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CAPACITY FOR SLIDING BETWEEN TENDON FASCICLES DECREASES WITH AGEING IN INJURY PRONE EQUINE TENDONS: A POSSIBLE MECHANISM FOR AGE-RELATED TENDINOPATHY?

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Abstract

Age-related tendinopathy is common in both humans and horses; the initiation and progression of which is similar between species. The majority of tendon injuries occur to high-strain energy storing tendons, such as the human Achilles tendon and equine superficial digital flexor (SDFT). By contrast, the low-strain positional human anterior tibialis tendon and equine common digital extensor (CDET) are rarely injured. It has previously been established that greater extension occurs at the fascicular interface in the SDFT than in the CDET; this may facilitate the large strains experienced during locomotion in the SDFT without damage occurring to the fascicles. This study investigated the alterations in whole tendon, fascicle and interfascicular mechanical properties in the SDFT and CDET with increasing age. It was hypothesised that the amount of sliding at the fascicular interface in the SDFT would decrease with increasing horse age, whereas the properties of the interface in the CDET would remain unchanged with ageing. Data support the hypothesis; there were no alterations in the mechanical properties of the whole SDFT or its constituent fascicles with increasing age. However, there was significantly less sliding at the fascicular interface at physiological loads in samples from aged tendons. There was no relationship between fascicle sliding and age in the CDET. The increase in stiffness of the interfascicular matrix in aged SDFT may result in the fascicles being loaded at an earlier point in the stress strain curve, increasing the risk of damage. This may predispose aged tendons to tendinopathy.

Keywords: Tendon, ageing; mechanics; equine; injury; interfascicular matrix; superficial digital flexor tendon.

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Introduction

Tendinopathies are characterised by pain and loss of function and are a common occurrence in the general population and those involved in sporting activities. Epidemiological studies have shown that the incidence of tendon injury and disease increases with increasing age. For example, Achilles tendon injuries are most prevalent in the fourth to fifth decade of life (Hess, 2010). A similar pattern is seen for patellar tendon injuries, but the peak occurs later in the seventh to eighth decades (Clayton and Court-Brown, 2008). These findings fit with the hypothesis that there is a gradual decrease in mechanical integrity of tendons due to accumulation of micro-damage to the extracellular matrix as individuals age, rather than an acute overstrain injury (Riley, 2008). However, previous studies have failed to show a clear reduction in tendon material properties with increasing age (Carroll *et al*., 2008; Faria *et al*., 2011; Kubo *et al*., 2003).

Epidemiological studies have also shown that some tendons are more prone to injury than others. In runners, the majority of injuries occur to the Achilles tendon (Knobloch *et al*., 2008), which is exposed to high levels of stress and strain during normal function. The patellar tendon is another common site of overuse injury. Naturally occurring tendon injuries are also seen in other species. In the horse, the superficial digital flexor tendon (SDFT, anatomical location shown in Fig. 1) is highly susceptible to injury, which is localised to the tendon core in the mid-metacarpal region (for a detailed review, refer to Thorpe *et al*. (2010a) and references therein). The equine SDFT, like the human Achilles, is subjected to high loads and strains *in vivo*. The initiation and progression of tendinopathy are similar between horses and humans (Innes and Clegg, 2010; Lui *et al*., 2010), and the prevalence of tendon injury increases with increasing subject age in both the human Achilles (Hess, 2010; Knobloch *et al*., 2008) and equine SDFT (Kasashima *et al*., 2004; Perkins *et al*., 2005). Therefore, the horse is often used as a model to study age-related changes that occur to the tendon tissue (Birch *et al*., 1999; Dudhia *et al*., 2007; Thorpe *et al*., 2010b) as it is difficult to obtain healthy samples from human subjects with a wide age range.

The high incidence of injury to high strain tendons such as the Achilles and equine SDFT may be partially explained by their function. The predominant function of most tendons is to transfer the forces generated by muscle contraction to bone, resulting in correct limb positioning for locomotion. These tendons are required

