in tendon structure and mechanical properties. *Matrix Biology*, 33(1):3–13.
- Duquette, J. J., Grigg, P., and Hoffman, A. H. (1996). The Effect of Diabetes on the Viscoelastic Properties of Rat Knee Ligaments. *Journal of Biomechanical Engineering*, 118(4):557–564.
- Dyment, N. A. and Galloway, J. L. (2015). Regenerative Biology of Tendon: Mechanisms for Renewal and Repair. *Current Molecular Biology Reports*, 1(3):124–131.

- Edama, M., Kubo, M., Onishi, H., Takabayashi, T., Inai, T., Yokoyama, E., Hiroshi, W., Satoshi, N., and Kageyama, I. (2015). The twisted structure of the human Achilles tendon. Scandinavian Journal of Medicine and Science in Sports, 25(5):e497–e503.
- Egom, E. E.-A., Bae, J. S. H., Capel, R., Richards, M., Ke, Y., Pharithi, R. B., Maher, V., Kruzliak, P., and Lei, M. (2016). Effect of sphingosine-1-phosphate on L-type calcium current and Ca^{2+} transient in rat ventricular myocytes. Molecular and Cellular Biochemistry, 419(1-2):83–92.
- Eliasson, P., Fahlgren, A., Pasternak, B., and Aspenberg, P. (2007). Unloaded rat Achilles tendons continue to grow, but lose viscoelasticity. Journal of Applied Physiology, 103:459–463.
- Ely, E. R., Verheyen, K. L. P., and Wood, J. L. N. (2004). Fractures and tendon injuries in National Hunt horses in training in the UK: a pilot study. Equine Veterinary Journal, 36(4):365–7.
- Eriksen, C., Svensson, R. B., Scheijen, J., Hag, A. M. F., Schalkwijk, C., Praet, S. F. E., Schjerling, P., Kjær, M., Magnusson, S. P., and Couppé, C. (2014). Systemic stiffening of mouse tail tendon is related to dietary advanced glycation end products but not high-fat diet or cholesterol. Journal of Applied Physiology, 117(8):840–7.
- Etherington, D. J. and Bailey, A. J. (1982). Metabolism of rat skin collagen: the effect of arrested development and subsequent catch-up growth on the stability of the connective tissue fibers. Collagen and Related Research, 2(6):507–22.
- Fang, F. and Lake, S. P. (2016). Modelling approaches for evaluating multiscale tendon mechanics. Interface Focus, 6(1):1–13.
- Fessel, G., Li, Y., Diederich, V., Guizar-Sicairos, M., Schneider, P., Sell, D. R., Monnier, V. M., and Snedeker, J. G. (2014). Advanced Glycation End-Products Reduce Collagen Molecular Sliding to Affect Collagen Fibril Damage Mechanisms but Not Stiffness. PLoS ONE, 9(11):e110948.
- Fessel, G. and Snedeker, J. G. (2009). Evidence against proteoglycan mediated collagen fibril load transmission and dynamic viscoelasticity in tendon. Matrix Biology, 28(8):503–10.
- Folkhard, W., Mosler, E., Geercken, W., Knörzer, E., Nemetschek-Gansler, H., Nemetschek, T., and Koch, M. H. J. (1987). Quantitative analysis of the molecular sliding mechanisms in native tendon collagen - time-resolved dynamic studies using synchrotron radiation. International Journal of Biological Macromolecules, 9(3):169–175.
- Fox, A. J. S., Bedi, A., Deng, X.-H., Ying, L., Harris, P. E., Warren, R. F., and Rodeo, S. A. (2011). Diabetes mellitus alters the mechanical properties of the native tendon in an experimental rat model. Journal of Orthopaedic Research, 29(6):880–885.
- Franchi, M., Trirè, A., Quaranta, M., Orsini, E., and Ottani, V. (2007). Collagen structure of tendon relates to function. The Scientific World Journal, 7(1):404–20.
- Franz, J. R., Slane, L. C., Rasske, K., and Thelen, D. G. (2015). Non-uniform in vivo deformations of the human Achilles tendon during walking. Gait and Posture, 41(1):192–197.
- Franz, J. R. and Thelen, D. G. (2016). Imaging and simulation of Achilles tendon dynamics: implications for walking performance in the elderly. Journal of Biomechanics, 49(9):1403–1410.
- Fraser, R. D. B., MacRae, T. P., Miller, A., and Suzuki, E. (1983). Molecular conformation and packing in collagen fibrils. Journal of Molecular Biology, 167:497–521.
- Fraser, R. D. B., MacRae, T. P., and Suzuki, E. (1979). Chain conformation in the collagen molecule. Journal of Molecular Biology, 129:463–481.
- Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H., and Bernstorff, S. (1997). Fibrillar structure and mechanical properties of collagen. Journal of Structural Biology, 122(1):119–22.
- Freedman, B. R., Sarver, J. J., Buckley, M. R., Voleti, P. B., and Soslowsky, L. J. (2014). Biomechanical and structural response of healing Achilles tendon to fatigue loading following acute injury. Journal of Biomechanics, 47(9):2028–2034.
- Freedman, B. R., Zuskov, A., Sarver, J. J., Buckley, M. R., and Soslowsky, L. J. (2015). Evaluating changes in tendon crimp with fatigue loading as an ex vivo structural assessment of tendon damage. Journal of Orthopaedic Research, 33(6):904–910.
- Frisén, M., Mägi, M., Sonnerup, I., and Viidik, A. (1969). Rheological analysis of soft collagenous tissue. Part I: theoretical considerations. Journal of biomechanics, 2(1):13–20.
- Frost, P., Bonde, J. P. E., Mikkelsen, S., Andersen, J. H., Fallentin, N., Kaergaard, A., and Thomsen, J. F. (2002). Risk of Shoulder Tendinitis in Relation to Shoulder Loads in Monotonous Repetitive Work. American Journal of Industrial Medicine, 41:11–18.

Bibliography

- Fujimoto, D., Moriguchi, T., Ishida, T., and Hayashi, H. (1978). The structure of pyridinoline, a collagen crosslink. Biochemical and Biophysical Research Communications, 84(1):52–57.
- Fung, D. T., Wang, V. M., Andarawis-Puri, N., Basta-Pljakic, J., Li, Y., Laudier, D. M., Sun, H. B., Jepsen, K. J., Schaffler, M. B., and Flatow, E. L. (2010). Early response to tendon fatigue damage accumulation in a novel in vivo model. Journal of Biomechanics, 43(2):274–9.
- Fung, D. T. C., Wang, V. M., Laudier, D. M., Shine, J. H., Basta-Pljakic, J., Jepsen, K. J., Schaffler, M. B., and Flatow, E. L. (2009). Subrupture tendon fatigue damage. Journal of Orthopaedic Research, 27(2):264–273.
- Fung, Y.-C. (2004). Biomechanics: Mechanical Properties of Living Tissues. Springer, New Delhi, 2nd ed. edition.
- Galeski, A., Kastelic, J., Baer, E., and Kohn, R. R. (1977). Mechanical and structural changes in rat tail tendon induced by alloxan diabetes and aging. Journal of Biomechanics, 10(11):775–82.
- Gautieri, A., Vesentini, S., Redaelli, A., and Ballarini, R. (2013). Modeling and measuring visco-elastic properties: From collagen molecules to collagen fibrils. International Journal of Non-Linear Mechanics, 56:25–33.
- Giacomozzi, C., D’Ambrogio, E., Uccioli, L., and Macellari, V. (2005). Does the thickening of Achilles tendon and plantar fascia contribute to the alteration of diabetic foot loading? Clinical Biomechanics, 20(5):532–539.
- Gibbon, W. W., Cooper, J. R., and Radcliffe, G. S. (1999). Sonographic incidence of tendon microtears in athletes with chronic Achilles tendinosis. British journal of sports medicine, 33(2):129–130.
- Giddings, V. L., Beaupré, G. S., Whalen, R. T., and Carter, D. R. (2000). Calcaneal loading during walking and running. Medicine and Science in Sports and Exercise, 32(3):627–634.
- Gillies, H. and Chalmers, J. (1970). The management of fresh ruptures of the tendo achillis. The Journal of bone and joint surgery. American volume, 52(2):337–43.
- Girod, I., Valensi, P., Laforêt, C., Moreau-Defarges, T., Guillon, P., and Baron, F. (2003). An economic evaluation of the cost of diabetic foot ulcers: results of a retrospective study on 239 patients. Diabetes & metabolism, 29(3):269–277.
- Gomes, A. A., Ackermann, M., Ferreira, J. P., Orselli, M. I. V., and Sacco, I. C. N. (2017). Muscle force distribution of the lower limbs during walking in diabetic individuals with and without polyneuropathy. Journal of NeuroEngineering and Rehabilitation, 14(11):1–13.
- Gomoll, A. H., Katz, J. N., Warner, J. J. P., and Millett, P. J. (2004). Rotator cuff disorders: recognition and management among patients with shoulder pain. Arthritis & Rheumatism, 20(12):3751–3761.
- Gonzalez, A. D., Gallant, M. A., Burr, D. B., and Wallace, J. M. (2014). Multiscale analysis of morphology and mechanics in tail tendon from the ZDSD rat model of type 2 diabetes. Journal of Biomechanics, 47(3):681–686.
- Gosline, J., Lillie, M., Carrington, E., Guerette, P., Ortlepp, C., and Savage, K. (2002). Elastic proteins: biological roles and mechanical properties. Philosophical Transactions of the Royal Society B: Biological Sciences, 357(1418):121–132.
- Grant, T. M., Thompson, M. S., Urban, J., and Yu, J. (2013). Elastic fibres are broadly distributed in tendon and highly localized around tenocytes. Journal of Anatomy, 222:573–579.
- Grant, W. P., Foreman, E. J., Wilson, A. S., Jacobus, D. A., and Kukla, R. M. (2005). Evaluation of Young’s modulus in Achilles tendons with diabetic neuroarthropathy. Journal of the American Podiatric Medical Association, 95(3):242–6.
- Grant, W. P., Sullivan, R., Sonenshine, D. E., Adam, M., Slusser, J. H., Carson, K. A., and Vinik, A. I. (1997). Electron microscopic investigation of the effects of diabetes mellitus on the Achilles tendon. The Journal of Foot and Ankle Surgery, 36(4):272–8.
- Graziano, A. M. and Raulin, M. L. (2014). Research Methods: A Process of Inquiry. Pearson Education, Harlow, 8th edition.
- Greenhalgh, D. G., Sprugel, K. H., Murray, M. J., and Ross, R. (1990). PDGF and FGF stimulate wound healing in the genetically diabetic mouse. The American Journal of Pathology, 136(6):1235–1246.
- Gross, C. E. and Nunley II, J. A. (2016). Acute Achilles tendon ruptures. Foot & Ankle International, 37(2):233–9.
- Gross, J. (1963). Comparative Biochemistry, Vol. 5. Academic Press, New York, NY.
- Grytz, R. and Meschke, G. (2009). Constitutive modeling of crimped collagen fibrils in soft tissues. Journal of the Mechanical Behavior of Biomedical Materials, 2(5):522–533.

- Gupta, H. S., Messmer, P., Roschger, P., Bernstorff, S., Klaushofer, K., and Fratzl, P. (2004). Synchrotron diffraction study of deformation mechanisms in mineralized tendon. Physical Review Letters, 93(15):2–5.
- Gupta, H. S., Seto, J., Krauss, S., Boesecke, P., and Screen, H. R. C. (2010). In situ multi-level analysis of viscoelastic deformation mechanisms in tendon collagen. Journal of Structural Biology, 169(2):183–191.
- Gusachenko, I., Tran, V., Houssen, Y. G., and Allain, J.-M. (2012). Polarization-Resolved Second-Harmonic Generation in Tendon upon Mechanical Stretching. Biophysical Journal, 102(9):2220–2229.
- Gutsmann, T., Fantner, G. E., Venturoni, M., Ekani-Nkodo, A., Thompson, J. B., Kindt, J. H., Morse, D. E., Fygenon, D. K., and Hansma, P. K. (2003). Evidence that collagen fibrils in tendons are inhomogeneously structured in a tubelike manner. Biophysical Journal, 84(4):2593–2598.
- Gwynne-Jones, D. P., Sims, M., and Handcock, D. (2011). Epidemiology and Outcomes of Acute Achilles Tendon Rupture with Operative or Nonoperative Treatment Using an Identical Functional Bracing Protocol. Foot & Ankle International, 32(04):337–343.
- Han, W. M., Heo, S. J., Driscoll, T. P., Smith, L. J., Mauck, R. L., and Elliott, D. M. (2013). Macro- to microscale strain transfer in fibrous tissues is heterogeneous and tissue-specific. Biophysical Journal, 105(3):807–817.
- Handsfield, G. G., Slane, L. C., and Screen, H. R. C. (2016). Nomenclature of the tendon hierarchy: An overview of inconsistent terminology and a proposed size-based naming scheme with terminology for multi-muscle tendons. Journal of Biomechanics, 49(13):3122–3124.
- Hannafin, J. A. and Arnoczky, S. P. (1994). Effect of cyclic and static tensile loading on water content and solute diffusion in canine flexor tendons: An in Vitro study. Journal of Orthopaedic Research, 12(3):350–356.
- Hansen, K. A., Weiss, J. A., and Barton, J. K. (2002). Recruitment of tendon crimp with applied tensile strain. Journal of Biomechanical Engineering, 124(1):72–7.
- Hanson, A. F. (2004). The Rat’s Tail.
- Haraldsson, B. T., Aagaard, P., Qvortrup, K., Bojsen-Møller, J., Krogsgaard, M. R., Koskinen, S., Kjær, M., and Magnusson, S. P. (2008). Lateral force transmission between human tendon fascicles. Matrix Biology, 27(2):86–95.
- Harrison, M. J. G. and Faris, I. B. (1976). The neuropathic factor in the aetiology of diabetic foot ulcers. Journal of the Neurological Sciences, 28(2):217–223.
- Haut, T. L. and Haut, R. C. (1997). The state of tissue hydration determines the strain-rate-sensitive stiffness of human patellar tendon. Journal of Biomechanics, 30(1):79–81.
- Hearn, E. J. (1997). Mechanics of Materials 2. Butterworth-Heinemann, Oxford, 3rd edition.
- Heinemeier, K. M., Schjerling, P., Heinemeier, J., Magnusson, S. P., and Kjær, M. (2013). Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb 14C. The FASEB Journal, 27(5):2074–2079.
- Herod, T. W., Chambers, N. C., and Veres, S. P. (2016). Collagen fibrils in functionally distinct tendons have differing structural responses to tendon rupture and fatigue loading. Acta Biomaterialia, 42:296–307.
- Herod, T. W. and Veres, S. P. (2018). Development of overuse tendinopathy: A new descriptive model for the initiation of tendon damage during cyclic loading. Journal of Orthopaedic Research, 36(1):467–476.
- Hex, N., Bartlett, C., Wright, D., Taylor, M., and Varley, D. (2012). Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs. Diabetic Medicine, 29(7):855–862.
- Hodgson, R. J., O’Connor, P. J., and Grainger, A. J. (2012). Tendon and ligament imaging. The British Journal of Radiology, 85:1157–72.
- Hollister, S. J. (2007). Section 9: Ligament/Tendon Structure-Function.
- Holmes, G. B. and Lin, J. (2006). Etiologic factors associated with symptomatic Achilles tendinopathy. Foot & Ankle International, 27(11):952–959.
- Hooley, C. J., McCrum, N. G., and Cohen, R. E. (1980). The viscoelastic deformation of tendon. Journal of Biomechanics, 13(6):521–8.
- Hsiao, M.-Y., Chen, Y.-C., Lin, C.-Y., Chen, W.-S., and Wang, T.-G. (2015). Reduced Patellar Tendon Elasticity with Aging: InVivo Assessment by Shear Wave Elastography. Ultrasound in Medicine & Biology, 41(11):2899–2905.

Bibliography

- Huang, H., Zhang, J., Sun, K., Zhang, X., and Tian, S. (2011). Effects of repetitive multiple freeze-thaw cycles on the biomechanical properties of human flexor digitorum superficialis and flexor pollicis longus tendons. Clinical Biomechanics, 26(4):419–423.
- Ivatt, R. J., editor (1984). The Biology of Glycoproteins. Plenum Press, New York, NY, 1st editio edition.
- Jackson, S. J., Andrews, N., Ball, D., Bellantuono, I., Gray, J., Hachoumi, L., Holmes, A., Latcham, J., Petrie, A., Potter, P., Rice, A., Ritchie, A., Stewart, M., Strepka, C., Yeoman, M., and Chapman, K. (2017). Does age matter? The impact of rodent age on study outcomes. Laboratory Animals, 51(2):160–169.
- Järvinen, M. (1992). Epidemiology of tendon injuries in sports. Clinics in Sports Medicine, 11(3):493–504.
- Jones, E. R., Jones, G. C., Legerlotz, K., and Riley, G. P. (2013). Cyclical strain modulates metalloprotease and matrix gene expression in human tenocytes via activation of TGF β . BBA - Molecular Cell Research, 1833(12):2596–2607.
- Jozsa, L., Kvist, M., Balint, B. J., Reffy, A., Jarvinen, M., Lehto, M., and Barzo, M. (1989). The role of recreational sport activity in Achilles tendon rupture. A clinical, pathoanatomical, and sociological study of 292 cases. American Journal of Sports Medicine, 17(3):338–343.
- Józsa, L. G. and Kannus, P. (1997). Human Tendons: Anatomy, Physiology and Pathology. Human Kinetics Publishers, Champaign, IL.
- Kamalakkannan, N. and Prince, P. S. M. (2006). Antihyperglycaemic and antioxidant effect of Rutin, a polyphenolic flavonoid, in streptozotocin-induced diabetic wistar rats. Basic & Clinical Pharmacology & Toxicology, 98(1):97–103.
- Kannus, P. (2000). Structure of the tendon connective tissue. Scandinavian Journal of Medicine & Science in Sports, 10(6):312–20.
- Kannus, P. and Jozsa, L. (1991). Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. The Journal of Bone and Joint Surgery, 73(10):1507–1525.
- Kastelic, J., Galeski, A., and Baer, E. (1978). The multicomposite structure of tendon. Connective tissue research, 6(December):11–23.
- Kastelic, J., Palley, I., and Baer, E. (1980). A structural mechanical model for tendon crimping. Journal of Biomechanics, 13(10):887–893.
- Kaux, J. F., Forthomme, B., le Goff, C., Crielaard, J. M., and Croisier, J. L. (2011). Current opinions on tendinopathy. Journal of Sports Science and Medicine, 10(2):238–253.
- Ker, R. F. (1981). Dynamic tensile properties of the plantaris tendon of sheep (*Ovis aries*). The Journal of Experimental Biology, 93(1):283–302.
- Ker, R. F. (2007). Mechanics of tendon, from an engineering perspective. International Journal of Fatigue, 29(6):1001–1009.
- Ker, R. F., Wang, X. T., and Pike, A. V. (2000). Fatigue quality of mammalian tendons. The Journal of Experimental Biology, 203(8):1317–27.
- Khan, K. M., Cook, J. L., Bonar, F., Harcourt, P., and Åstrom, M. (1999). Histopathology of Common Tendinopathies. Sports Medicine, 27(6):393–408.
- Killian, M. L., Cavinatto, L., Galatz, L. M., and Thomopoulos, S. (2012). The Role of Mechanobiology in Tendon Healing. Journal of Shoulder and Elbow Surgery, 21(2):228–237.
- King, A. J. F. (2012). The use of animal models in diabetes research. British Journal of Pharmacology, 166(3):877–94.
- Kjær, M., Langberg, H., Miller, B. F., Boushel, R., Crameri, R. M., Koskinen, S., Heinemeier, K. M., Olesen, J. L., Døssing, S., Hansen, M., Pedersen, S. G., Rennie, M. J., and Magnusson, S. P. (2005). Metabolic activity and collagen turnover in human tendon in response to physical activity. Journal of Musculoskeletal and Neuronal Interactions, 5(1):41–52.
- Knorzer, E., Folkhard, W., Geercken, W., Boschert, C., Koch, M. H. J., Hilbert, B., Krah, H., Mosler, E., Nemetschek-Gansler, H., and Nemetschek, T. (1986). New aspects of the etiology of tendon rupture. Archives of Orthopaedic and Traumatic Surgery, 105:113–120.
- Komi, P. V. (1990). Relevance of in vivo force measurements to human biomechanics. Journal of Biomechanics, 23:23–34.
- Koob, T. J. and Vogel, K. G. (1987). Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon. Journal of Orthopaedic Research, 5(3):414–24.