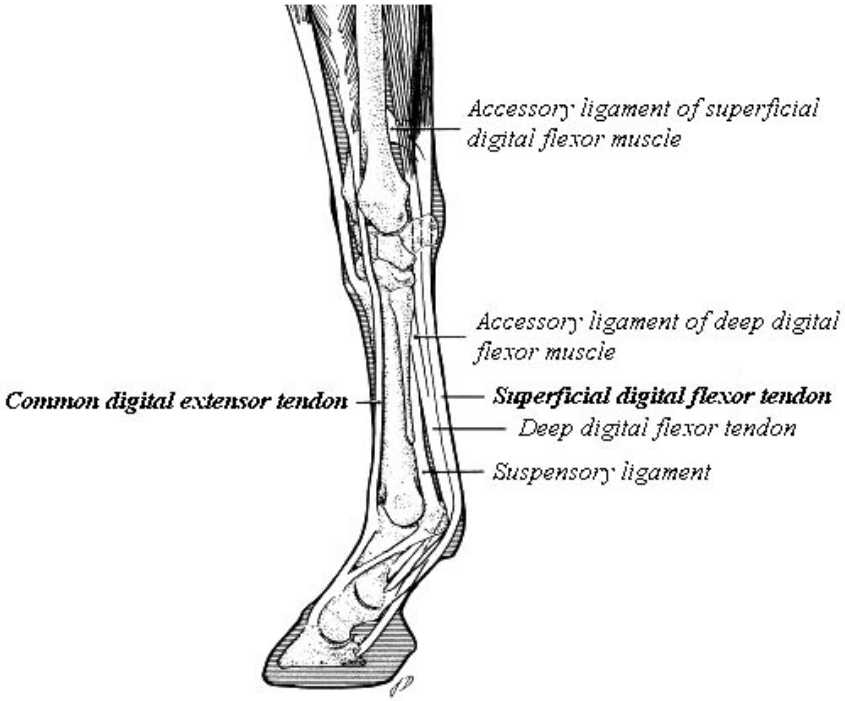

Fig. 1. Diagram showing anatomy of the equine forelimb and the location of the SDFT and CDET. Reproduced from Smith *et al.* (2002), with permission from Elsevier.