- Kujala, U. M., Sarna, S., and Kaprio, J. (2005). Cumulative Incidence of Achilles Tendon Rupture and Tendinopathy in Male Former Elite Athletes. *Clinical Journal of Sport Medicine*, 15(3):133–135.
- Kumar, S., Ashe, H. A., Parnell, L. N., Fernando, D. J. S., Tsigos, C., Young, R. J., Ward, J. D., and Boulton, A. J. M. (1994). The Prevalence of Foot Ulceration and its Correlates in Type 2 Diabetic Patients: a Population-based Study. *Diabetic Medicine*, 11(5):480–484.
- LaCroix, A. S., Duenwald-Kuehl, S. E., Lakes, R. S., and Vanderby Jr, R. (2013). Relationship between tendon stiffness and failure: a metaanalysis. *Journal of Applied Physiology*, 115(1):43–51.
- Lagier, P. R. and Exer, B. (1960). Etude de la composition chimique de tissus humains de nature conjonctive en rapport avec l'âge: derme et aponévrose de la paroi abdominale, tendon d'Achille. *Gerontology*, 4:39–59.
- Lampi, M. C. and Reinhart-King, C. A. (2018). Targeting extracellular matrix stiffness to attenuate disease: From molecular mechanisms to clinical trials. *Science Translational Medicine*, 10(422):1–15.
- Langberg, H., Olesen, J. L., Gemmer, C., and Kjær, M. (2002). Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans. *Journal of Physiology*, 542(3):985–990.
- Lantto, I., Heikkinen, J., Flinkkilä, T., Ohtonen, P., and Leppilahti, J. (2015). Epidemiology of Achilles tendon ruptures: Increasing incidence over a 33-year period. *Scandinavian Journal of Medicine & Science in Sports*, 25(1):e133–e138.
- Laurent, G. (1987). Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. *American Journal of Physiology-Cell Physiology*, 252:C1–C9.
- Le Page, L. M., Rider, O. J., Lewis, A. J., Ball, V., Clarke, K., Johansson, E., Carr, C. A., Heather, L. C., and Tyler, D. J. (2015). Increasing pyruvate dehydrogenase flux as a treatment for diabetic cardiomyopathy: A combined ¹³C hyperpolarized magnetic resonance and echocardiography study. *Diabetes*, 64(8):2735–2743.
- Lee, A. H. and Elliott, D. M. (2017). Freezing does not alter multiscale tendon mechanics and damage mechanisms in tension. *Annals of the New York Academy of Sciences*, 1409(1):85–94.
- Legerlotz, K., Jones, G. C., Riley, G. P., and Screen, H. R. C. (2011). Cyclic loading of tendon fascicles using a novel fatigue loading system increases interleukin-6 expression. *International Journal of Experimental Pathology*, 92(3):A1–A38.
- Legerlotz, K., Riley, G. P., and Screen, H. R. C. (2010). Specimen dimensions influence the measurement of material properties in tendon fascicles. *Journal of Biomechanics*, 43(12):2274–2280.
- Legerlotz, K., Riley, G. P., and Screen, H. R. C. (2013). GAG depletion increases the stress-relaxation response of tendon fascicles, but does not influence recovery. *Acta Biomaterialia*, 9(6).
- Lemaitre, J. (1984). How to use damage mechanics. *Nuclear Engineering and Design*, 80(2):233–245.
- Lenzen, S. (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*, 51(2):216–226.
- Li, Y., Fessel, G., Georgiadis, M., and Snedeker, J. G. (2013). Advanced glycation end-products diminish tendon collagen fiber sliding. *Matrix Biology*, 32(3):169–177.
- Like, A. A. and Rossini, A. A. (1976). Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science*, 193(4251):415–7.
- Lin, T. W., Cardenas, L., and Soslowsky, L. J. (2004). Biomechanics of tendon injury and repair. *Journal of Biomechanics*, 37:865–877.
- Liu, S. H., Yang, R.-S., Al-Shaikh, R., and Lane, J. M. (1995). Collagen in tendon, ligament, and bone healing: a current review. *Clinical Orthopaedics and Related Research*, 3(318):265–278.
- Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). *Molecular Cell Biology*. W. H. Freeman, New York, NY, 4th editio edition.
- Loots, M. A. M., Lamme, E. N., Mekkes, J. R., Bos, J. D., and Middelkoop, E. (1999). Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. *Archives of Dermatological Research*, 291(2-3):93–99.
- López-Guerra, E. A. and Solares, S. D. (2014). Modeling viscoelasticity through spring-dashpot models in intermittent-contact atomic force microscopy. *Beilstein Journal of Nanotechnology*, 5(1):2149–2163.
- Lui, P. P. Y. (2017). Tendinopathy in diabetes mellitus patients - Epidemiology, pathogenesis, and management. *Scandinavian Journal of Medicine & Science in Sports*, 27(8):776–787.

Bibliography

- Lutgers, H. L., Gerrits, E. G., Graaff, R., Links, T. P., Sluiter, W. J., Gans, R. O., Bilo, H. J., and Smit, A. J. (2009). Skin autofluorescence provides additional information to the UK Prospective Diabetes Study (UKPDS) risk score for the estimation of cardiovascular prognosis in type 2 diabetes mellitus. *Diabetologia*, 52(5):789–797.
- Lynch, H. A., Johannessen, W., Wu, J. P., Jawa, A., and Elliott, D. M. (2003). Effect of fiber orientation and strain rate on the nonlinear uniaxial tensile material properties of tendon. *Journal of Biomechanical Engineering*, 125(5):726–31.
- Maeda, E. and Ohashi, T. (2015). Mechano-regulation of gap junction communications between tendon cells is dependent on the magnitude of tensile strain. *Biochemical and Biophysical Research Communications*, 465(2):281–286.
- Maffulli, N., Testa, V., Capasso, G., Ewen, S. W., Sullo, A., Benazzo, F., and King, J. B. (2004). Similar Histopathological Picture in Males with Achilles and Patellar Tendinopathy. *Medicine and Science in Sports and Exercise*, 36(9):1470–1475.
- Maffulli, N., Waterston, S., and Squair, J. (1999). Changing incidence of Achilles tendon rupture in Scotland: a 15-year study. *Clinical Journal of*.
- Maffulli, N., Wong, J., and Almekinders, L. C. (2003). Types and epidemiology of tendinopathy. *Clinics in Sports Medicine*, 22(4):675–92.
- Magnusson, S. P. and Kjær, M. (2003). Region-specific differences in Achilles tendon cross-sectional area in runners and non-runners. *European Journal of Applied Physiology*, 90(5-6):549–553.
- Magnusson, S. P., Langberg, H., and Kjær, M. (2010). The pathogenesis of tendinopathy: balancing the response to loading. *Nature Publishing Group*, 6(10):262–8.
- Malaviya, P., Butler, D. L., Boivin, G. P., Smith, F. N., Barry, F. P., Murphy, J. M., and Vogel, K. G. (2000). An in vivo model for load-modulated remodelling in the rabbit flexor tendon. *Journal of Orthopaedic Research*, 18:116–125.
- Mansel, C., Gardiner, N. J., Kuzma-Kuzniarska, M., Hulley, P. A., and Thompson, M. S. (2015). Static fatigue properties of tendons in a rat model of diabetes. In *British Society of Matrix Biology*, London, UK.
- Mansford, K. R. L. and Opie, L. (1968). Comparison of metabolic abnormalities in diabetes mellitus induced by streptozotocin or by alloxan. *The Lancet*, 291(7544):670–671.
- Mansor, L. S., Gonzalez, E. R., Cole, M. A., Tyler, D. J., Beeson, J. H., Clarke, K., Carr, C. A., and Heather, L. C. (2013). Cardiac metabolism in a new rat model of type 2 diabetes using high-fat diet with low dose streptozotocin. *Cardiovascular Diabetology*, 12(1):1–10.
- Masic, A., Bertinetti, L., Schuetz, R., Chang, S.-W., Metzger, T. H., Buehler, M. J., and Fratzl, P. (2015). Osmotic Pressure Induced Tensile Forces in Tendon Collagen. *Nature communications*, 6:5942.
- Mazzone, M. F. and McCue, T. (2002). Common conditions of the achilles tendon. *American Family Physician*, 65(9):1805–1810.
- McLennan, S., Yue, D. K., Marsh, M., Swanson, B., Delbridge, L., Reeve, T., and Turtle, J. R. (1986). The prevention and reversibility of tissue non-enzymatic glycosylation in diabetes. *Diabetic Medicine*, 3(2):141–146.
- Meerwaldt, R., Graaff, R., Oomen, P. H. N., Links, T. P., Jager, J. J., Alderson, N. L., Thorpe, S. R., Baynes, J. W., Gans, R. O. B., and Smit, A. J. (2004). Simple non-invasive assessment of advanced glycation endproduct accumulation. *Diabetologia*, 47(7):1324–1330.
- Meyers, M. and Chawla, K. K. (2009). *Mechanical Behaviour of Materials*. Cambridge University Press, Cambridge, 2nd ed. edition.
- Miller, K. S., Connizzo, B. K., Feeney, E., and Soslowsky, L. J. (2012). Characterizing local collagen fiber re-alignment and crimp behavior throughout mechanical testing in a mature mouse supraspinatus tendon model. *Journal of Biomechanics*, 45(12):2061–2065.
- Misof, K., Rapp, G., and Fratzl, P. (1997a). A new molecular model for collagen elasticity based on synchrotron X-ray scattering evidence. *Biophysical Journal*, 72(3):1376–1381.
- Misof, K., Rapp, G., Landis, W. J., Klaushofer, K., Hulmes, D. J. S., and Fratzl, P. (1997b). Mechanical properties of normal and defective collagen-implications for osteogenesis imperfecta. *Bone*, 20(4):11.
- Moller, A. and Westlin, N. E. (1996). Increasing incidence of Achilles tendon rupture. *Acta Orthop Scand*, 67(5):479–481.
- Monnier, V. M., Kohn, R. R., and Cerami, A. (1984). Accelerated age-related browning of human collagen in diabetes mellitus. *Proceedings of the National Academy of Sciences*, 81(2):583–587.

- Monnier, V. M., Sell, D. R., Nagaraj, R. H., Miyata, S., Grandhee, S., Odetti, P., and Ibrahim, S. A. (1992). Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia. Diabetes, 41(SUPPL. 2):36–41.
- Mosler, E., Folkhard, W., Knörzer, E., Nemetschek-Gansler, H., Nemetschek, T., and Koch, M. H. J. (1985). Stress-induced molecular rearrangement in tendon collagen. Journal of Molecular Biology, 182(4):589–596.
- Nahata, A. (2011). Spectrofluorimetry as an Analytical Tool. Pharmaceutica Analytica Acta, 02(08):8–10.
- Nakama, L. H., King, K. B., Abrahamsson, S., and Rempel, D. M. (2005). Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. Journal of Orthopaedic Research, 23(5):1199–1205.
- Nemetschek, T., Jonak, R., Meinel, A., Nemetschek-Gansler, H., and Riedl, H. (1977). Knickdeformationen an Kollagen* Kinking Deformities in Collagen. Arch. orthop. Unfall-Chir, 89:249–257.
- Neuberger, A. (1955). Fibrous Proteins and Their Biological Significance. Symposia of the Society for Experimental Biology, 9:72–84.
- Newton, V. L., Guck, J. D., Cotter, M. A., Cameron, N. E., and Gardiner, N. J. (2017). Neutrophils Infiltrate the Spinal Cord Parenchyma of Rats with Experimental Diabetic Neuropathy. Journal of Diabetes Research, 2017.
- Nimni, M. E. and Harkness, R. D. (1988). Molecular structure and functions of collagen. In Nimni, M. E., editor, Collagen, pages 1 – 77. CRC Press, Boaca Raton, FL, 1st editio edition.
- Nyyssönen, T., Lüthje, P., and Kröger, H. (2008). The increasing incidence and difference in sex distribution of Achilles tendon rupture in Finland in 1987/1999. Scandinavian Journal of Surgery, 97(3):272–275.
- O'Brien, M. (1997). Structure and metabolism of tendons. Scandinavian Journal of Medicine & Science in Sports, 7(2):55–61.
- Orgel, J. P. R. O., Antipova, O., Sagi, I., Bitler, A., Qiu, D., Wang, R., Xu, Y., and San Antonio, J. D. (2011). Collagen fibril surface displays a constellation of sites capable of promoting fibril assembly, stability, and hemostasis. Connective Tissue Research, 52(1):18–24.
- Orgel, J. P. R. O., Irving, T. C., Miller, A., and Wess, T. J. (2006). Microfibrillar structure of type I collagen in situ. Proceedings of the National Academy of Sciences of the United States of America, 103(24):9001–9005.
- Parry, D. A. D., Barnes, G. R. G., and Craig, A. S. (1978). A Comparison of the Size Distribution of Collagen Fibrils in Connective Tissues as a Function of Age and a Possible Relation between Fibril Size Distribution and Mechanical Properties. Proceedings of the Royal Society B: Biological Sciences, 203(1152):305–321.
- Parry, D. A. D. and Craig, A. S. (1978). Collagen fibrils and elastic fibers in rattail tendon: an electron microscopic investigation. Biopolymers, 17(4):843–55.
- Patel, D., Sharma, S., Screen, H. R. C., and Bryant, S. J. (2018). Effects of cell adhesion motif, fiber stiffness, and cyclic strain on tenocyte gene expression in a tendon mimetic fiber composite hydrogel. Biochemical and Biophysical Research Communications, 499(3):642–647.
- Peffer, M. J., Thorpe, C. T., Collins, J. A., Eong, R., Wei, T. K. J., Screen, H. R. C., and Clegg, P. D. (2014). Proteomic analysis reveals Age-related changes in tendon matrix composition, with Age-and injury-specific matrix fragmentation. Journal of Biological Chemistry, 289(37):25867–25878.
- Pensalfini, M., Duenwald-Kuehl, S. E., Kondratko-Mittnacht, J., Lakes, R., and Vanderby, R. (2014). Evaluation of Global Load Sharing and Shear-Lag Models to Describe Mechanical Behavior in Partially Lacerated Tendons. Journal of Biomechanical Engineering, 136(9):091006.
- Petruska, J. A. and Hodge, A. J. (1964). A subunit model for the tropocollagen macromolecule. Proc. Natl. Acad. Sci. USA, 51:871–876.
- Pierre-Jerome, C., Moncayo, V., and Terk, M. R. (2010). MRI of the Achilles tendon: A comprehensive review of the anatomy, biomechanics, and imaging of overuse tendinopathies. Acta Radiologica, 51(4):438–54.
- Pike, A. V. L., Ker, R. F., and Alexander, R. M. (2000). The Development of Fatigue Quality in High-and Low-Stressed Tendons of Sheep (Ovis Aries). The Journal of Experimental Biology, 203:2187–2193.
- Pingel, J., Lu, Y., Starborg, T., Fredberg, U., Langberg, H., Nedergaard, A., Weis, M., Eyre, D., Kjær, M., and Kadler, K. E. (2014). 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: Evidence of tenocyte and matrix buckling. Journal of Anatomy, 224(5):548–555.

Bibliography

- Pollock, C. and Shadwick, R. (1994). Allometry of muscle, tendon, and elastic energy storage capacity in mammals. American Journal of Physiology, 35(3):R1022–R1031.
- Praemer, A., Furner, S., Rice, D. P., and Kelsey, J. L. (1992). Musculoskeletal conditions in the United States. American Academy of Orthopaedic Surgeons, Park Ridge, IL, 1st edition.
- Probst, A., Palmes, D., Freise, H., Langer, M., Joist, A., and Spiegel, H. U. (2000). A New Clamping Technique for Biomechanical Testing of Tendons in Small Animals. Journal of Investigative Surgery, 13(6):313–8.
- Provenzano, P. P., Hurschler, C., and Vanderby, R. (2001). Microstructural Morphology in the Transition Region Between Scar and Intact Residual Segments of a Healing Rat Medial Collateral Ligament. Connective Tissue Research, 42(2):123–133.
- Provenzano, P. P. and Vanderby, R. (2006). Collagen fibril morphology and organization: Implications for force transmission in ligament and tendon. Matrix Biology, 25(2):71–84.
- Puxkandl, R., Zizak, I., Paris, O., Keckes, J., Tesch, W., Bernstorff, S., Purslow, P. P., and Fratzl, P. (2002). Viscoelastic properties of collagen: synchrotron radiation investigations and structural model. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 357(1418):191–197.
- Reddy, G. K. (2004). Cross-Linking in Collagen by Nonenzymatic Glycation Increases the Matrix Stiffness in Rabbit Achilles Tendon. Experimental Diabetes Research, 5(2):143–153.
- Reddy, G. K., Stehno-Bittel, L., and Enwemeka, C. S. (2002). Glycation-induced matrix stability in the rabbit achilles tendon. Archives of Biochemistry and Biophysics, 399(2):174–180.
- Reed, M. J., Meszaros, K., Entes, L. J., Claypool, M. D., Pinkett, J. G., Gadbois, T. M., and Reaven, G. M. (2000). A new rat model of type 2 diabetes: The fat-fed, streptozotocin-treated rat. Metabolism: Clinical and Experimental, 49(11):1390–1394.
- Rees, J. D., Wilson, A. M., and Wolman, R. L. (2006). Current concepts in the management of tendon disorders. Rheumatology, 45(5):508–21.
- Reese, S. P. and Weiss, J. A. (2013). Tendon fascicles exhibit a linear correlation between Poisson’s ratio and force during uniaxial stress relaxation. Journal of Biomechanical Engineering, 135(3):034501–1 to 034501–5.
- Reeves, N. D. (2005). Influence of 90-day simulated microgravity on human tendon mechanical properties and the effect of resistive countermeasures. Journal of Applied Physiology, 98(6):2278–2286.
- Reiber, G. E. (1996). The epidemiology of diabetic foot problems. Diabetic Medicine, 13(1):S6–11.
- Reiser, K. M. (1994). Influence of Age and Long-Term Dietary Restriction on Enzymatically Mediated Crosslinks and Nonenzymatic Glycation of Collagen in Mice. Journal of Gerontology: Biological Sciences, 49(2):71–79.
- Ricard-Blum, S. (2011). The Collagen Family. Cold Spring Harbor Perspectives in Biology, 3(1):1–19.
- Rigozzi, S. (2011). Structure and Function in Tendon. PhD thesis, ETH Zurich.
- Rigozzi, S., Müller, R., Stemmer, A., and Snedeker, J. G. (2013). Tendon glycosaminoglycan proteoglycan sidechains promote collagen fibril sliding-AFM observations at the nanoscale. Journal of Biomechanics, 46(4).
- Rigozzi, S., Stemmer, A., Müller, R., and Snedeker, J. G. (2011). Mechanical response of individual collagen fibrils in loaded tendon as measured by atomic force microscopy. Journal of Structural Biology, 176(1):9–15.
- Riley, G. P. (2008). Tendinopathy from basic science to treatment. Nature Clinical Practice Rheumatology, 4(2):82–9.
- Rosenqvist, U. (1984). An epidemiological survey of diabetic foot problems in the Stockholm County 1982. Acta Medica Scandinavica, 687:55–60.
- Ross, M. H., Kaye, G. I., and Pawlina, W. (2003). Histology: a text and atlas with cell and molecular biology. Lippincott Williams & Wilkins, Philadelphia, PA, 4th edition.
- Roukis, T. S., Hurless, J. S., and Page, J. C. (1996). Functional significance of torsion of the tendon of tibialis posterior. Journal of the American Podiatric Medical Association, 86(4):156–63.
- Rowe, R. W. D. (1985). The Structure of Rat Tail Tendon. Connective Tissue Research, 14(1):9–20.