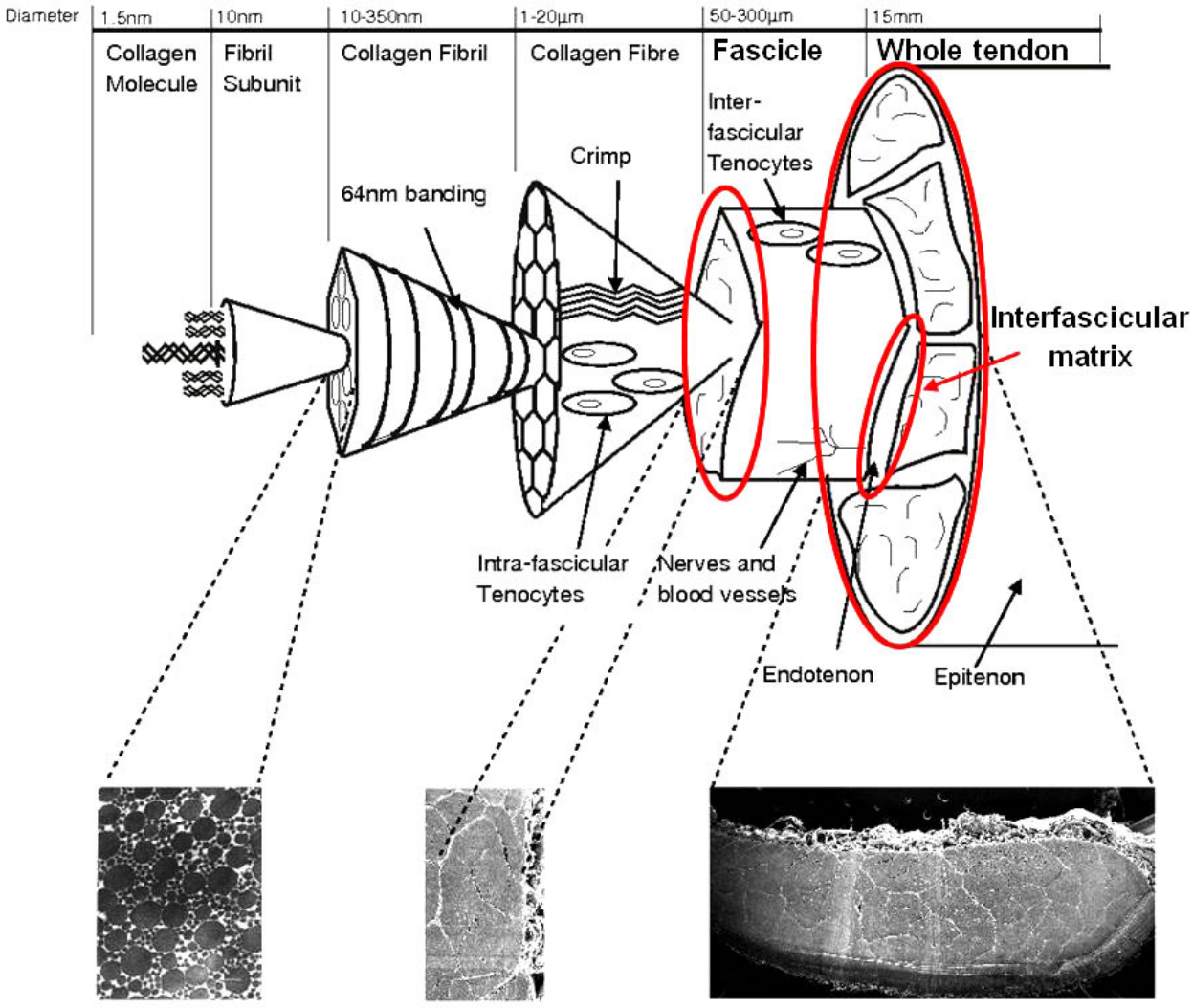

Fig. 2. Schematic showing the hierarchical structure of tendon tissue and illustrating the levels investigated in this study. Reproduced with kind permission from Thorpe *et al.* (2010a), Equine Veterinary Journal, Wiley Publishing.

to extend relatively small amounts for efficient function. However, specific tendons, including the human Achilles and equine SDFT also store and return energy during exercise, resulting in a reduction in the energetic cost of locomotion (Alexander, 1991; Biewener, 1998). For efficient energy storage, these tendons undergo a large amount of deformation; one legged hopping exercise can cause strain in the human Achilles to exceed 10 % (Lichtwark and Wilson, 2005), and strain in the equine SDFT during galloping exercise has been measured at 16 % (Stephens *et al*., 1989). These strains are close to the failure strains recorded for the respective tendons during *in vitro* mechanical testing (Batson *et al*., 2003; Wren *et al*., 2001), suggesting that energy storing tendons operate with low safety margins in order to maximise efficiency. In non-energy storing tendons, such as the human anterior tibialis tendon and equine common digital extensor tendon (CDET, anatomical location shown in Fig. 1), maximum *in vivo* strains in the region of 3 % have been reported (Birch *et al*., 2008; Maganaris and Paul, 1999). These values are much lower than the tendon failure strain (Batson *et al*., 2003) and may explain why positional tendons rarely suffer from overstrain injuries. Previous studies indicate a specialisation of tendon structure and mechanical properties to suit function (Birch, 2007) and suggest that these specialisations may be the focus of age-related alterations.

Tendon matrix is composed predominantly of type I collagen, with a small percentage of other collagens and non-collagenous proteins, such as proteoglycans. Type I collagen molecules are grouped together in a highly ordered fashion, forming fibrils, fibres and fascicles, with a small amount of proteoglycan-rich matrix interspersing each hierarchical level (Kastelic *et al*., 1978; Fig. 2). Previous work has established that, at the fibre level, this non-collagenous matrix governs tendon visco-elastic behaviour by enabling sliding between adjacent collagen fibres (Gupta *et al*., 2010; Screen, 2008). However, the role of the interfascicular matrix (IFM), which binds the fascicles together, is less well defined. The IFM is comprised of collagen type III and proteoglycans, and is synthesised and maintained by a small population of fibroblasts (Dahlgren *et al*., 2005; Fallon *et al*., 2002; Kannus, 2000) (Fig. 2). Little is known about the precise structure and function of the IFM, but previous studies suggest it may play an important role in tendon function. The glycoprotein lubricin, which is localised to the IFM (Funakoshi *et al*., 2008) may facilitate sliding between tendon fascicles (Kohrs *et al*., 2011). Elastin may also influence fascicle sliding and recoil; it has been reported that components of the elastic fibre are situated between fascicles in canine cruciate ligament (Smith *et al*., 2011), but their distribution within tendons is yet to be determined. The functionally distinct equine SDFT and CDET show a significant difference in gross tendon failure strain. Our previous work however showed that this is not due to differences in the mechanical properties of the constituent tendon fascicles (Thorpe *et al*., 2012). Instead, we reported significant differences in the amount of sliding behaviour between the tendon fascicles, with significantly more sliding between fascicles in the SDFT than in the CDET at physiological loads. These results suggest that, in the energy storing SDFT, the properties of the IFM may directly influence the mechanical properties of the whole tendon but are less influential in the low strain CDET, indicating that interfascicular sliding is critical for energy storing tendon function (Thorpe *et al*., 2012). We therefore hypothesise that the amount of sliding at the fascicular interface in the SDFT decreases with increasing horse age, whereas the properties of the IFM in the CDET remain unchanged with ageing.