- Roy, S., Boss, C., Rezakhaniha, R., and Stergiopoulos, N. (2011). Experimental characterization of the distribution of collagen fiber recruitment. Journal of Biorheology, 24:84.
- Roylance, D. (2001). Engineering Viscoelasticity. Technical report, Massachusetts Institute of Technology, Cambridge, MA.
- Salsich, G. B. B., Mueller, M. J. J., and Sahrmann, S. A. A. (2000). Passive ankle stiffness in subjects with diabetes and peripheral neuropathy versus an age-matched comparison group. Physical therapy, 80(4):352–362.
- Sarkar, S. K., Hiyama, Y., Niu, C. H., Young, P. E., Gerig, J. T., and Torchia, D. A. (1987). Molecular dynamics of collagen side chains in hard and soft tissues. A multinuclear magnetic resonance study. Biochemistry, 26(21):6793–6800.
- Sasaki, N. and Odajima, S. (1996a). Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy. Journal of Biomechanics, 29(9):1131–1136.
- Sasaki, N. and Odajima, S. (1996b). Stress-strain curve and Young's modulus of a collagen molecule as determined by the X-ray diffraction technique. Journal of Biomechanics, 29(5):655–658.
- Sasaki, N., Shukunami, N., Matsushima, N., and Izumi, Y. (1999). Time-resolved X-ray diffraction from tendon collagen during creep using synchrotron radiation. Journal of Biomechanics, 32(3):285–292.
- Schechtman, H. and Bader, D. L. (1997). In Vitro Fatigue of Human Tendons. Journal of Biomechanics, 30(8):829–835.
- Schechtman, H. and Bader, D. L. (2002). Fatigue damage of human tendons. Journal of Biomechanics, 35(3):347–353.
- Schiele, N. R., von Flotow, F., Tochka, Z. L., Hockaday, L. A., Marturano, J. E., Thibodeau, J. J., and Kuo, C. K. (2015). Actin cytoskeleton contributes to the elastic modulus of embryonic tendon during early development. Journal of Orthopaedic Research, 33(6):874–881.
- Schnedl, W. J., Ferber, S., Johnson, J. H., and Newgard, C. B. (1994). STZ transport and cytotoxicity: Specific enhancement in GLUT2-expressing cells. Diabetes, 43(11):1326–1333.
- Scott, J. E. (1988). Proteoglycan-fibrillar collagen interactions. The Biochemical Journal, 252(2):313–23.
- Screen, H. R. C. (2008). Investigating load relaxation mechanics in tendon. Journal of the Mechanical Behavior of Biomedical Materials, 1(1):51–58.
- Screen, H. R. C. (2009). Hierarchical approaches to understanding tendon mechanics. Journal of Biomechanical Science and Engineering, 4(4):481–499.
- Screen, H. R. C., Bader, D. L., Lee, D. A., and Shelton, J. C. (2004a). Local strain measurement within tendon. Strain, 40(4):157–163.
- Screen, H. R. C., Berk, D. E., Kadler, K. E., Ramirez, F., and Young, M. F. (2015). Tendon Functional Extracellular Matrix. Journal of Orthopaedic Research, 33(6):793–799.
- Screen, H. R. C. and Evans, S. L. (2009). Measuring strain distributions in the tendon using confocal microscopy and finite elements. The Journal of Strain Analysis for Engineering Design, 44(5):327–335.
- Screen, H. R. C., Lee, D. A., Bader, D. L., and Shelton, J. C. (2004b). An investigation into the effects of the hierarchical structure of tendon fascicles on micromechanical properties. Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine, 218(2):109–119.
- Screen, H. R. C., Seto, J., Krauss, S., Boesecke, P., and Gupta, H. S. (2011). Extrafibrillar diffusion and intrafibrillar swelling at the nanoscale are associated with stress relaxation in the soft collagenous matrix tissue of tendons. Soft Matter, 7(23):11243.
- Screen, H. R. C., Shelton, J. C., Bader, D. L., and Lee, D. A. (2005a). Cyclic tensile strain upregulates collagen synthesis in isolated tendon fascicles. Biochemical and Biophysical Research Communications, 336(2):424–429.
- Screen, H. R. C., Shelton, J. C., Chhaya, V. H., Kayser, M. V., Bader, D. L., and Lee, D. A. (2005b). The influence of noncollagenous matrix components on the micromechanical environment of tendon fascicles. Annals of Biomedical Engineering, 33(8):1090–9.
- Screen, H. R. C., Toorani, S., and Shelton, J. C. (2013). Microstructural stress relaxation mechanics in functionally different tendons. Medical Engineering and Physics, 35(1):96–102.
- Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. International Journal of Preventive Medicine, 4(6):624–630.

Bibliography

- Shadwick, R. E. (1990). Elastic energy storage in tendons: mechanical differences related to function and age. Journal of Applied Physiology, 68(3):1033–1040.
- Sharma, P. and Maffulli, N. (2005). Basic biology of tendon injury and healing. The Surgeon, 3(5):309–316.
- Sharon, N. (1988). Nomenclature of glycoproteins, glycopeptides and peptidoglycans (Recommendations 1985). Pure and Applied Chemistry, 60(9):1389–1394.
- Shen, Z. L., Dodge, M. R., Kahn, H., Ballarini, R., and Eppell, S. J. (2008). Stress-Strain Experiments on Individual Collagen Fibrils. Biophysical Journal, 95(8):3956–3963.
- Shen, Z. L., Kahn, H., Ballarini, R., and Eppell, S. J. (2011). Viscoelastic properties of isolated collagen fibrils. Biophysical Journal, 100(12):3008–3015.
- Shepherd, J. H., Legerlotz, K., Demirci, T., Klemm, C., Riley, G. P., and Screen, H. R. C. (2014a). Functionally distinct tendon fascicles exhibit different creep and stress relaxation behaviour. Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine, 228(1):49–59.
- Shepherd, J. H., Riley, G. P., and Screen, H. R. C. (2014b). Early stage fatigue damage occurs in bovine tendon fascicles in the absence of changes in mechanics at either the gross or micro-structural level. Journal of the Mechanical Behavior of Biomedical Materials, 38:163–72.
- Shepherd, J. H. and Screen, H. R. C. (2013). Fatigue loading of tendon. International Journal of Experimental Pathology, 94(4):260–270.
- Sherby, O. D. and Burke, P. M. (1968). Mechanical behavior of crystalline solids at elevated temperature. Progress in Materials Science, 13:323–390.
- Sims, T. J., Avery, N. C., and Bailey, A. J. (2000). Quantitative determination of collagen crosslinks. Extracellular Matrix Protocols, pages 11–26.
- Smith, B. L., Schäffer, T. E., Viani, M., Thompson, J. B., Frederick, N. A., Kindt, J. H., Belcher, A., Stucky, G. D., Morse, D. E., and Hansma, P. K. (1999). Molecular mechanistic origin of the toughness of natural adhesives, fibres and composites. Nature, 399(6738):761–763.
- Smith, K. D., Vaughan-Thomas, A., Spiller, D. G., Innes, J. F., Clegg, P. D., and Comerford, E. J. (2011). The organisation of elastin and fibrillins 1 and 2 in the cruciate ligament complex. Journal of Anatomy, 218(6):600–7.
- Smith, T. J. (2006). The relationship between tendon morphology and function. PhD thesis, University of London.
- Snedeker, J. G. (2016). How High Glucose Levels Affect Tendon Homeostasis. In Ackermann, P. W. and Hart, D. A., editors, Metabolic Influences on Risk for Tendon Disorder, chapter 18, pages 191–198. Springer International Publishing.
- Snedeker, J. G. and Gautieri, A. (2014). The role of collagen crosslinks in ageing and diabetes - the good, the bad, and the ugly. Muscles, Ligaments and Tendons Journal, 4(3):303–8.
- Sode, J., Obel, N., Hallas, J., and Lassen, A. (2007). Use of fluroquinolone and risk of Achilles tendon rupture: a population-based cohort study. European Journal of Clinical Pharmacology, 63(5):499–503.
- Sonksen, P. and Sonksen, J. (2000). Insulin: understanding its action in health and disease. British Journal of Anaesthesia, 85(1):69–79.
- Soslowsky, L. J., Thomopoulos, S., Tun, S., Flanagan, C. L., Keefer, C. C., Mastaw, J., and Carpenter, J. E. (2000). Neer Award 1999: Overuse activity injures the supraspinatus tendon in an animal model: A histologic and biomechanical study. Journal of Shoulder and Elbow Surgery, 9:79–84.
- Spadaro, J. A., Becker, R. O., and Bachman, C. H. (1970). The distribution of trace metal ions in bone and tendon. Calcified Tissue Research, 6(1):49–54.
- Steven, F. S., Minns, R. J., and Finlay, J. B. (1975). Evidence for the local denaturation of collagen fibrils during the mechanical rupture of human tendons. Injury, 6:317–319.
- Svensson, R. B., Hansen, P., Hassenkam, T., Haraldsson, B. T., Aagaard, P., Kovanen, V., Krogsaard, M. R., Kjær, M., and Magnusson, S. P. (2012). Mechanical properties of human patellar tendon at the hierarchical levels of tendon and fibril. Journal of Applied Physiology, 112(26):419–426.
- Svensson, R. B., Hassenkam, T., Hansen, P., Kjær, M., and Magnusson, S. P. (2011). Tensile force transmission in human patellar tendon fascicles is not mediated by glycosaminoglycans. Connective Tissue Research, 52(5):415–21.

- Sverdlík, A. and Lanir, Y. (2002). Time-dependent mechanical behavior of sheep digital tendons, including the effects of preconditioning. Journal of biomechanical engineering, 124(1):78–84.
- Szaro, P., Witkowski, G., Smigielski, R., Krajewski, P., and Ciszek, B. (2009). Fascicles of the adult human Achilles tendon - An anatomical study. Annals of Anatomy, 191(6):586–593.
- Szczesny, S. E. and Elliott, D. M. (2014a). Incorporating plasticity of the interfibrillar matrix in shear lag models is necessary to replicate the multiscale mechanics of tendon fascicles. Journal of the Mechanical Behavior of Biomedical Materials, 40:325–338.
- Szczesny, S. E. and Elliott, D. M. (2014b). Interfibrillar shear stress is the loading mechanism of collagen fibrils in tendon. Acta Biomaterialia, 10(6):2582–2590.
- Szczesny, S. E., Fetchko, K. L., Dodge, G. R., and Elliott, D. M. (2017). Evidence that interfibrillar load transfer in tendon is supported by small diameter fibrils and not extrafibrillar tissue components. Journal of Orthopaedic Research, pages 1–8.
- Szczesny, S. E., Peloquin, J. M., Cortes, D. H., Kadlowec, J. A., Soslowsky, L. J., and Elliott, D. M. (2012). Biaxial tensile testing and constitutive modeling of human supraspinatus tendon. Journal of Biomechanical Engineering, 134(2):021004–1 – 9.
- Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiological Research, 50(6):537–46.
- Taconic Biosciences (2018). Sprague Dawley.
- Takács, Á., Rudas, I. J., and Haidegger, T. (2015). Surface deformation and reaction force estimation of liver tissue based on a novel nonlinear massspringdamper viscoelastic model. Medical & Biological Engineering & Computing, 54(10):1553–1562.
- Tallon, C., Maffulli, N., and Ewen, S. (2001). Ruptured Achilles tendons are significantly more degenerated than tendinopathic tendons. Medicine and science in sports and exercise, 33(12):1983–1990.
- Tang, S., Zeenath, U., and Vashishth, D. (2007). Effects of non-enzymatic glycation on cancellous bone fragility. Bone, 40(4):1144–1151.
- Tempelhof, S., Rupp, S., and Seil, R. (1999). Age-related prevalence of rotator cuff tears in asymptomatic shoulders. Journal of Shoulder and Elbow Surgery, 8(4):296–299.
- Thompson, J. B., Kindt, J. H., Drake, B., Hansma, H. G., Morse, D. E., and Hansma, P. K. (2001). Bone indentation recovery time correlates with bond reforming time. Nature, 414(6865):773–776.
- Thompson, J. I. and Czernuszka, J. T. (1995). The Effect of Two Types of Cross-Linking on Some Mechanical Properties of Collagen. Bio-Medical Materials and Engineering, 5(1):37–48.
- Thornton, G. M., Schwab, T. D., and Oxland, T. R. (2007). Fatigue is more damaging than creep in ligament revealed by modulus reduction and residual strength. Annals of Biomedical Engineering, 35(10):1713–1721.
- Thornton, G. M., Shrive, N. G., and Frank, C. B. (2001). Altering ligament water content affects ligament pre-stress and creep behaviour. Journal of Orthopaedic Research, 19(5):845–851.
- Thorpe, C. T., Chaudhry, S., Lei, I. I., Varone, A., Riley, G. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2015a). Tendon overload results in alterations in cell shape and increased markers of inflammation and matrix degradation. Scandinavian Journal of Medicine & Science in Sports, 25(4):e381–e391.
- Thorpe, C. T., Godinho, M. S. C., Riley, G. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2015b). The interfascicular matrix enables fascicle sliding and recovery in tendon, and behaves more elastically in energy storing tendons. Journal of the Mechanical Behavior of Biomedical Materials, 52:85–94.
- Thorpe, C. T., Karunaseelan, K. J., Ng, J., Riley, G. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2015c). The composition of the interfascicular matrix is specialised in energy storage tendons to facilitate elastic behaviour. Bone & Joint Journal, 97-B(SUPP 11):19.
- Thorpe, C. T., Peffers, M. J., Simpson, D., Halliwell, E., Screen, H. R. C., and Clegg, P. D. (2016). Anatomical heterogeneity of tendon: fascicular and interfascicular tendon compartments have distinct proteomic composition. Nature Publishing Group, 6(February):1–12.
- Thorpe, C. T., Riley, G. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2014a). Effect of fatigue loading on structure and functional behaviour of fascicles from energy-storing tendons. Acta Biomaterialia, 10(7).

Bibliography

- Thorpe, C. T., Riley, G. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2014b). Fascicles from energy-storing tendons show an age-specific response to cyclic fatigue loading. *Journal of the Royal Society*, 11(92):20131058.
- Thorpe, C. T., Spiesz, E. M., Chaudhry, S., Screen, H. R. C., and Clegg, P. D. (2015d). Science in brief: Recent advances into understanding tendon function and injury risk. *Equine Veterinary Journal*, 47(2):137–140.
- 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. *Journal of Biological Chemistry*.
- Thorpe, C. T., Udeze, C. P., Birch, H. L., Clegg, P. D., and Screen, H. R. C. (2012). Specialization of tendon mechanical properties results from interfascicular differences. *Journal of the Royal Society, Interface*, 9(76):3108–17.
- Torp, S., Baer, E., and Friedman, B. (1975). Effects of age and of mechanical deformation on the ultrastructure of tendon. *Structure of Fibrous Biopolymers*, 26:223–50.
- Tresoldi, I., Oliva, F., Benvenuto, M., Fantini, M., Masuelli, L., Bei, R., and Modesti, A. (2013). Tendon's ultrastructure. *Muscles, Ligaments and Tendons Journal*, 3(1):2–6.
- US Bureau of Labor Statistics (2016). R31: Number of nonfatal occupational injuries and illnesses involving days away from work by event or exposure leading to injury or illness and selected natures of injury or illness, private industry. Technical report, USBL.
- Vailas, A. C., Pedrini, V. A., Pedrini-Mille, A., and Holloszy, J. O. (1985). Patellar tendon matrix changes associated with aging and voluntary exercise. *Journal of Applied Physiology*, 58(5):1572–6.
- Vanhoutte, G., Verhoye, M., Raman, E., Roberts, M., and van der Linden, A. (2002). In-vivo non-invasive study of the thermoregulatory function of the blood vessels in the rat tail using magnetic resonance angiography. *NMR in Biomedicine*, 15(4):263–269.
- Veres, S. P., Harrison, J. M., and Lee, J. M. (2013a). Cross-link stabilization does not affect the response of collagen molecules, fibrils, or tendons to tensile overload. *Journal of Orthopaedic Research*, 31(12):1907–1913.
- Veres, S. P., Harrison, J. M., and Lee, J. M. (2013b). Repeated subrupture overload causes progression of nanoscaled discrete plasticity damage in tendon collagen fibrils. *Journal of Orthopaedic Research*, 31(5):731–737.
- Veres, S. P. and Lee, J. M. (2012). Designed to fail: a novel mode of collagen fibril disruption and its relevance to tissue toughness. *Biophysical Journal*, 102(12):2876–84.
- Vergari, C., Pourcelot, P., Holden, L., Ravary-Plumioën, B., Gerard, G., Laugier, P., Mitton, D., and Crevier-Denoix, N. (2011). True stress and Poisson's ratio of tendons during loading. *Journal of Biomechanics*, 44(4):719–724.
- Viidik, A. (1966). Biomechanics and functional adaption of tendons and joint ligaments. In Evans, F. G., editor, *Studies on the Anatomy and Function of Bone and Joints*, pages 17–39. Springer-Verlag, New York, NY, 1st editio edition.
- Viidik, A. (1972). Simultaneous Mechanical and Light Microscopic Studies of Collagen Fibers. *Zeitschrift für Anatomie und Entwicklungsgeschichte*, 136:204–212.
- Vincent, J. (2012). Basic Elasticity and Viscoelasticity. In *Structural Biomaterials*, chapter Chapter 1, pages 1–26. Princeton University Press, Princeton, NJ, 3rd editio edition.
- Vogt, B. W., Schleicher, E. D., and Wieland, O. H. (1982). ϵ -amino-lysine-bound glucose in human tissues obtained at autopsy: increase in diabetes mellitus. *Diabetes*, 31(12):1123–7.
- Volper, B. D., Huynh, R. T., Arthur, K. A., Noone, J., Gordon, B. D., Zacherle, E. W., Munoz, E., Sørensen, M. A., Svensson, R. B., Broderick, T. L., Magnusson, S. P., Howden, R., Hale, T. M., and Carroll, C. C. (2015). The influence of acute and chronic streptozotocin-induced diabetes on rat tendon extracellular matrix and mechanical properties. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 309(1):1135–43.
- Waggett, A. D., Benjamin, M., and Ralphs, J. R. (2006). Connexin 32 and 43 gap junctions differentially modulate tenocyte response to cyclic mechanical load. *European Journal of Cell Biology*, 85(11):1145–54.
- Walden, G., Liao, X., Donell, S., Raxworthy, M. J., Riley, G. P., and Saeed, A. (2017). A clinical, biological, and biomaterials perspective into tendon Injuries and regeneration. *Tissue Engineering*, 23(1):44–58.
- Wall, M. E., Weinhold, P. S., Siu, T., Brown, T. D., and Banes, A. J. (2007). Comparison of cellular strain with applied substrate strain in vitro. *Journal of Biomechanics*, 40(1):173–181.