Materials and Methods

Sample collection

The right and left forelimbs distal to the carpus were collected from full or half Thoroughbred horses (n = 17) aged 3 to 20 years (12.28 ± 5.61, median = 13), euthanased at a commercial equine abattoir. The horses were divided into 3 groups, based on age. Young group: age 3 to 8 years (representing young, but skeletally mature horses), n = 5. Middle group: age 9 to 14 years (middle aged horses), n = 5. Old group: age 15 to 20 years (geriatric horses (Ireland *et al*., 2012)), n = 7. The tendons showed no evidence of injury at post-mortem examination. The SDFT and CDET were dissected free from the limbs at the level of the metacarpophalangeal joint, wrapped in tissue paper dampened with phosphate-buffered saline and stored frozen at -20 °C wrapped in tin foil. The properties of whole tendons, fascicles and IFM were determined for all horses as described below.

Protocol for whole tendon testing

The SDFT and CDET from the left forelimb of each horse were allowed to thaw at room temperature before the cross-sectional area (CSA) and mechanical properties were measured. The CSA was measured at the mid-metacarpal level of the SDFT and CDET, as the tendons are thinnest in this region (Birch *et al*., 2002). Tendon CSA was determined by making a cast of the mid-metacarpal region of the tendon, while unloaded, using an alginate paste (Goodship and Birch, 2005). The tendon was removed and a digital image taken of a transverse section through the cast. Image analysis software was used to measure the area that had been occupied by the tendon.

Tendons were mounted vertically, in a servo-hydraulic materials testing machine (Dartec Ltd, Stourbridge, UK) with a 50 kN load cell, gripped using cryoclamps cooled with liquid CO_2 (Riemersa and Schamhardt, 1982). The clamps were set at 10 cm apart and the mid-metacarpal region of the tendon was centred between the clamps, providing a homogeneous length of tendon for testing. The tendons were pre-loaded to 100 N (SDFT) or 25 N (CDET), which represents a negligible load of approximately 1-2 % of the failure load and allows determination of a resting length. The pre-load was held for approximately 1 min while the distance between the two freeze lines was measured to give the effective gauge length of the tendon (Batson *et al*., 2003). The freeze lines were marked with ink, and circular dots were drawn onto the surface of the tendon at approximately 10 mm intervals.