- Wang, J. H.-C. (2006). Mechanobiology of Tendon. Journal of Biomechanics, 39(9):1563–1582.
- Wang, X. T. and Ker, R. F. (1995). Creep rupture of wallaby tail tendons. Journal of Experimental Biology, 198(3):831–845.
- Wang, X. T., Ker, R. F., and Alexander, R. M. (1995). Fatigue rupture of wallaby tail tendons. The Journal of Experimental Biology, 198(3):847–852.
- Wenger, M. P. E., Horton, M. A., and Mesquida, P. (2008). Nanoscale scraping and dissection of collagen fibrils. Nanotechnology, 19(38):1–11.
- Wenstrup, R. J., Florer, J. B., Brunskill, E. W., Bell, S. M., Chervoneva, I., and Birk, D. E. (2004). Type V collagen controls the initiation of collagen fibril assembly. Journal of Biological Chemistry, 279(51):53331–53337.
- Wess, T. J., Hammersley, A. P., Wess, L., and Miller, A. (1998a). A consensus model for molecular packing of type I collagen. Journal of Structural Biology, 122(1-2):92–100.
- Wess, T. J., Hammersley, A. P., Wess, L., and Miller, A. (1998b). Molecular packing of type I collagen in tendon. Journal of Molecular Biology, 275(2):255–67.
- Wiens, R., Findlay, C. R., Baldwin, S. G., Kreplak, L., Lee, J. M., Veres, P., and Gough, K. M. (2016). High spatial resolution (1.1 mm and 20 nm) FTIR polarization contrast imaging reveals pre-rupture disorder in damaged tendon. Faraday Discussions, 187:555–573.
- Willett, T. L., Labow, R. S., Avery, N. C., and Lee, J. M. (2007). Increased proteolysis of collagen in an in vitro tensile overload tendon model. Annals of Biomedical Engineering, 35(11):1961–1972.
- Woo, S. L.-Y., Debski, R. E., Zeminski, J., Abramowitch, S. D., Chan Saw, S. S., and Fenwick, J. A. (2000). Injury and repair of ligaments and tendons. Annual Review of Biomedical Engineering, 2(1):83–118.
- Woo, S. L.-Y., Gomez, M. A., Woo, Y. K., and Akeson, W. H. (1982). Mechanical properties of tendons and ligaments II: the relationships of immobilization and exercise on tissue remodeling. Biorheology, 19(3):397–408.
- Woo, S. L.-Y., Ritter, M. A., Amiel, D., Sanders, T. M., Gomez, M. A., Kuei, S. C., Garfin, S. R., and Akeson, W. H. (1980). The biomechanical and biochemical properties of swine tendons - long term effects of exercise on the digital extensors. Connective Tissue Research, 7(3):177–83.
- World Health Organisation (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. Technical report, WHO, Geneva.
- World Health Organisation (2013). Diabetes.
- World Health Organisation (2015). Fact sheet N404: Ageing and health. Technical report, World Health Organisation.
- Wren, T. A. L., Lindsey, D. P., Beaupré, G. S., and Carter, D. R. (2003). Effects of creep and cyclic loading on the mechanical properties and failure of human Achilles tendons. Annals of Biomedical Engineering, 31(6):710–717.
- Wren, T. A. L., Yerby, S. A., Beaupré, G. S., and Carter, D. R. (2001). Mechanical properties of the human achilles tendon. Clinical Biomechanics, 16:245–251.
- Yahia, L.-H. and Drouin, G. (1989). Microscopical investigation of canine anterior cruciate ligament and patellar tendon: Collagen fascicle morphology and architecture. Journal of Orthopaedic Research, 7(2):243–251.
- Yanagishita, M. (1993). Function of proteoglycans in the extracellular matrix. Acta Pathologica Japonica, 43(6):283–93.
- Yeung, C.-Y. C., Garva, R., Pickard, A., Chang, J., Holmes, D. F., Lu, Y., Mallikarjun, V., Swift, J., Adamson, A., Calverley, B., Jun Meng, Q., and Kadler, K. E. (2018). Circadian Clock Regulation of the Secretory Pathway. bioRxiv, 304014.
- Zhang, M., Lv, X.-Y., Li, J., Xu, Z.-G., and Chen, L. (2008). The Characterization of High-Fat Diet and Multiple Low-Dose Streptozotocin Induced Type 2 Diabetes Rat Model. Experimental Diabetes Research, pages 1–9.

Appendices

A. Viscoelasticity Models: Derivations	157
A.1. Kelvin-Voigt Model	157
A.2. Maxwell Model	160
A.3. Standard Linear Solid Model - Stress-Based Configuration	163
A.4. Standard Linear Solid Model - Strain-Based Configuration	168
A.5. Sliding and Pullout Model	168
B. Rat Dissection: Video Protocol	171
C. The Susceptibility of Tendon to Damage: Software Protocol	173
D. The Susceptibility of Tendon to Damage: Written Protocol	177
E. The Susceptibility of Tendon to Damage: Mechanistic Markers	181
E.1. Materials and Methods	181
E.2. Results	183
E.2.1. STZ Model vs Healthy Controls	183
E.2.2. Energy-Storage vs Positional Functional Roles	183
E.3. Discussion	186
E.3.1. The Standard Linear Solid Model	186
E.3.2. STZ-Model vs Healthy Controls	189
E.3.3. Energy-Storage vs Positional Functional Roles	189
F. The Susceptibility of Tendon to Damage: Effect of Rat Strain	191
G. Steady-State Strain Rate as a Measure of Damage: Video Protocol	193
H. Quantification of AGEs: Written Protocol	195

Appendices

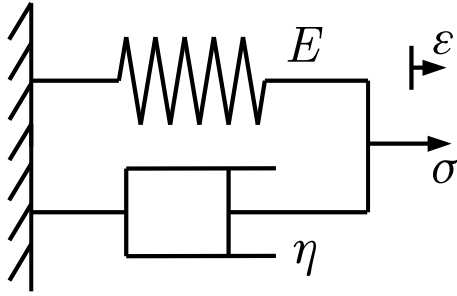
H.1. Preparation	195
H.2. Day 1	196
H.3. Day 2	197
H.4. Day 3	197
I. Quantification of AGEs: Video Protocol	199

A. Viscoelasticity Models: Derivations

In this section, some calculations were performed using Wolfram Alpha (www.wolframalpha.com).

A.1. Kelvin-Voigt Model

The collagenous matrix is modelled as a spring s with loading modulus E and the ground substance as a dashpot d with viscosity η . These components are placed in parallel and subjected to a stress σ and a strain ϵ over time t .



$$\sigma = \sigma_s + \sigma_d \quad (\text{A.1})$$

$$\epsilon = \epsilon_s = \epsilon_d \quad (\text{A.2})$$

$$\sigma_s = E\epsilon_s \quad (\text{A.3})$$

$$\sigma_d = \eta\dot{\epsilon}_d \quad (\text{A.4})$$

Stress Response

Substituting A.3, A.4 and A.2 into A.1:

$$\sigma = E\epsilon + \eta\dot{\epsilon}$$

which is shown in Figure 2.4a.

Strain Creep

Given loading parameter $\sigma = \sigma_0$ (i.e. constant stress applied as a step response at $t = 0$), shown in Figure 2.4b, and rearranging the previous equation:

$$\begin{aligned}\frac{\sigma_0 - E\epsilon}{\eta} &= \frac{d\epsilon}{dt} \\ \therefore 1dt &= \frac{\eta}{\sigma_0 - E\epsilon} d\epsilon \\ \therefore 1dt &= \frac{\eta}{E} \frac{1}{\frac{\sigma_0}{E} - \epsilon} d\epsilon\end{aligned}$$

Integrating with regards to time:

$$\begin{aligned}t &= \frac{-\eta}{E} \ln \left(\frac{\sigma_0}{E} - \epsilon \right) + C \\ \therefore \frac{-E}{\eta} t - C &= \ln \left(\frac{\sigma_0}{E} - \epsilon \right) \\ \therefore e^{\left(\frac{-E}{\eta} t - C \right)} &= \frac{\sigma_0}{E} - \epsilon \\ \therefore e^{\left(\frac{-E}{\eta} t \right)} \div e^C &= \frac{\sigma_0}{E} - \epsilon\end{aligned}$$

Let $K = \frac{1}{e^C}$:

$$\therefore \epsilon = \frac{\sigma_0}{E} + Ke^{\left(\frac{-E}{\eta} t \right)}$$

Substituting in boundary condition $\epsilon = 0$ @ $t = 0$:

$$\begin{aligned}0 &= \frac{\sigma_0}{E} + K \Rightarrow K = \frac{-\sigma_0}{E} \\ \therefore \epsilon &= \frac{\sigma_0}{E} \left(1 - e^{\left(\frac{-E}{\eta} t \right)} \right)\end{aligned}$$

which is shown in Figure 2.4c. As $t \rightarrow \infty$, $\epsilon \rightarrow \frac{\sigma_0}{E}$, which is also shown in Figure 2.4c.

Hysteresis

Given loading parameter $\sigma = \bar{\sigma} \sin(\omega t) + \bar{\sigma}$ (i.e. cyclic tensile loading where the average load is $\bar{\sigma}$, the peak load is $2\bar{\sigma}$ and the minimum load is 0 MPa, as shown in Figure 2.4h)

for many loading cycles and substituting into the stress response equation:

$$\bar{\sigma} \sin(\omega t) + \bar{\sigma} = E\epsilon + \eta \dot{\epsilon}$$

$$\therefore 0 = -\bar{\sigma} \sin(\omega t) - \bar{\sigma} + E\epsilon + \eta \dot{\epsilon}$$

The solution to this first-order linear ordinary differential equation is:

$$\epsilon = C e^{\frac{-Et}{\eta}} + \bar{\sigma} \times \frac{\omega^2 \eta^2 - \omega E \eta \cos(\omega t) + E^2 \sin(\omega t) + E^2}{E^3 + \omega^2 E \eta^2}$$

Substituting in boundary condition $\epsilon = 0$ @ $t = 0$:

$$0 = C + \bar{\sigma} \times \frac{\omega^2 \eta^2 - \omega E \eta + E^2}{E^3 + \omega^2 E \eta^2}$$

$$\therefore \epsilon = \frac{\omega E \eta - \omega^2 \eta^2 - E^2}{E^3 + \omega^2 E \eta^2} \bar{\sigma} e^{\frac{-Et}{\eta}} + \frac{\omega^2 \eta^2 + E^2}{E^3 + \omega^2 E \eta^2} \bar{\sigma} + \frac{\omega \eta}{E^2 + \omega^2 \eta^2} \bar{\sigma} \cos(\omega t) + \frac{E}{E^2 + \omega^2 \eta^2} \bar{\sigma} \sin(\omega t)$$

Note that the sine and cosine terms represent the in-phase (energy storage) and out-of-phase (energy dissipation) components of the response, respectively. Now σ can be plotted against ϵ over t :

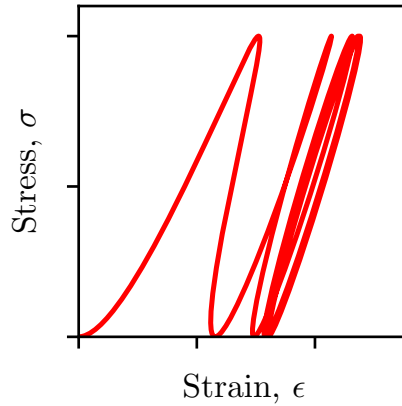
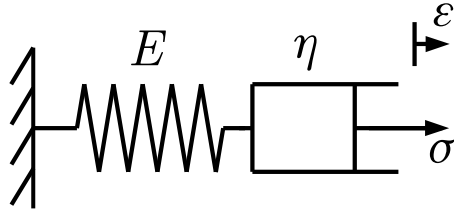


Figure A.1: *Cyclic loading response of the Kelvin-Voigt model*

A.2. Maxwell Model

The collagenous matrix is modelled as a spring s with elasticity E and the ground substance as a dashpot d with viscosity η . These components are placed in series and subjected to a stress σ and a strain ϵ over time t .



$$\sigma = \sigma_s = \sigma_d \quad (\text{A.5})$$

$$\epsilon = \epsilon_s + \epsilon_d \quad (\text{A.6})$$

$$\sigma_s = E\epsilon_s \quad (\text{A.7})$$

$$\sigma_d = \eta\dot{\epsilon}_d \quad (\text{A.8})$$

Strain Response

Taking the temporal derivative of A.6 gives:

$$\dot{\epsilon} = \dot{\epsilon}_s + \dot{\epsilon}_d$$

Taking the temporal derivative of A.7 and substituting in A.5 gives:

$$\dot{\sigma}_s = \dot{\sigma} = E\dot{\epsilon}_s$$

Substituting this in, along with A.8, gives:

$$\dot{\epsilon} = \frac{1}{E}\dot{\sigma} + \frac{1}{\eta}\sigma \quad (\text{A.9})$$

which is shown in Figure 2.4d.

Stress Relaxation

Given loading parameter $\epsilon = \epsilon_0$ (i.e. constant strain applied as a step response at $t = 0$), shown in Figure 2.4e:

$$\dot{\epsilon} = 0$$

Substituting into A.9:

$$0 = \frac{1}{E} \frac{d\sigma}{dt} + \frac{1}{\eta} \sigma$$

Rearranging:

$$\begin{aligned} \frac{-E}{\eta} &= \frac{1}{\sigma} \frac{d\sigma}{dt} \\ \frac{-E}{\eta} dt &= \frac{1}{\sigma} d\sigma \end{aligned}$$

Integrating both sides with respect to time:

$$\frac{-E}{\eta} t = \ln(\sigma) + C \quad (\text{A.10})$$

Substituting the material's elastic response in as a boundary condition: $\sigma = E\epsilon_0$ @ $t = 0$

$$0 = \ln(E\epsilon_0) + C \Rightarrow C = -\ln(E\epsilon_0)$$

Substituting into A.10:

$$\begin{aligned} \frac{-E}{\eta} t &= \ln(\sigma) - \ln(E\epsilon_0) \\ \therefore e^{\frac{-E}{\eta} t} &= \frac{\sigma}{E\epsilon_0} \\ \therefore E\epsilon_0 e^{\frac{-E}{\eta} t} &= \sigma \end{aligned}$$

which is shown in Figure 2.4f. As $t \rightarrow \infty$, $\sigma \rightarrow 0$, which is also shown in Figure 2.4f.

Hysteresis

Given loading parameter $\sigma = \bar{\sigma} \sin(\omega t) + \bar{\sigma}$ (i.e. cyclic tensile loading where the average load is $\bar{\sigma}$, the peak load is $2\bar{\sigma}$ and the minimum load is 0 MPa, as shown in Figure 2.4h)

for many loading cycles:

$$\sigma = \bar{\sigma} \sin(\omega t) + \bar{\sigma}$$

$$\therefore \dot{\sigma} = \bar{\sigma} \omega \cos(\omega t)$$

Substituting into the strain response equation (Equation A.9):

$$\dot{\epsilon} = \frac{\bar{\sigma} \omega}{E} \cos(\omega t) + \frac{\bar{\sigma}}{\eta} \sin(\omega t) + \frac{\bar{\sigma}}{\eta}$$

Integrating gives:

$$\epsilon = \frac{\bar{\sigma}}{E} \sin(\omega t) - \frac{\bar{\sigma}}{\omega \eta} \cos(\omega t) + \frac{\bar{\sigma} t}{\eta} + C$$

Substituting in boundary condition $\epsilon = 0$ @ $t = 0$:

$$0 = \frac{-\bar{\sigma}}{\omega \eta} + C \Rightarrow C = \frac{\bar{\sigma}}{\omega \eta}$$

$$\therefore \epsilon = \frac{\bar{\sigma}}{E} \sin(\omega t) - \frac{\bar{\sigma}}{\omega \eta} \cos(\omega t) + \frac{\bar{\sigma} t}{\eta} + \frac{\bar{\sigma}}{\omega \eta}$$

Now σ can be plotted against ϵ over t . Note the similarity to the multi-cycle response of the Kelvin-Voigt model, except now the increase in strain continues steadily instead of decreasing. This is thus a better model of cyclic loading when the load is large, whilst Kelvin-Voigt is better when the load is low.