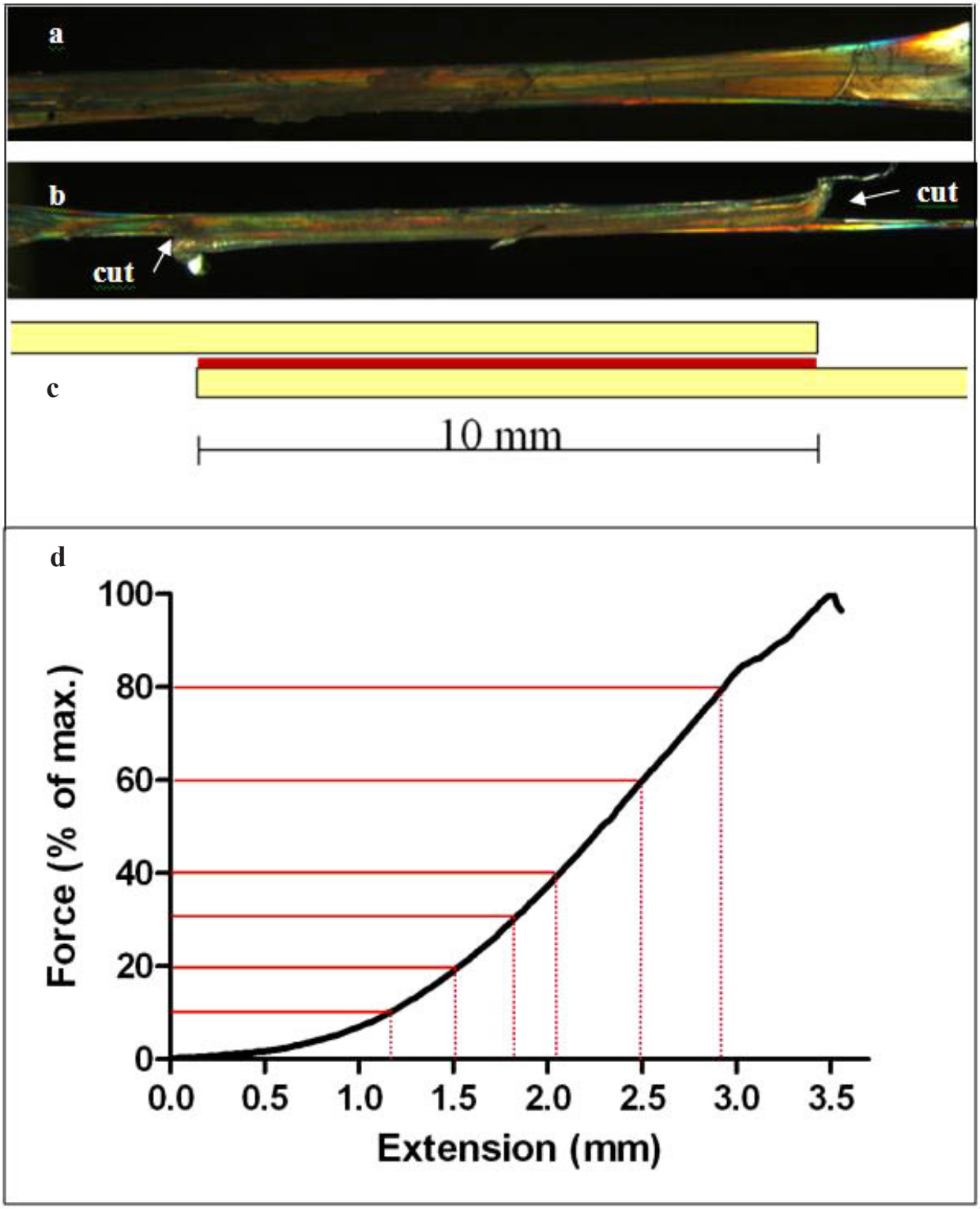

Fig. 3. An illustration of fascicle dissection for testing of the IFM. **(a)** Two intact fascicles bound by IFM viewed under polarising light; **(b)** one end of each fascicle has been cut, leaving a section of intact IFM of 10 mm length; and **(c)** schematic of fascicle dissection, with fascicles in yellow and IFM in red. **(d)** Calculation of the amount of IFM extension at different percentages of failure force from the force extension curve. Adapted from Thorpe *et al*. (2012).

Tendons were pre-conditioned to reach a steady state, using a method adapted from Batson *et al*. (2003) with 20 cycles between 0 % and 5.25 % strain (approximately 24 % and 28 % of failure strain in the SDFT and CDET, respectively) using a sine wave at a frequency of 0.5 Hz. At the end of the preconditioning step the load was removed so that slack was visible in the tendon. Tendons were then tested at room temperature at a speed of 5 %/s until failure.

Protocol for tendon fascicle testing

Fascicles (approx. 35 mm in length) were dissected from the core ($n = 6$ from each tendon) and periphery ($n = 6$ from each tendon) of the mid-metacarpal region of the SDFT and CDET from the right forelimb of each horse. Fascicles were isolated by cutting with a scalpel longitudinally though the tendon using previously established protocols (Legerlotz *et al*., 2010; Legerlotz *et al*., 2011). The diameter of each fascicle was determined by a non-contact laser micrometer (LSM-501, Mitotuyo, Japan; resolution = 0.5 μm) at multiple points along a 10 mm region in the middle of the fascicle. The smallest diameter recorded was used to calculate CSA, assuming a circular shape. Fascicles were secured in a materials testing machine (Bionix100, MTS, Cirencester, UK; crosshead resolution = 0.001 mm, linearity < 0.25 %) 50 N load cell (resolution = 0.001 N, linearity = ± 0.07 in the 1 N range) by pneumatically driven grips with a serrated surface which exerted a gripping pressure of 3 GPa. The grip to grip distance was set to 20 mm. The fascicles were pre-loaded to 0.1 N (Legerlotz *et al*., 2010), which represents a load of approximately 1-2 % of fascicle failure load, and the resulting grip to grip distance was taken as the effective gauge length. The fascicle surface was marked with ink dots spaced every 4 mm. Fascicles were pre-conditioned to reach a steady state, using the same protocol as for whole tendons, with 20 cycles between 0 % and 5.25 % strain (approximately 40 % and 33 % of failure strain in the SDFT and CDET fascicles respectively) using a sine wave at a frequency of 0.5 Hz. At the end of the preconditioning step the load was removed so that slack was visible in the fascicles. Fascicles were then tested at room temperature at a strain rate of 5 %/s until failure. During dissection and testing, specimens were kept moist by continually spraying with phosphate-buffered saline solution.

Calculation of tendon and fascicle mechanical and material properties

Force and displacement data were continuously recorded at 100 Hz during preconditioning and the test to failure. The displacement at which the initial pre-load was reached was taken as the start point for the test to failure in all specimens. Tests were videoed (Panasonic SDR-550, 30 fps) and strain at failure calculated using the surface markers and compared to the machine-derived data. The method used did not result in a significant difference in failure strain in either whole tendon or fascicles; hence, failure strain was calculated from the raw machine-derived displacement data for all the samples. Engineering stress and strain were calculated using the CSA and effective gauge length for each sample. A continuous modulus was calculated across every 5 data points of each stress strain curve and smoothed using a 5-point moving average filter (Legerlotz *et al*., 2010). From these data, a maximum modulus value was determined.

Assessment of mechanics at the fascicular interface

In order to investigate the mechanics of the fascicular interface, groups of 2 fascicles bound together by the IFM were dissected from the core ($n = 6$ from each tendon) or periphery ($n = 6$ from each tendon) of the mid-metacarpal region of the SDFT and CDET from the right forelimb of each horse. The fascicles were secured into a custom-made dissection rig, which was placed under a stereomicroscope fitted with an analyser and rotatable polarising lens (Leica, Wetzlar, Germany). This generates elliptically polarised light, which enables clear visualisation of the individual collagen fascicles. The opposing end of each fascicle was cut transversely, leaving a consistent 10 mm length of intact IFM (Fig. 3a-c).

After removal from the dissection rig, the intact end of each opposing fascicle was secured in a materials testing machine (Bionix100, MTS) with a grip to grip distance of 20 mm and the fascicles pulled apart to failure at a speed of 1 mm/s. Force and extension data were recorded at 100 Hz during the test, and from these data the point at which the load reached 0.02 N was defined as the test start point. Extension was measured as grip to grip displacement. A force-extension curve was derived for each sample, from which the amount of IFM extension was calculated at different percentages of IFM failure load (Fig. 3d). The area under the curve was calculated from the normalised curves and expressed as a percentage of the maximum area.

Scanning electron microscopy (SEM)

SEM was used to assess alterations in fascicle packing with increasing age in the SDFT. A 10 mm transverse section was harvested from the mid-metacarpal region of an additional group of SDFTs and CDETs from young (age range: 2 to 5 years, $n = 4$) and old (age: 20 years, $n = 3$) horses that had not undergone mechanical testing. The sections were snap frozen in hexane and stored at -70 °C, wrapped in cling film. Each sample was then lyophilised in a freeze drier overnight, mounted onto an aluminium stub, coated with gold-palladium and viewed under the SEM at 35x magnification (Jeol JMS 550OLV). Two images were taken of each sample. For each image, fascicle edges were identified and the image thresholded to produce a binary image (Image J, 1.34s, National Institute of Health, USA). The percentage area occupied by the IFM was then measured. It was not possible to identify a sufficient number of fascicle edges in CDET samples to allow calculation of IFM area in this tendon, and so values are only reported for the SDFT.

Statistical analysis

Data were tested for normality using a Kolmogorov-Smirnov test. All the data followed a normal distribution. Correlation of mechanical properties with horse age was determined using Pearson product moment correlation analysis and linear mixed effects with horse as a fixed factor in SPlus (Insightful, Seattle, WA, USA). Significant differences between the age groups were determined using linear mixed effects analysis in SPlus. Statistical significance was taken as $p < 0.05$.

Results

The elastic modulus, failure stress and failure strain of the SDFT and CDET as whole tendons were not significantly different between the age groups (Table 1). Statistical

Table 1. Material properties of the SDFT and CDET and their constituent fascicles from horses separated into 3 groups based on age. Data are displayed as mean ± SD. Significant difference compared to young age group: [a] $p < 0.05$, [c] $p < 0.001$.