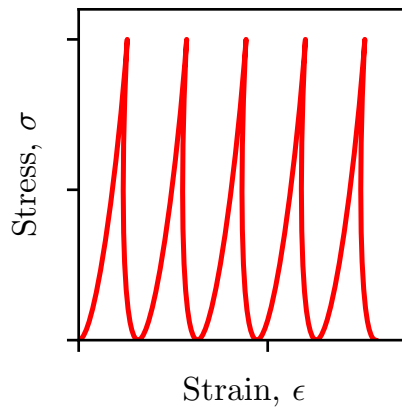
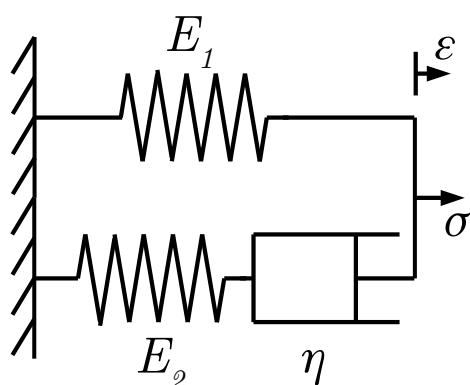


Figure A.2: *Cyclic load response of the Maxwell model*

A.3. Standard Linear Solid Model - Stress-Based Configuration

The tendon is modelled as a combination of the Kelvin-Voigt and the Maxwell models. A spring s_1 with elasticity E_1 is placed in parallel with a ‘Maxwell element’ m , comprising of a spring s_2 with elasticity E_2 and a dashpot d with viscosity η . These components are subjected to a stress σ and a strain ϵ over time t .



$$\sigma = \sigma_m + \sigma_{s_1} \quad (\text{A.11})$$

$$\epsilon = \epsilon_m = \epsilon_{s_1} \quad (\text{A.12})$$

$$\sigma_m = \sigma_d = \sigma_{s_2} \quad (\text{A.13})$$

$$\epsilon_m = \epsilon_d + \epsilon_{s_2} \quad (\text{A.14})$$

Response

The response of the system is governed by the following equation:

$$(E_1 + E_2)\dot{\epsilon} + \frac{E_1 E_2}{\eta}\epsilon = \dot{\sigma} + \frac{E_2}{\eta}\sigma \quad (\text{A.15})$$

Strain Creep

Given loading parameter $\sigma = \sigma_0$ (i.e. constant stress applied as a step response at $t = 0$), shown in Figure 2.5b:

$$\dot{\sigma} = 0$$

Substituting into A.15:

$$(E_1 + E_2)\dot{\epsilon} + \frac{E_1 E_2}{\eta}\epsilon = \frac{E_2}{\eta}\sigma_0$$

This is a first-order linear ordinary differential equation, the solution to which is as follows:

$$\epsilon = C e^{\left(\frac{-E_1 E_2}{\eta(E_1 + E_2)} t\right)} + \frac{\sigma_0}{E_1}$$

Substituting in the initial, elastic load response as a boundary condition: $\sigma_0 = (E_1 + E_2)\epsilon @ t = 0$

$$\frac{\sigma_0}{E_1 + E_2} = C + \frac{\sigma_0}{E_1} \Rightarrow C = \frac{\sigma_0}{E_1 + E_2} - \frac{\sigma_0}{E_1}$$

$$\therefore \epsilon = \left(\frac{\sigma_0}{E_1 + E_2} - \frac{\sigma_0}{E_1} \right) \times e^{\left(\frac{-E_1 E_2}{\eta(E_1 + E_2)} t\right)} + \frac{\sigma_0}{E_1} \quad (\text{A.16})$$

Thus, when $t = 0$, $\epsilon = \frac{\sigma_0}{E_1 + E_2}$, which is shown on Figure 2.5c. As $t \rightarrow \infty$, $\epsilon \rightarrow \frac{\sigma_0}{E_1}$, which is also shown in Figure 2.5c. If a time constant τ is introduced such that $\tau = \frac{\eta(E_1 + E_2)}{E_1 E_2}$, then substituting all of these into Equation A.16 gives:

$$\epsilon = (\epsilon_0 - \epsilon_\infty) \times e^{\left(\frac{-t}{\tau}\right)} + \epsilon_\infty \quad (\text{A.17})$$

Which is the form that appears in Figures 2.5c and 2.11. Stated explicitly, the constants are:

$$\epsilon_0 = \frac{\sigma_0}{E_1 + E_2} \quad (\text{A.18})$$

$$\epsilon_\infty = \frac{\sigma_0}{E_1} \quad (\text{A.19})$$

$$\tau = \frac{\eta(E_1 + E_2)}{E_1 E_2} \quad (\text{A.20})$$

which, when re-arranged, give:

$$E_1 = \frac{\sigma_0}{\epsilon_\infty} \quad (\text{A.21})$$

$$E_2 = \frac{\sigma_0}{\epsilon_0} - \frac{\sigma_0}{\epsilon_\infty} \quad (\text{A.22})$$

$$\eta = \tau_v \left(\frac{\sigma_0}{\epsilon_0} - \frac{\sigma_0}{\epsilon_\infty} \right) \quad (\text{A.23})$$

Also relevant is:

$$\epsilon_\infty - \epsilon_0 = \epsilon_{\infty-0} = \frac{\sigma_0}{E_1} - \frac{\sigma_0}{E_1 + E_2} \quad (\text{A.24})$$

Names are assigned to these constants as follows:

Table A.1: Nomenclature associated with the model

Symbol	Name
ϵ_0	Elastic strain
ϵ_∞	Viscoelastic strain
$\epsilon_{\infty-0}$	Viscous strain
τ	Viscoelastic time constant
E_1	Kelvin-Voigt stiffness
E_2	Maxwell stiffness
η	Viscosity

These can be depicted pictorially as below:

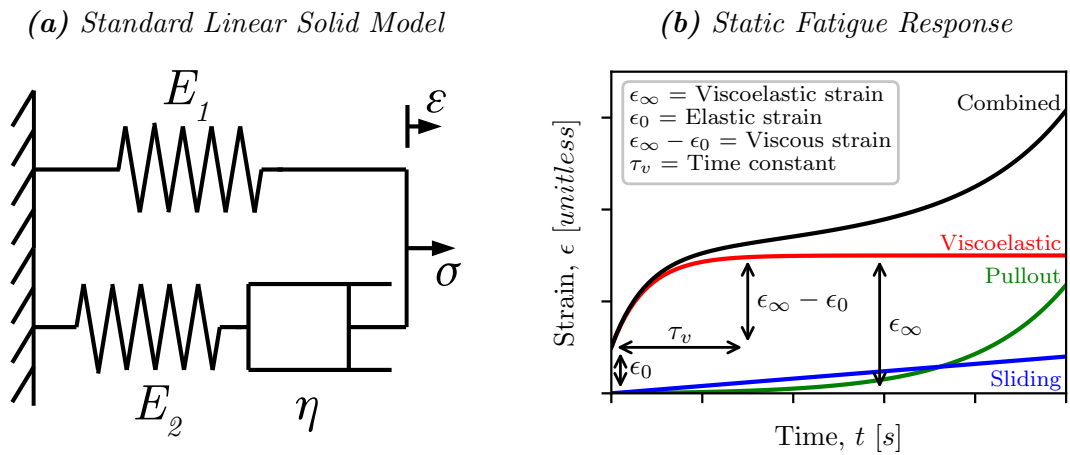


Figure A.3: Visual definition of the markers

Stress Relaxation

Given loading parameter $\epsilon = \epsilon_0$ (i.e. constant strain applied as a step response at $t = 0$), shown in Figure 2.5e:

$$\dot{\epsilon} = 0$$

Substituting into A.15:

$$\frac{E_1 E_2}{\eta} \epsilon_0 = \dot{\sigma} + \frac{E_2}{\eta} \sigma$$

This is a first-order linear ordinary differential equation, the solution to which is as follows:

$$\sigma = E_1 \epsilon_0 + C e^{\frac{-E_2}{\eta} t}$$

Substituting in the initial, elastic load response as a boundary condition: $\sigma = (E_1 + E_2) \epsilon_0$ @ $t = 0$

$$(E_1 + E_2) \epsilon_0 = E_1 \epsilon_0 + C \Rightarrow C = (E_1 + E_2) \epsilon_0 - E_1 \epsilon_0$$

$$\therefore \sigma = E_1 \epsilon_0 + ((E_1 + E_2) \epsilon_0 - E_1 \epsilon_0) \times e^{\frac{-E_2}{\eta} t} \quad (\text{A.25})$$

Thus, when $t = 0$, $\sigma = (E_1 + E_2) \epsilon_0$, which is shown in Figure 2.5f. As $t \rightarrow \infty$, $\sigma \rightarrow E_1 \epsilon_0$, which is also shown in Figure 2.5f. If a time constant τ is introduced such that $\tau = \frac{\eta}{E_2}$, then substituting all of these into Equation A.25 gives:

$$\sigma = \sigma_\infty + (\sigma_0 - \sigma_\infty) \times e^{\frac{-t}{\tau}}$$

Which is the form that appears in Figures 2.5f.

Hysteresis

Given loading parameter $\sigma = \bar{\sigma} \sin(\omega t) + \bar{\sigma}$ (i.e. cyclic tensile loading where the average load is $\bar{\sigma}$, the peak load is $2\bar{\sigma}$ and the minimum load is 0 MPa, as shown in Figure 2.5h) for many loading cycles:

$$\dot{\sigma} = \bar{\sigma} \omega \cos(\omega t)$$

Substituting into A.15:

$$(E_1 + E_2)\dot{\epsilon} + \frac{E_1 E_2}{\eta}\epsilon = \bar{\sigma}\omega \cos(\omega t) + \frac{\bar{\sigma} E_2}{\eta} \sin(\omega t) + \frac{\bar{\sigma} E_2}{\eta}$$

This is a first-order linear ordinary differential equation, the solution to which is as follows for $\omega = 1$:

$$\begin{aligned} \epsilon = & \frac{\bar{\sigma} (E_1 E_2^2 + E_2 \eta^2 + E_1 \eta^2)}{E_1^2 (E_2^2 + \eta^2) + 2E_1 E_2 \eta^2 + E_2^2 \eta^2} \sin(t) - \\ & \frac{\bar{\sigma} E_2^2 \eta}{E_1^2 (E_2^2 + \eta^2) + 2E_1 E_2 \eta^2 + E_2^2 \eta^2} \cos(t) + \\ & \bar{\sigma} \frac{\frac{E_2^2 \eta^2}{E_1} + E_1 E_2^2 + 2E_2 \eta^2 + E_1 \eta^2}{E_1^2 (E_2^2 + \eta^2) + 2E_1 E_2 \eta^2 + E_2^2 \eta^2} + \\ & C e^{\left(\frac{-E_1 E_2 t}{\eta(E_1 + E_2)}\right)} \end{aligned}$$

Substituting in a boundary condition: $\epsilon = 0$ @ $t = 0$

$$\begin{aligned} 0 = & \bar{\sigma} \frac{-E_2^2 \eta + \frac{E_2^2 \eta^2}{E_1} + E_1 E_2^2 + 2E_2 \eta^2 + E_1 \eta^2}{E_1^2 (E_2^2 + \eta^2) + 2E_1 E_2 \eta^2 + E_2^2 \eta^2} + C \\ \Rightarrow C = & \bar{\sigma} \frac{E_2^2 \eta - \frac{E_2^2 \eta^2}{E_1} - E_1 E_2^2 - 2E_2 \eta^2 - E_1 \eta^2}{E_1^2 (E_2^2 + \eta^2) + 2E_1 E_2 \eta^2 + E_2^2 \eta^2} \end{aligned}$$

This can now be plotted:

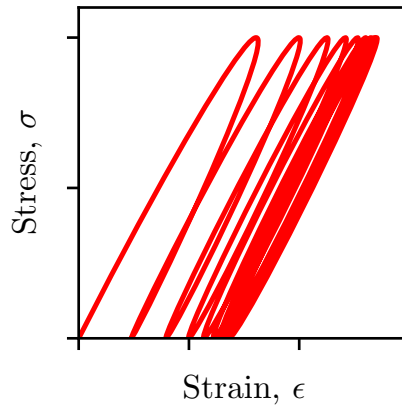
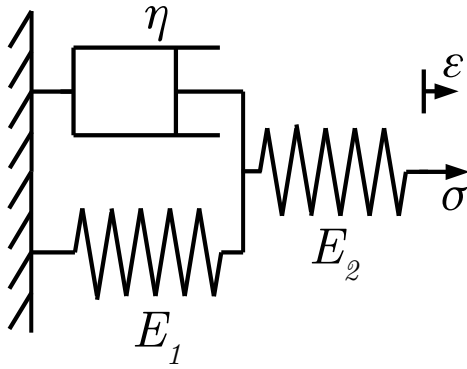


Figure A.4: Cyclic loading response of the SLSM

A.4. Standard Linear Solid Model - Strain-Based Configuration

This configuration is equivalent to a Kelvin-Voigt model in series with a spring. A ‘Kelvin-Voigt’ element kv , comprising a spring s_1 with elasticity E_1 and a dashpot d with viscosity η , is placed in series with a spring s_2 with elasticity E_2 . These components are subjected to a stress σ and a strain ϵ over time t .



$$\sigma = \sigma_{kv} = \sigma_{s_2} \quad (\text{A.26})$$

$$\epsilon = \epsilon_{kv} + \epsilon_{s_2} \quad (\text{A.27})$$

$$\sigma_{kv} = \sigma_d + \sigma_{s_1} \quad (\text{A.28})$$

$$\epsilon_{kv} = \epsilon_d = \epsilon_{s_1} \quad (\text{A.29})$$

Strain Creep

The response of the system is equal to that of a Kelvin-Voigt model with a spring in series:

$$\epsilon = \frac{\sigma_0}{E_1} \left(1 - e^{\left(\frac{-E_1}{\eta} t \right)} \right) + \frac{\sigma_0}{E_2}$$

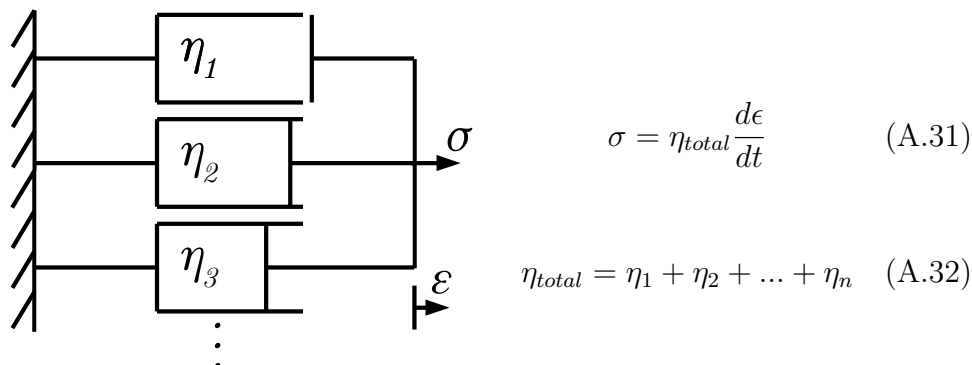
Thus, when $t = 0$, $\epsilon = \frac{\sigma_0}{E_2}$. As $t \rightarrow \infty$, $\epsilon \rightarrow \frac{\sigma_0}{E_1} + \frac{\sigma_0}{E_2}$. If a time constant τ is introduced such that $\tau = \frac{\eta}{E_1}$, then substituting all of these in the previous equation gives:

$$\epsilon = (\epsilon_\infty - \epsilon_0) \times e^{\left(\frac{-t}{\tau} \right)} + \epsilon_0 \quad (\text{A.30})$$

A.5. Sliding and Pullout Model

The mechanisms of permanent viscoelastic deformation - molecular, fibril and fibre sliding - can be modelled as n dashpots in parallel (where n is large but finite) each with viscosity

η_i . These components are subjected to a constant stress σ over time t which causes a strain ϵ .



Non-Permanent Response

To ensure consistency with the Standard Linear Solid Model outlined in A.3, the dashpots are all assumed to have a static friction. Thus, below a certain force threshold, the dashpots will not strain at all. This means that the model is identical to an SLSM at low forces.

Sliding Response

From Equation A.31:

$$\sigma dt = \eta_{total} d\epsilon$$

Integrating both sides:

$$\sigma t = \eta_{total} \epsilon$$

$$\epsilon = \frac{\sigma t}{\eta_{total}}$$

Thus, as σ and η_{total} are constant, strain will increase linearly over time.

Pullout Response

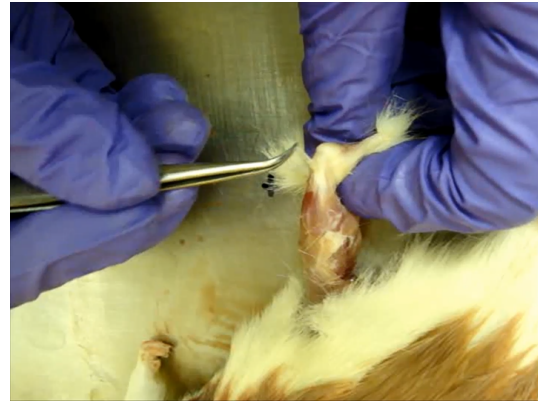
Eventually, as the dashpots continue extending, they will reach their maximum lengths. After this, the plungers will pull free from their dashpots (in the same way that a fibril will pull free from another after sufficient sliding). Each dashpot will ‘pull out’ at a different value of ϵ , and once they have pulled out they will no longer contribute to the

model. Thus, from Equation A.32, η_{total} will reduce each time a dashpot pulls out and the gradient of the strain-time response (given by the last ‘sliding response’ equation) will thus increase, resulting in the plot seen in Figure 2.10b. This will continue to happen until all the dashpots have pulled out (i.e. rupture).