		Whole SDFT	Whole CDET	SDFT fascicle	CDET fascicle
CSA (mm^2)	3 - 8 years	68.3 ± 20.0	19.7 ± 3.5	0.13 ± 0.02	0.16 ± 0.08
	9 - 14 years	97.5 ± 18.3	29.1 ± 8.7	0.13 ± 0.02	0.15 ± 0.02
	15- 20 years	98.0 ± 40.6	27.2 ± 6.7	0.11 ± 0.04	0.16 ± 0.03
Failure Stress (MPa)	3 - 8 years	116.9 ± 19.4	148.5 ± 33.7	36.72 ± 4.74	38.84 ± 5.80
	9 - 14 years	130.7 ± 31.0	145.4 ± 33.1	38.98 ± 1.53	42.22 ± 7.79
	15- 20 years	104.0 ± 21.7	160.3 ± 24.8	34.93 ± 8.67	36.71 ± 6.03
Strain at Failure (%)	3 - 8 years	21.1 ± 4.8	15.3 ± 3.0	12.38 ± 0.83	14.76±1.23
	9 - 14 years	24.8 ± 6.0	18.8 ± 3.4	13.12 ± 1.93	16.27 ± 1.11[a]
	15- 20 years	21.7 ± 3.3	19.7 ± 3.7	12.67 ± 0.90	17.75 ± 1.35[c]
Elastic Modulus (MPa)	3 - 8 years	676.3 ± 142.2	1036.8 ± 176.0	331.05 ± 29.81	336.42 ± 78.21
	9 - 14 years	626.8 ± 97.7	935.9 ± 175.6	338.82 ± 49.87	338.53 ± 70.28
	15- 20 years	586.2 ± 93.7	1024.0 ± 124.1	318.91 ± 74.62	268.26 ± 46.25

Table 2. Ultimate properties of the interfascicular matrix in the SDFT and CDET from horses separated into 3 groups based on age. Data are displayed as Mean ± SD. Significant difference between tendon types: [b] $p < 0.01$, [c] $p < 0.001$. Significant difference in the old aged group compared to the young and middle aged groups in the SDFT: [1] $p < 0.05$, [2] $p < 0.01$.

	SDFT			CDET		
	Young	**Middle**	**Old**	**Young**	**Middle**	**Old**
Extension at failure (mm)	2.81 ± 0.70	2.45 ± 0.66	2.61 ± 0.72	2.81 ± 0.70	2.95 ± 0.83	3.07 ± 0.99[b]
Force at failure (N)	1.49 ± 1.09	1.57 ± 0.78	1.50 ± 0.93	1.38 ± 0.97	1.76 ± 1.07	1.61 ± 1.11
Area under curve (%)	45.27 ± 5.38[1]	43.71 ± 7.58[2]	49.67 ± 6.44	52.44 ± 4.87[c]	54.12 ± 6.75[c]	52.50 ± 5.25

analysis did not show any differences in the mechanical properties between fascicles from the core and periphery of either tendon, so these data were combined. Fascicles from the SDFT showed no age-related alterations in material properties. In the CDET, the elastic modulus and failure stress of fascicles decreased slightly with increasing age. Although this was not significant, the corresponding increase in CDET fascicle failure strain was significant in the old and middle age groups compared to the young age group ($p \leq 0.049$; Table 1).

Failure properties for the interface in the SDFT and CDET in each of the age groups are shown in Table 2. Considering the failure point, there was no alteration in IFM extension or force with increasing horse age in either tendon. However, a small, non-significant increase in the IFM extension at failure in the CDET with increasing age resulted in IFM failure extension being significantly lower in the SDFT than in the CDET in the old age group ($p = 0.005$).

Whilst there are small differences in IFM failure properties between tendon types, the shape of the force extension curves for the IFM are quite different, as illustrated by the significantly smaller area under the curve in the SDFT compared to the CDET in the young and middle aged groups ($p < 0.0001$). Furthermore, the shape of the force extension curve for the SDFT changes greatly with age demonstrating increased IFM stiffness during a loading cycle (Fig. 4), and increased area under the curve compared to the young and middle aged groups ($p = 0.019$). In the young age group, there was significantly more extension at the fascicular interface at and below 60 % of failure load in the SDFT than in the CDET ($p \leq 0.0004$). In the middle age group, extension at the interface was significantly greater in the SDFT at and below 30 % of failure load ($p \leq 0.04$), whereas in the aged group the difference between tendon types was completely lost, and there was no difference in the amount of extension at the interface at any percentage of failure load (Fig. 5). In addition, the amount of IFM extension at 10, 30 and 60 % of failure load showed a significant negative correlation with increasing horse age in SDFT samples ($p \leq 0.048$; Fig. 6). This was accompanied by an increase in IFM stiffness at 1 mm extension (the amount of extension previously shown to account for the difference between fascicle and tendon failure strain in the SDFT (Thorpe *et al.*, 2012)). IFM stiffness in the SDFT showed a significant positive