B. Rat Dissection: Video Protocol



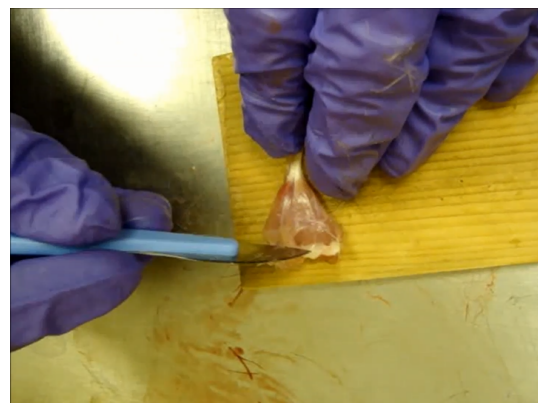
(a) Skin the leg completely



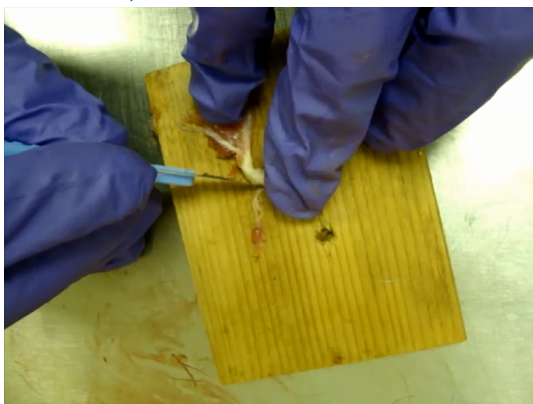
(b) Cut away at the sheath surrounding the Achilles tendon



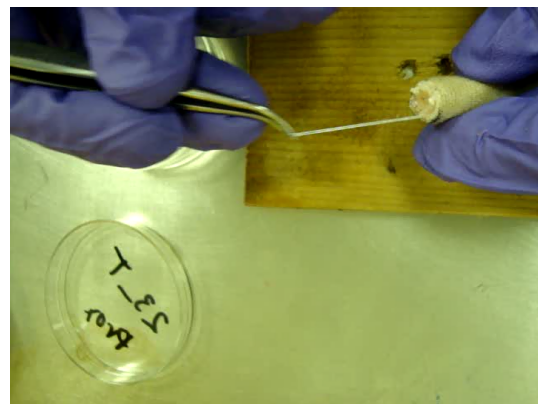
(c) Resect the forepaw (leaving the calcaneus) and separate tendon from tibia



(d) Use blunt dissection to remove muscle



(e) Continue to remove extraneous material to obtain the Achilles tendon

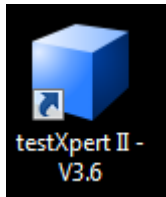


(f) To obtain tails tendons, slide these out of the tail

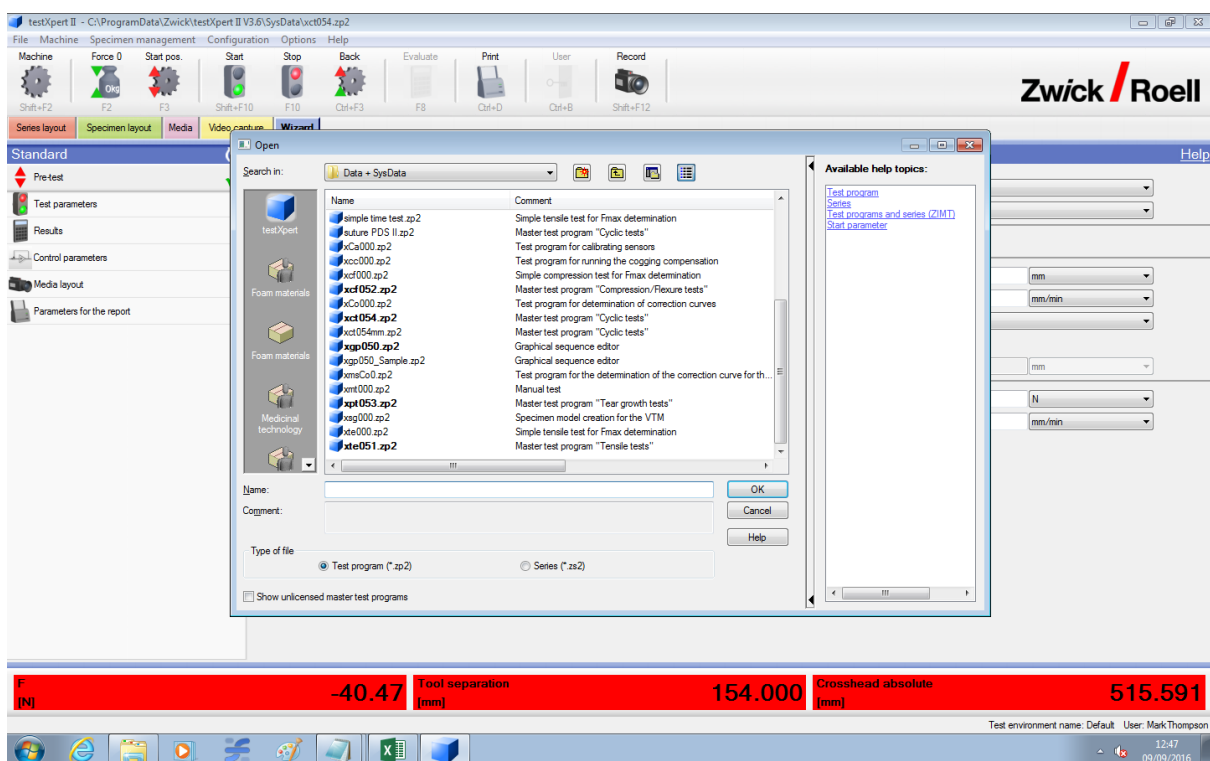
Figure B.1: Selected frames from the instructional video

C. The Susceptibility of Tendon to Damage: Software Protocol

1. Open testXpert II - V3.6



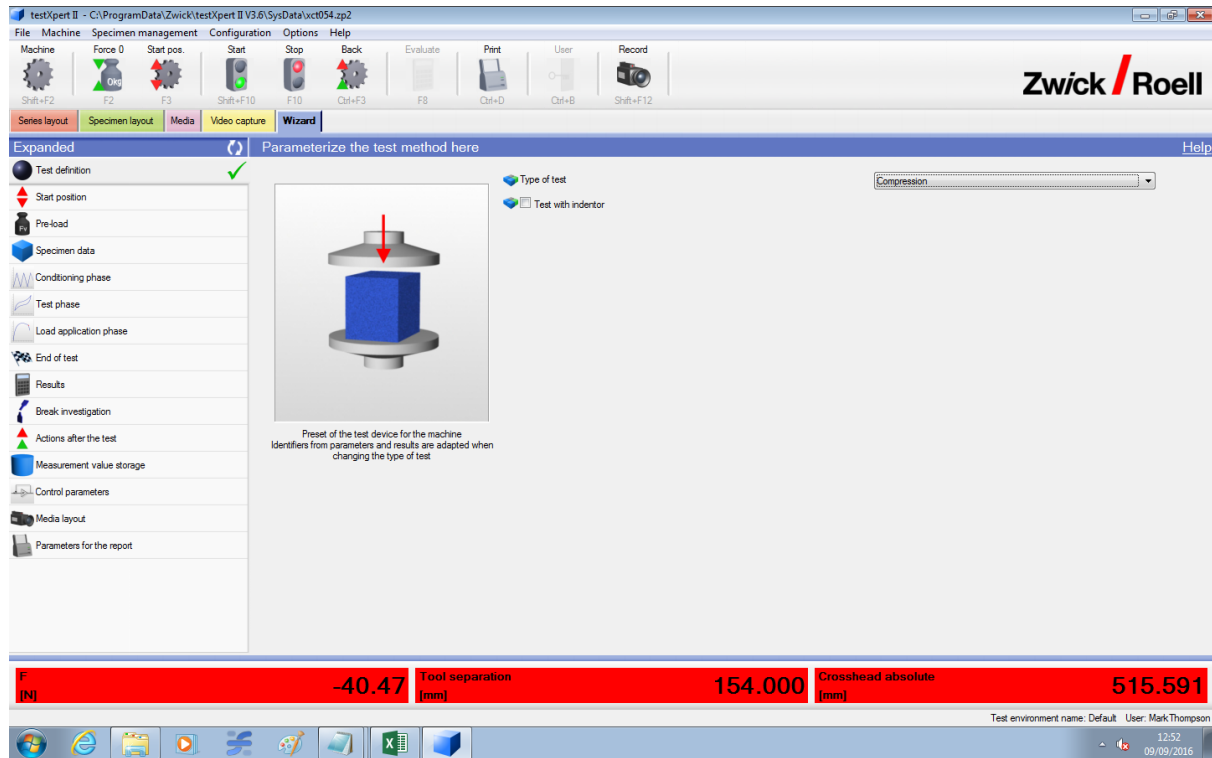
2. File → Open → Search in C:\ProgramData\Zwick\testXpert II V3.6\<Data + SysData> → select the 'Master test program' most aligned with the intended test. xct054.zp2 was used for this experiment (a template for creep tests, both static and cyclic)



3. Select 'Neutral' terminology

4. Five coloured tabs will be visible: 'Series layout', 'Specimen layout', 'Media', 'Video

Capture' and 'Wizard'. In the 'Wizard' tab, click the circular arrows (located below the 'Video Capture' tag) to change from Standard to Expanded.



5. Save as a 'Test program' (.zp2). Save the parameters via Options → Export Wizard parameters or Export all parameters.

6. Change more parameters at Configuration → Parameters. A parameter assigned to the 'Series level' will affect all samples tested while one assigned to the 'Specimen level' will change for each run.

7. Setup the exporting of data via File → Export interfaces:

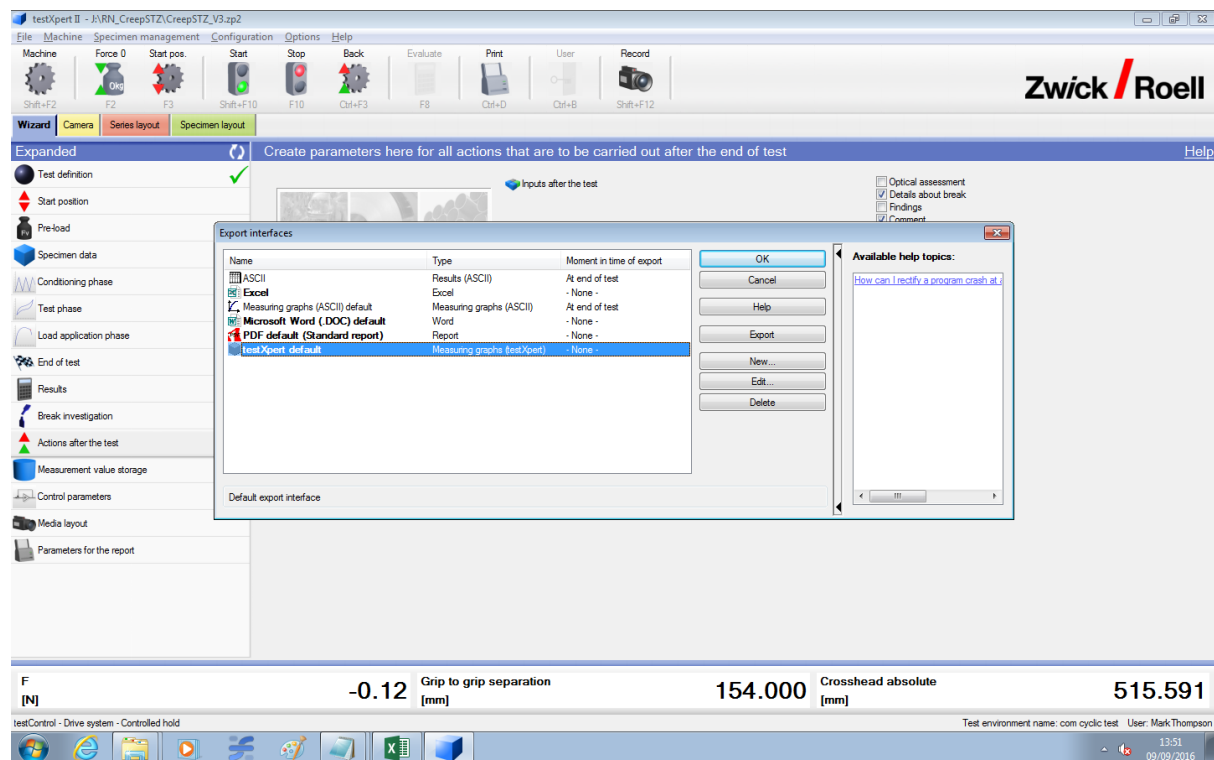
7.1 the 'Results' as defined in the Wizard (in this experiment they are the 'Details about break' and 'Comment' text inputs) are exported via ASCII → Moment at time of export → change to 'At end of test' → click 'Edit' → Export file → Export to a File → Freely configurable path and filename → Edit → Edit the path and filename as you wish (e.g. include the specimen number in the filename and set file extension to .txt) → can edit the Format settings, Export mode, etc

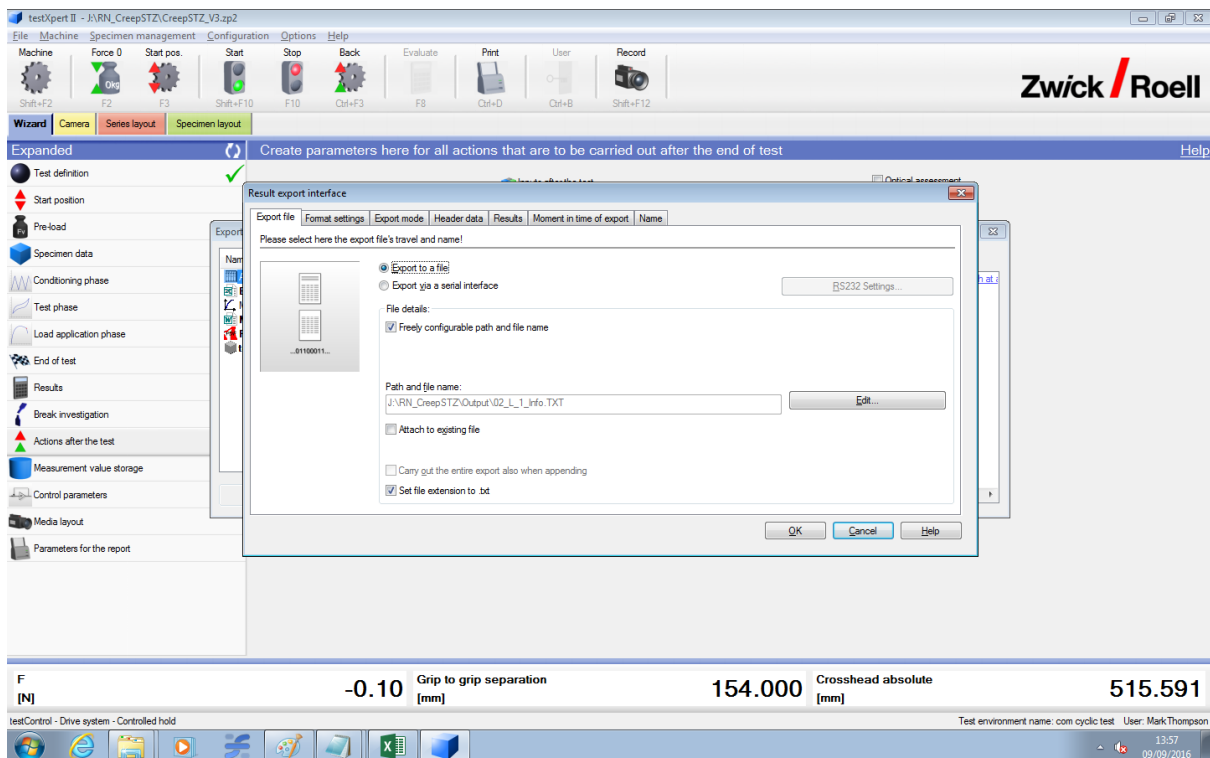
7.2 Measuring graphs (ASCII) default: this exports all the Time, Force and Grip-to-Grip

Separation data in a .txt file. Change ‘Moment in time of export’ to ‘At end of test’ and click ‘Edit’ to change the filename and edit the other settings as before.

7.3 The data can also be exported to Excel. While this is slower, it automatically plots a graph of the data which is useful in providing immediate overall feedback as to the success of the run; it can be used to confirm that the correct graph shape is being achieved. Once the protocol is finalised and a few samples have been run without incident, there is no reason to export to Excel and it should be stopped (the files are relatively large and thus should not be exported without good reason).

7.4 If a webcam is plugged into the USB port it should connect automatically. Edit the settings for the video exports.





8. Turn the Zwick machine on at the wall → flick the switch at the back of the tower → press the ‘On’ button at the front of the tower (testXpert II needs to be opened and loaded before doing this). The bar at the bottom of the testXpert software will change from red to white.

9. To start a test, load a specimen (this might involve moving the gripper up and down via editing the ‘Start position’ parameter in the ‘Wizard’) then press ‘Force 0’ (F2) and ‘Start’ (Shift+F10)

D. The Susceptibility of Tendon to Damage: Written Protocol

Before Starting

- File → Export interfaces → ASCII, Excel Default, Measuring graphs (ASCII) default → At end of test
- Extract video frames using Python (videos must be converted to mp4 first)

Apparatus Setup

- Computer on, RN_CreepSTZ folder, Info Sheet.txt, RN_CreepSTZ_V3.xls open
- Load cell installed and calibrated
- Micrometer setup with retort stand
- Webcam on retort stand and plugged in
- Setup instruments for sample use 1: double layer blue towel, small petri dish
- Setup instruments for sample use 2: double layer blue towel, screws in lid, allen key, thin tweezers, scissors, fineliner, both grippers, plates, rubber parts, one of the box's long screws
- Setup instruments for apparatus use: wedges, Perspex box (empty or fill & in blue box), the other one of the box's long screws, bluetack, ruler, cell phone, cell phone cover, gloves, PBS, squeeze bottle with fresh PBS, funnel
- Place camera against base, square to it (use 2xblue tack to mark position)
- Place retort stand for camera with white backing attached

Apparatus Preparation

- Lights in room on
- Zwick machine on at wall, black switch hidden in the back of the Zwick's tower → wait a bit
- Open Creep_STZ_V3.zp2 → wait a few seconds
- Zwick tower's front 'ON' switch → bottom bar in Creep_STZ_V3.zp2 turns white

Sample Preparation

- Place sample in small petri dish, return others to storage
- Open 'RN_CreepSTZ_V3.xls' → enter details of new sample
- Turn on laser micrometre
- If ACHILLES tendon, run sample over tissue to get rid of excess PBS
- If TAIL tendon, mark off a section with a fineliner
- Hang sample from retort stand → take 2 measurements at right angles at 5 points along length
- Turn off micrometer
- If ACHILLES tendon, put sample on paper towel → make 2 dots with fineliner
- If TAIL tendon, rehydrate sample in PBS → remove excess PBS on towel → make 4 dots with fineliner → return sample to PBS

Preparation for Data Analysis/Calibration

- Load the Perspex box (hook piece front, wedged, lowermost right screw thread entirely away from clamp body, head half-away) → half-fill with PBS
- Place ruler into centre of Perspex box, where the sample would run
- Focus camera (right click, configure, device settings, camera control, focus) → take manual photo → don't touch camera from this point

Sample Loading

- Use nuts and screw to attach grippers
- Load distal end of sample into lower gripper
- Load upper gripper into upper clamp, holding both grippers (250mm G-G is a good distance to move)
- Lower gripper until holes line up (165mm for sandwich-to-sandwich; 160mm for wedge-to-sandwich)
- Attach lower gripper to box, unattach grippers
- Add PBS to fill line
- Check that grippers are vertically aligned and proximal one will move upwards without an obstacle → use the retort stand from the micrometer if needed (recommended for ACHILLES tendon)

Test Preparation

- Calculate smallest CSA of an ellipsis → get required force for 30 MPa for tail tendons, 10 MPa for Achilles tendon (and 2,5 MPa for tail tendon, 250 kPa for Achilles tendon)
- Enter the required Point of Load Application force into 'Wizard' - 'Test phase'
- Start phone stopwatch → put phone in sleeve → position phone
- Wait for phone light to dim
- Check camera focus
- Open screen capture software → check filepath → start recording

Test

- Force 0 → Start
- Enter 'Specimen ID', 'Type', Rat # T/L/R Run #
- Take screen shots to Paint
- Occasionally stop and re-start screen capture

After test

- Enter 'Detail about break' and 'Comment' wet zone/dry zone/at grip 'Break-Dots(2&3) PlasticDots(3&4) Y' → which are the BreakDots, PlasticDots and did you turn the sample around when measuring plastic deformation
- Close and exit screen capture software
- Save the paint files
- Close and then move the output files to new folder
- Leave sample in PBS for at least 2 min

Relaxation Test

- Unload sample: unattach lower gripper from box → hold both grippers → unclamp upper gripper
- Remove one part (note which dots are being looked at - Dot 1 is at the top - and if the sample is being turned upside down) → re-attach grippers
- Re-load (163 mm G-G separation is good)
- Re-position phone/restart stopwatch as needed

- Wait for phone light to dim
- Check camera focus
- ‘RN_CreepSTZ.ods’ → enter details of new run
- Open screen capture software → check filepath → start recording
- Force 0 → Start
- Enter ‘Specimen ID’, ‘Type’, Rat # T/L/R Run #
- Take screen shots to paint

After Test

- Enter ‘Detail about break’ and ‘Comment’ wet zone/dry zone/at grip ‘Break-Dots(2&3) PlasticDots(3&4) Y’ → which are the BreakDots, PlasticDots and did you turn the sample around when measuring plastic deformation
- Close and exit screen capture software
- Save the paint files
- Close and then move the output files to new folder

Final

- Close testXpertII → save data file to last run’s folder
- Unload grippers → throw sample away

E. The Susceptibility of Tendon to Damage: Mechanistic Markers

In addition to the phenomenological markers reported in Chapter 3 a number of mechanistic markers were calculated from the same data.

E.1. Materials and Methods

These mechanistic markers relate to the model that was used to fit the data and as such represent the physical processes that occurred within the samples. Specifically, this model is a modified version of the stress-based configuration of the Standard Linear Solid Model (shown in Figures 3.4a and E.1a) the static fatigue response to which is shown in Figures 3.4b and E.1b. The mathematical representation of this response - i.e. the equation that was fitted to the static fatigue data - is:

$$\epsilon = -(\epsilon_{\infty} - \epsilon_0) \times e^{\left(\frac{-t}{\tau_v}\right)} + \epsilon_{\infty} + m \times t + A \left(e^{\left(\frac{t}{\tau_d}\right)} - 1 \right) \quad (\text{E.1})$$

where ϵ_0 = initial viscoelastic strain (also called ‘elastic strain’), $(\epsilon_{\infty} - \epsilon_0)$ = marginal viscoelastic strain (also called ‘viscous strain’) and τ_v = viscoelastic time constant. These three constants are the equation-based mechanistic markers that are reported here. Further, the relationships between these and the components of the SLSM (the Kelvin-Voigt spring with stiffness E_1 , the Maxwell spring with stiffness E_2 and the dashpot with viscosity η) are as follows:

$$E_1 = \frac{\sigma_0}{\epsilon_{\infty}} \quad (\text{E.2})$$

$$E_2 = \frac{\sigma_0}{\epsilon_0} - \frac{\sigma_0}{\epsilon_{\infty}} \quad (\text{E.3})$$

$$\eta = \tau_v \left(\frac{\sigma_0}{\epsilon_0} - \frac{\sigma_0}{\epsilon_\infty} \right) \quad (\text{E.4})$$

with these having been derived in Appendix A.3 (Equations A.21 to A.23). These three constants are the model-based mechanistic markers that are reported here.

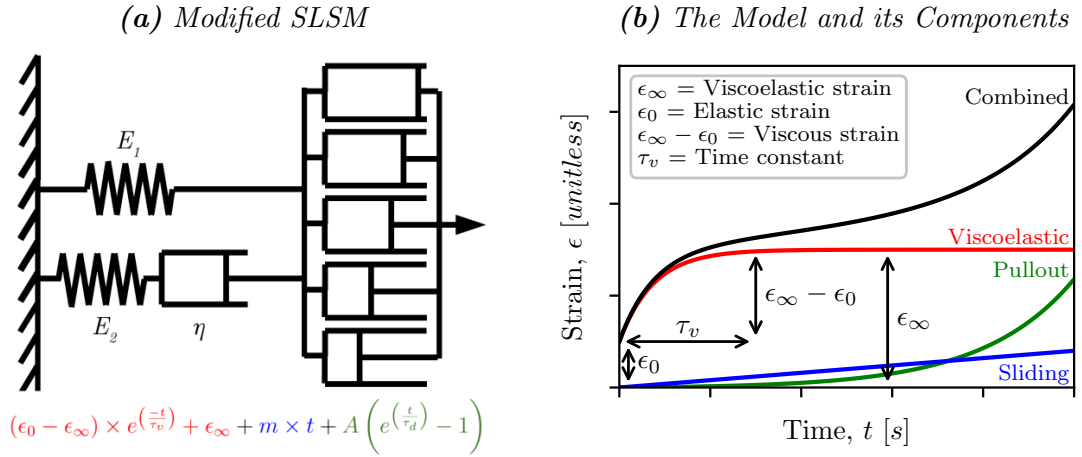


Figure E.1: Details of the model applied to the static fatigue data

Statistical analysis was performed on all six mechanistic markers in the same manner as was done on the six phenomenological markers. One instance of outlier rejection occurred (the viscoelastic time constant of an STZ-treated Achilles tendon) and one result had all categories deemed Normal (the stiffness of the Kelvin-Voigt spring, E_1). The uncertainty of each of the markers was calculated by first considering the process whereby the model's equation had been fitted to the data. As this fitting was performed using Python's `least_squares` subroutine it was thus possible to also calculate the relevant Jacobian matrix. This could subsequently be converted into an approximation of the Hessian matrix and then into an approximation of the covariance matrix. This was then multiplied by the residual variance before the square roots of the diagonal elements were taken, yielding the standard deviations of the fitted parameters. From these, the uncertainties of the mechanistic markers were estimated. The smallest was 0.00208% (viscous strain) and the largest was 0.0640% (viscosity).

E.2. Results

Values are reported in Table E.1 and represented graphically in Figure E.2.

E.2.1. STZ Model vs Healthy Controls

STZ treatment caused significant increases in the values of the viscoelastic times constants and of tail tendons' viscosities, while it caused a decrease in the Maxwell spring stiffness of Achilles tendons.

E.2.2. Energy-Storage vs Positional Functional Roles

Significant differences due to functional role were found in all markers and all tendon types apart from the viscoelastic time constants and viscous strains of STZ tails. In the markers derived from the fitted equation, these differences were decreases (in tail tendons compared to Achilles) whilst in markers derived from the springs and dashpots they were increases.

Table E.1: Data is reported as median $\pm$ IQR and p -values (Mann-Whitney U test) are shown in italics alongside the experimental groups they refer to. Significance ($p \leq 0.05$) is denoted with a star ($\star$) when it refers to STZ and with a circle ($\circ$) when it refers to functional role. For sample sizes, see Figure E.2

<i>Viscoelastic Time Constant, τ_v [s]</i>			
	STZ	Control	<i>Significance</i>
Tail	541 $\pm$ 827	27.0 $\pm$ 58.3	$p=0.004\star$
Achilles	932 $\pm$ 304	134 $\pm$ 407	$p=0.007\star$
<i>Significance</i>	$p=0.62$	$p=0.001\circ$	
<i>Elastic Strain, ϵ_0 [%]</i>			
	STZ	Control	<i>Significance</i>
Tail	4.24 $\pm$ 3.05	2.59 $\pm$ 2.27	$p=0.74$
Achilles	80.0 $\pm$ 56.5	36.9 $\pm$ 23.9	$p=0.08$
<i>Significance</i>	$p=0.003\circ$	$p=0.00001\circ$	
<i>Viscous Strain, $\epsilon_0 - \epsilon_\infty$ [%]</i>			
	STZ	Control	<i>Significance</i>
Tail	1.19 $\pm$ 0.505	1.19 $\pm$ 0.501	$p=0.96$
Achilles	3.07 $\pm$ 8.99	4.39 $\pm$ 12.8	$p=0.65$
<i>Significance</i>	$p=0.22$	$p=0.01\circ$	
<i>Viscosity, η [GPa $\cdot$ s]</i>			
	STZ	Control	<i>Significance</i>
Tail	156 $\pm$ 230	1.46 $\pm$ 7.02	$p=0.006\star$
Achilles	0.308 $\pm$ 0.373	0.300 $\pm$ 0.350	$p=0.49$
<i>Significance</i>	$p=0.003\circ$	$p=0.02\circ$	
<i>Kelvin-Voigt Spring Stiffness, E_1 [MPa]</i>			
	STZ	Control	<i>Significance</i>
Tail	440 $\pm$ 214	595 $\pm$ 520	$p=0.81$
Achilles	12.2 $\pm$ 11.4	20.1 $\pm$ 16.6	$p=0.32$
<i>Significance</i>	$p=0.003\circ$	$p=0.0002\circ$	
<i>Maxwell Spring Stiffness, E_2 [MPa]</i>			
	STZ	Control	<i>Significance</i>
Tail	207 $\pm$ 711	336 $\pm$ 405	$p=0.89$
Achilles	0.685 $\pm$ 0.753	1.81 $\pm$ 2.22	$p=0.02\star$
<i>Significance</i>	$p=0.003\circ$	$p=0.00001\circ$	

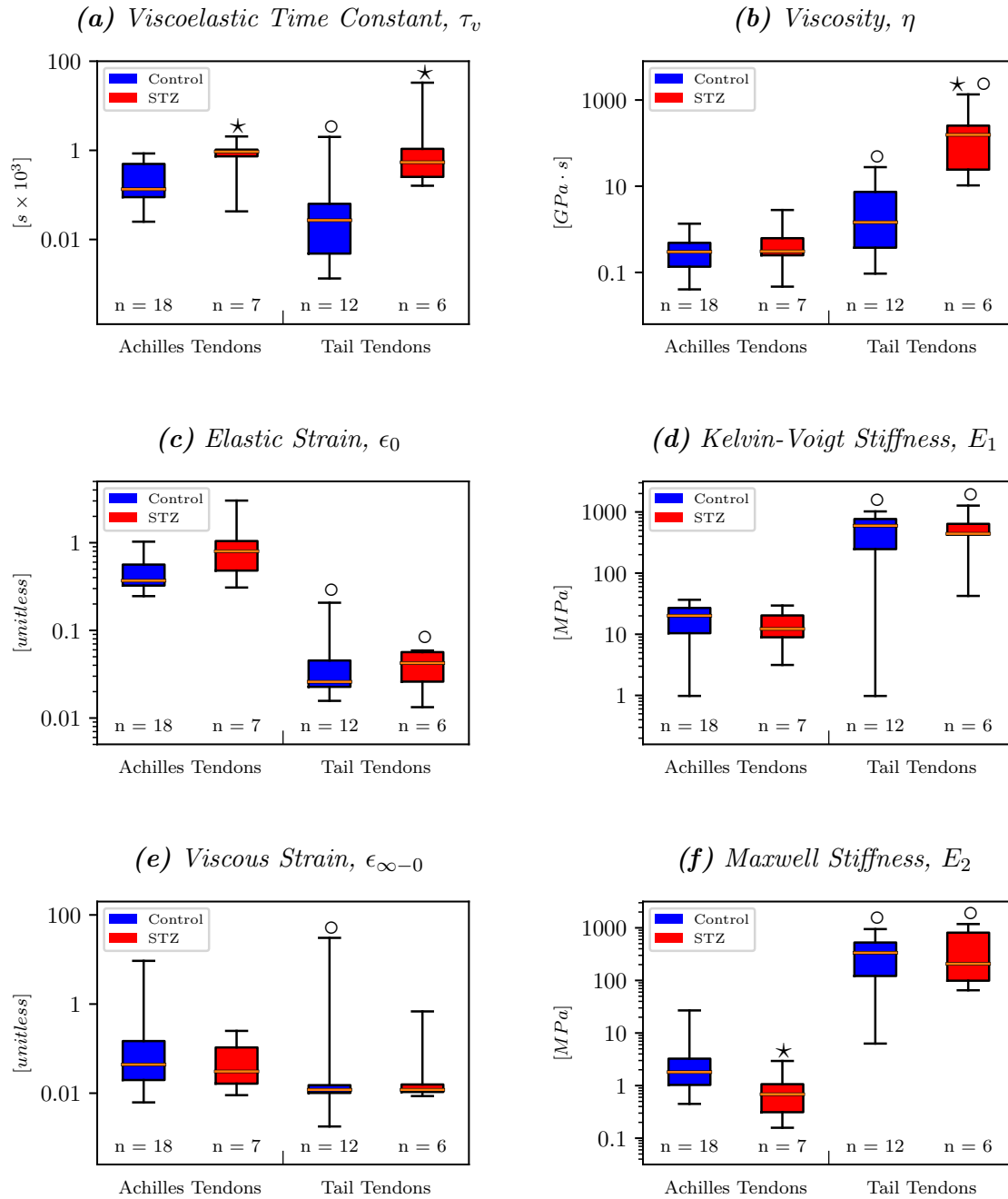


Figure E.2: Box-and-whisker plots of the characteristics of the applied model, representative of mechanistic markers. Significant differences ($p \leq 0.05$, Mann-Whitney) due to STZ treatment are seen in the viscoelastic time constants (a), viscosities of tail tendons (b) and Maxwell spring stiffnesses of Achilles tendons (f).

E.3. Discussion

The values of the uncertainties related to the calculations of the results - which ranged from 0.0021% (viscous strain) to 0.064% (Kelvin-Voigt stiffness) - were considered too small relative to the inter-quartile ranges to be relevant. Note also that these mechanistic markers are only those that relate to the viscoelastic portion of the model (as opposed to the sliding or damaging portions). This is because this component is the least stochastic of the three and thus it is the most representative of innate material properties.

E.3.1. The Standard Linear Solid Model

In this investigation, the mechanisms behind the strain response were not measured directly but rather modelled (see Figure 2.11). This model consisted of three parts - pullout, sliding and viscoelasticity - only the last of which is reported in the results. The reason for not reporting the pullout portion of the model is its high stochasticity, while the sliding portion was not reported due to its similarity with the steady-state strain rate measurement which is already included in the main results. As such, after the model's equation (Equation E.1) was fitted to the data, the elastic strain ϵ_0 , the viscous strain $\epsilon_\infty - \epsilon_0$ and the viscoelastic time constant τ_v were determined from the viscoelastic portion's constants, see Figure E.1b. These represent the following:

- The length of the viscoelastic process (viscoelastic time constant)
- The magnitude of the viscoelastic process (viscous strain)
- The initial elastic response, which is independent of viscoelasticity (elastic strain)

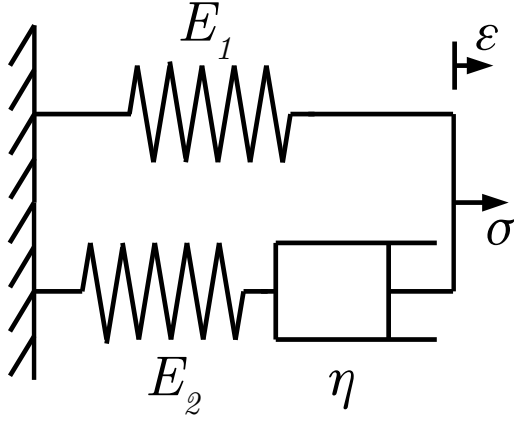
Once these constants were calculated, the standard linear solid model (SLSM) from which the viscoelastic portion of the model is derived could be considered (see Figure E.1a). While it is possible to represent the SLSM in two different (but mathematically equivalent) ways - as a Maxwell model in parallel with a spring (Figure E.3a) or as a Kelvin-Voigt model in series with a spring (Figure E.3c), see Appendix A.3 and A.4 - only the former arrangement was analysed here (yielding values for its viscosity η , its Kelvin-Voigt spring's stiffness E_1 and its Maxwell spring's stiffness E_2 , see Figure E.3b). The latter arrangement

was not used because its results would have effectively been the same as ϵ_0 , $\epsilon_\infty - \epsilon_0$ and τ_v (they would merely have differed by constants, see Figure E.3d), and thus they would not have added additional insight. More to the point, the difference between the two SLSM arrangements is that one models how the tendon dissipates stress while the other models how it distributes strain. Therefore, because the equation used to fit the strain vs time data is a model of strain, it is unsurprising that the results derived from it are similar to those derived from the strain-based SLSM. As such, the SLSM arrangement chosen for this experiment is instead that which models how a tendon dissipates stress. In particular, the way in which a tendon dissipates stress is as follows: initially, load is taken up by both the viscous and the elastic tendon components but, over time, the viscous stress reduces to zero whilst the elastic stress increases to 100% of that applied. These are represented in the SLSM by the Maxwell element and the Kelvin-Voigt spring, respectively. Note that it is the viscous and elastic *stresses* that are being modelled as opposed to the viscous and elastic components themselves. This is because the strains of the individual springs and dashpot do not match up with those of physical components (the opposite would be true in the strain-based SLSM arrangement: the stresses would not match up). Also note that the SLSM represents the non-damaging viscoelastic process, and as such the mechanisms being modelled are recoverable. Specifically, these mechanisms are:

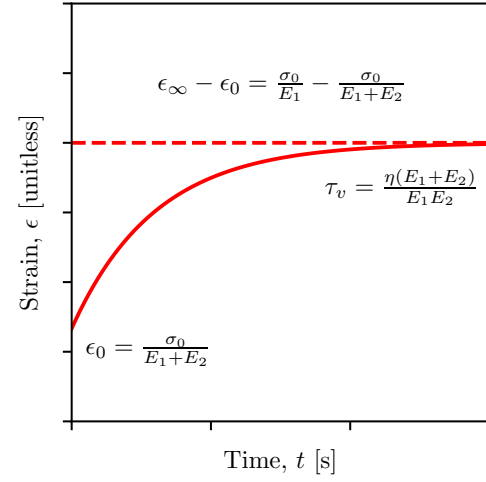
- Stress dissipation via the (elastic) stretching of collagen molecules, fibrils and fibres (this is modelled by the Kelvin-Voigt spring)
- The initial stress absorption by water molecules when load is first applied (modelled by the Maxwell spring)
- The resistance to the transfer of stress from the water to the collagen (modelled by the dashpot), i.e. the resistance to the movement of water molecules and to collagen sliding

With this established, the effects of the STZ model and of functional role can now be analysed.

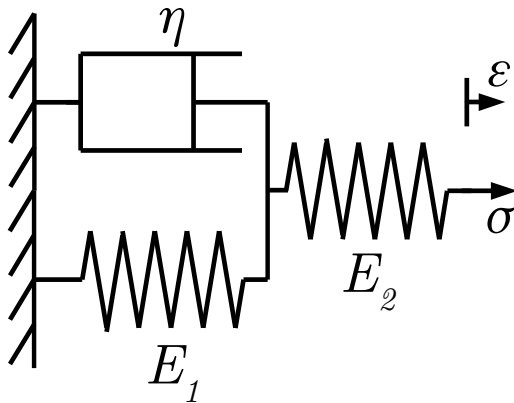
(a) Stress-based SLSM configuration



(b) Strain response of stress-based SLSM



(c) Strain-based SLSM configuration



(d) Strain response of strain-based SLSM

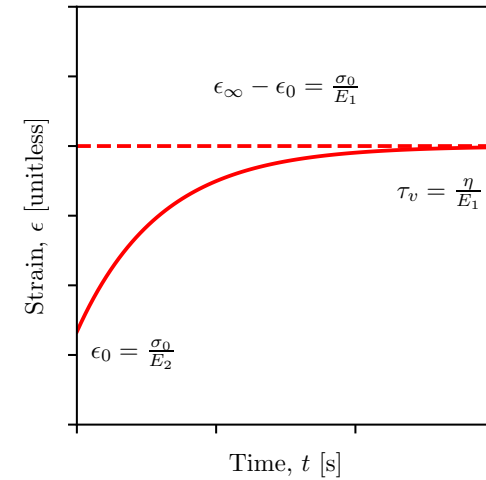


Figure E.3: The two different configurations of the SLSM give mathematically equivalent responses but different values for viscous strain ($\epsilon_\infty - \epsilon_0$), elastic strain (ϵ_0) and viscoelastic time constant (τ_v). In particular, the values for these three results as given by the strain-based model are very similar to its values for E_1 , E_2 and η , respectively. Indeed, as shown by the equations in (d), they merely differ by constants. Thus, this model adds less insight into the mechanisms behind the creep response compared to the stress-based model, and as such it was not used.

E.3.2. STZ-Model vs Healthy Controls

Again, as it was with the phenomenological markers, the viscous components appear to be more affected by the STZ model than the elastic components are and, again, the effect in question is a lengthening of the viscoelastic process. This is seen in the significant increases of both tendons' viscoelastic time constants, in the increase of the tail's viscosity and in the decrease of the Achilles's Maxwell spring stiffness - these being the three viscous-based results. Conversely, the three elastic-based results - elastic strain, Kelvin-Voigt stiffness and viscous strain (the magnitude of which, despite the name, depends heavily on elastic components) - show no significance. In other words, STZ causes net damage (the PoI) to be delayed by slowing down the viscoelastic response (although the magnitude of the viscoelastic response remains the same). In a strain vs time plot, this manifests as a less tight curve (i.e. a larger time constant) the start (elastic strain) and height increase (viscous strain) of which remain unchanged. In tail tendons, this is achieved by increasing the resistance to the transfer of stress from the initially-loaded water molecules onto the collagen (i.e. increased viscosity). In Achilles tendons, this is achieved by not loading the water molecules to as high a stress in the first place (i.e. decreased Maxwell spring stiffness) which decreases the forces that cause them to move. The stresses taken up by the elastic components - which cause the elastic and viscous strains - do not change.

E.3.3. Energy-Storage vs Positional Functional Roles

It is known that viscoelasticity has an energy absorption effect (literally: damping), and thus it is expected that the results related to viscoelasticity will be significantly different across functional roles. Specifically, in taut tail tendons compared to spring-like Achilles tendons, the time constant should be lower, the viscosity higher and the viscous strain lower. Whilst this is indeed the case in general, it appears that STZ has a masking effect on this: out of the three, only viscosity is significant in STZ-treated tail tendons while all are significant in control tail tendons. The results that are independent of (or less-dependent on) viscoelasticity - i.e. elastic strain, Kelvin-Voigt stiffness and Maxwell stiffness - are all significantly different in tails compared to Achilles. This is because,

in vivo, a tail tendon strains considerably less and over shorter periods of time than an Achilles, and thus ϵ_0 , E_1 and E_2 need to be smaller, larger and larger, respectively.

F. The Susceptibility of Tendon to Damage: Effect of Rat Strain

Table F.1: Results from the control samples only, sorted by rat strain. Data is reported as median $\pm$ IQR with significance due to rat strain ($p \leq 0.05$; Mann-Whitney U test) denoted with a circle ($\circ$). For sample sizes, see Figure F.1

<i>Steady-State Strain Rate, $\dot{\epsilon}_{PoI}$ [$\mu\text{strain/s}$]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	15.7 $\pm$ 235	44.4 $\pm$ 56.1	$p=0.59$
Tail	571 $\pm$ 1440	908 $\pm$ 1009	$p=0.97$
<i>Loading Modulus, E [MPa]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	48.5 $\pm$ 56.8	72.0 $\pm$ 38.5	$p=0.20$
Tail	361 $\pm$ 503	177 $\pm$ 643	$p=0.18$
<i>Permanent Strain, ϵ_p [%]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	1.89 $\pm$ 2.56	4.86 $\pm$ 5.26	$p=0.96$
Tail	0.758 $\pm$ 1.35	2.83 $\pm$ 5.02	$p=0.58$
<i>Strain Energy at the PoI, U_{PoI} [J]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	35.5 $\pm$ 26.7	50.9 $\pm$ 21.4	$p=0.42$
Tail	9.19 $\pm$ 9.23	0.995 $\pm$ 0.313	$p=0.36$
<i>Time at the PoI, t_{PoI} [min]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	10.7 $\pm$ 33.1	11.5 $\pm$ 41.0	$p=0.59$
Tail	1.10 $\pm$ 2.93	0.515 $\pm$ 1.77	$p=0.58$
<i>Strain at the PoI, ϵ [%]</i>			
	Wistar	Sprague-Dawley	<i>Significance</i>
Achilles	60.3 $\pm$ 66.3	39.3 $\pm$ 17.1	$p=0.24$
Tail	12.4 $\pm$ 8.21	6.17 $\pm$ 5.24	$p=0.36$

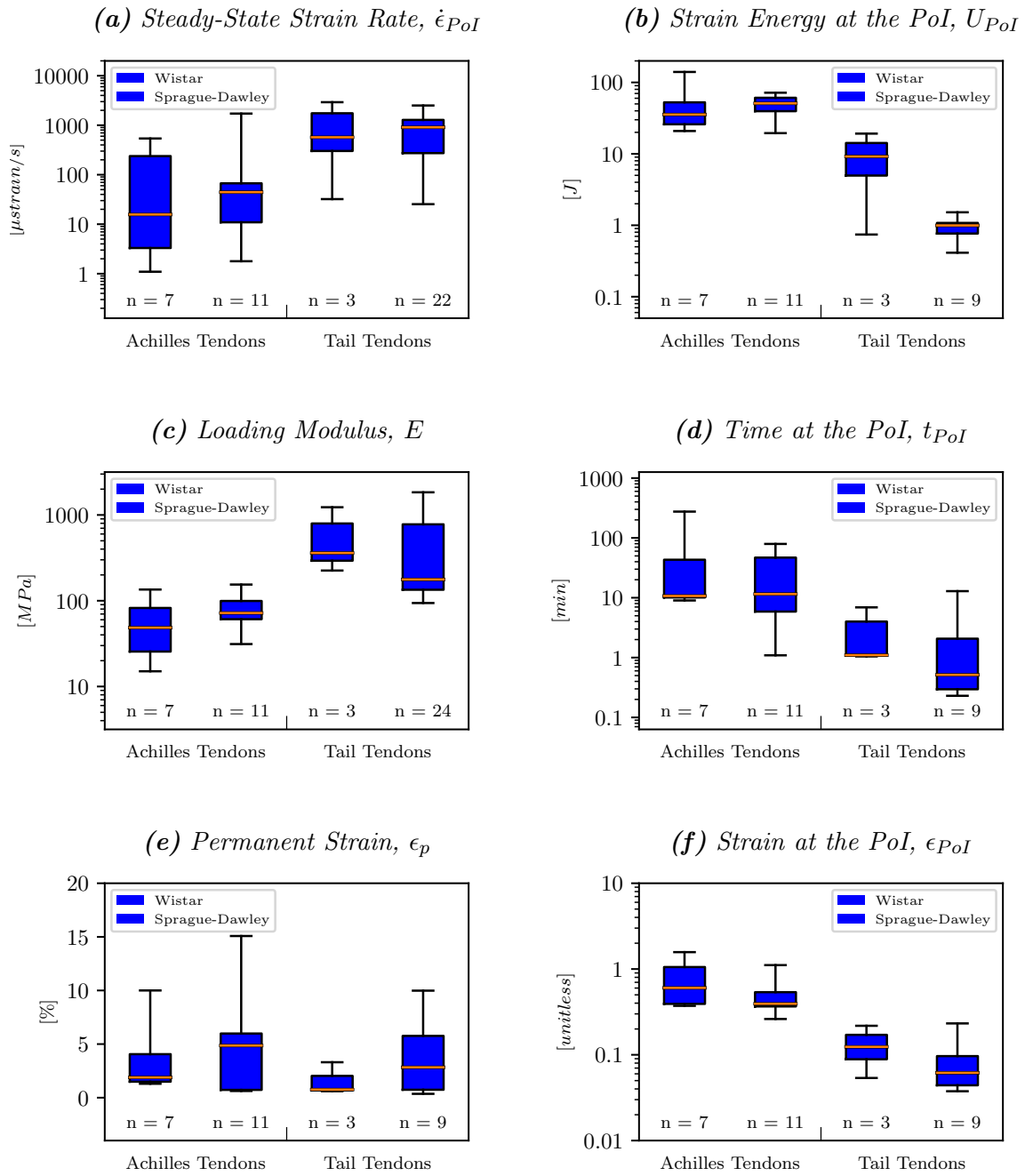
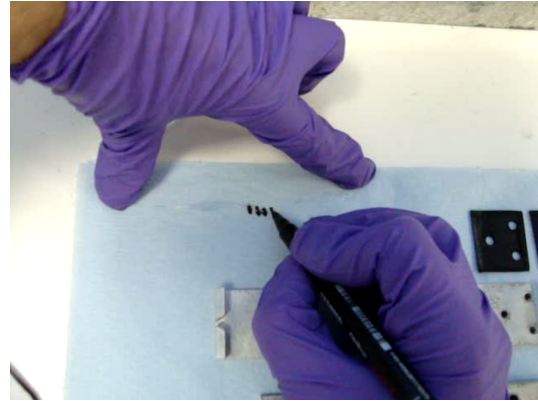


Figure F.1: Box-and-whisker plots of the phenomenological markers, only including control samples and separated by rat strain. No significant differences are seen between any groups.

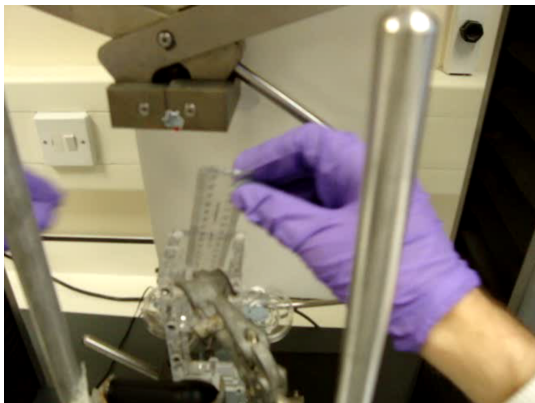
G. Steady-State Strain Rate as a Measure of Damage: Video Protocol



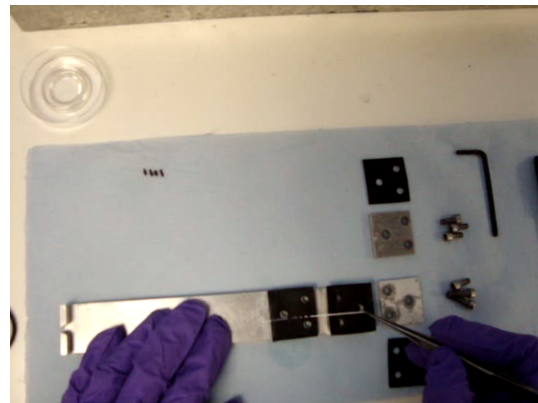
(a) Diameter measurement using an optical micrometer



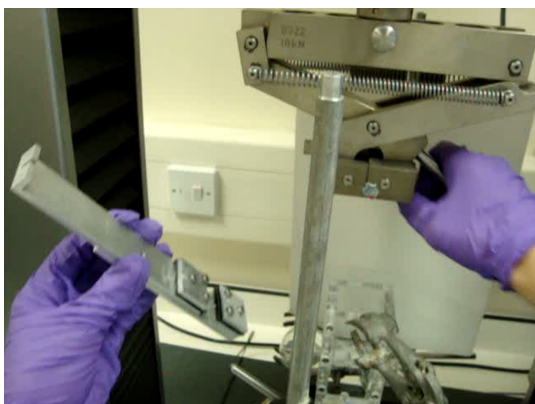
(b) Marking dots on the sample



(c) Placing ruler for still frame



(d) Loading sample into grippers



(e) Loading upper gripper into clamp



(f) Fastening lower gripper to Perspex box

Figure G.1: Selected frames from the instructional video

H. Quantification of AGEs: Written Protocol

H.1. Preparation

Hydrochloric Acid

The protocol calls for HCl with a concentration of 6M. The undiluted HCl available in the lab is labelled as ‘37%, $r = 1.18$.’ These represent v/v% concentration (i.e. there is 0.37 mL HCl per mL of solution) and (relative) density (i.e. 1.18 g/mL) respectively. Given that the molar mass of HCl is 36.46 g/mol, the following formula can be used:

$$\text{Molar Concentration} = v/v \text{ Concentration} \times \text{Relative Density} \times \frac{1}{\text{Molar Mass}}$$

This reveals that the concentrate HCl has a molarity of 11.97 M (mol/L). However, the protocol calls for 6 M HCl. Thus, adding the concentrated HCl to water until a ratio of 1:1 is reached will achieve this.

Phosphate Buffered Saline

The stock PBS in the lab is 10X. Thus 10 ml of this should be added to 90 ml of water to get a concentration of 1X.

Eppendorf Tubes

- The Eppendorf tubes in the Thompson bay are not sufficiently robust for this experiment (for reference, their ordering details are: Centrifuge tubes FB74031 Capacity 1.5ml)
- Either ask to use more robust tubes from another group or order some; order from <http://www.sigmaaldrich.com/labware.html>, code T9661-1000EA
- The masses of the tubes must be known beforehand (there are scales in the lab that can measure to the hundredth microgram or better; use these)

H.2. Day 1

Apparatus

- PPA: lab coat, gloves, glasses
- Flask for liquid nitrogen
- Pestle & mortar
- Petri dishes
- Fine point forceps
- Eppendorf tubes with known masses (nearest hundred micrograms)
- Eppendorf tube holder
- Cell scraper
- 6 M hydrochloric acid, decanted in glass jar
- Pipette
- Phosphate buffered saline, concentration of 1X, decanted in glass jar
- Hot block
- Samples: rat tail tendons already harvested and stored in PBS (in a test tube or similar)

Procedure

1. Take pestle & mortar, petri dishes, Eppendorf tubes in their holder, cell scraper, HCl, pipette and the samples in PBS to the fume hood
2. Pour tendons into a petri dish, still in PBS
3. Prepare flask of liquid nitrogen, carry it to the fume hood
4. Take 5 tendons from the petri dish and place into the mortar
5. Return the remaining tendons to the test tube
6. Pour in nitrogen, wait until it has almost boiled off, pour in more, wait until this has boiled off completely, grind tendons with pestle until near-powdered (additional pours of nitrogen might be needed)
7. Scrape the contents of the mortar into an Eppendorf tube using the cell scraper
8. Pipette a few drops of 6 M HCl into the Eppendorf tube (just enough to cover the sample)
9. Place the Eppendorf tubes into the hot block in the fume hood at 110°C

10. Leave the samples for 24 hours

H.3. Day 2

1. Turn the hot block down to 35°C
2. Open the lids of the Eppendorf tubes
3. Leave overnight (until the samples are dry)

H.4. Day 3

Phosphate Buffered Saline

1. Weigh the Eppendorf tubes with the samples inside
2. Calculate the masses of the samples (current mass - initial mass of empty tube)
3. 1 ml of 1X PBS needs to be added to each Eppendorf tube for every 14 mg of sample it contains; calculate how much PBS each needs and add it. This creates a suspension.

Centrifuge

1. Load tubes into the centrifuge (orientated so that the hinges of the tubes are on the outside)
2. The loading should be such that the tubes are equally spaced around the circumference of the centrifuge. If this is not possible, fill a spare Eppendorf tube with water to approximately the same level as the others and load it along with the others; the centrifuge must be balanced!
3. Centrifuge for 10 min at 2500 g

Spectrofluorimetry

1. Open a new well plate with at least 3 times the number of wells as there are samples (96 wells is recommended)
2. Create three aliquots of each supernatant
3. (optional) Create a 'border' around these wells using either PBS or water (or a combination of these). In certain circumstances it is recommended to do this when using a microplate reader, but in this instance it is almost certainly overkill. Nonetheless, it can still be useful to have these 'non-samples' present in the well

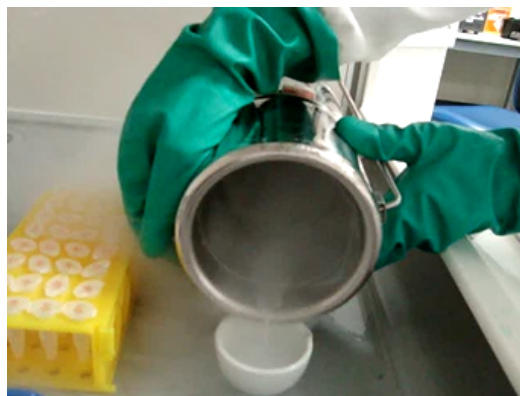
plate as they can be referred to when tweaking the setup of the microplate reader (i.e. when setting the gain and choosing a base sample).

4. Assay for AGE fluorescence (excitation wavelengths of 370, emission wavelengths of 440 nm) and amino acid content (glycine: 229 nm excitation, 448 nm emission) using the microplate spectrofluorometer:
 - From the desktop of the microplate reader's computer: Open 'Omega' → select 'USER' → 'Run' → Open the plate slot with 'Plate Out' → load the well plate (without its lid) → close it again
 - 'Manage Protocols' → select 'rowan', then 'Edit' → 'Basic Parameters': Microplate (COSTAR 96), Excitation (355), Emission (460) → 'Layout': select the wells that are fill and set them to 'Sample' → 'Concentrations': leave as is
 - 'Start Measurement' → select protocol to use ('rowan') → 'Start measurement' → leave settings as is → 'Start measurement'
 - From desktop, open 'Omega Data Analysis' → 'USER' → 'Run' → find and open the run just completed (it will be at the top of the list) → export to Excel
5. Dispose of samples
6. Calculate proportion of AGEs to amino acid content per mg of sample, and compare this value for STZ vs control
7. Fluorescence is reported with units of aFU/mg (arbitrary fluorescence units per milligram of substance being imaged)

I. Quantification of AGEs: Video Protocol



(a) Apparatus required



(b) Pouring of liquid nitrogen onto samples



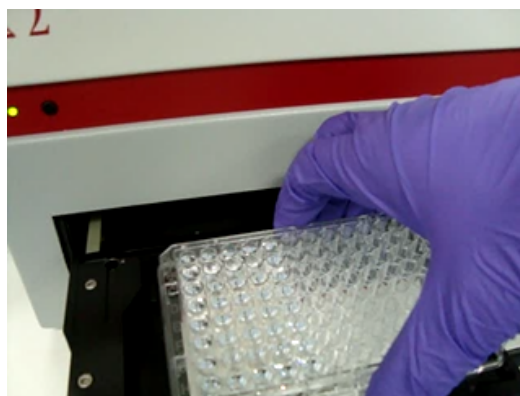
(c) Grinding into powder



(d) Addition of phosphate buffered saline



(e) Loading the centrifuge



(f) Aliquots placed into the microplate reader

Figure I.1: Selected frames from the instructional video