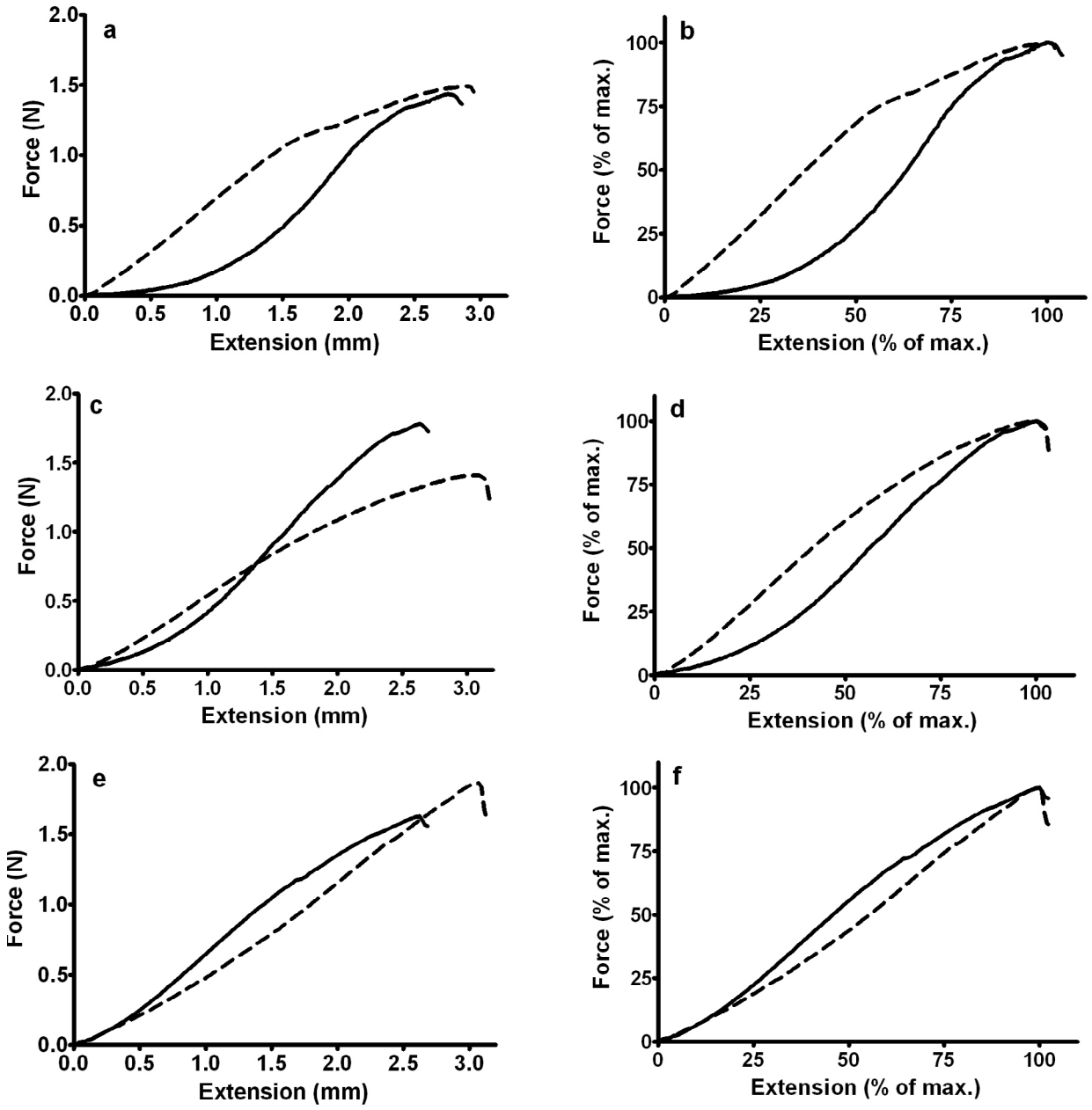

Fig. 4. Representative force extension curves for the fascicular interface in the SDFT (———) and CDET (– – – –) from a 3 year old horse **(a,b)**, a 10 year old horse **(c,d)**, and a 20 year old horse **(e,f)**. For comparison, non-normalised (a,c,e) and normalised curves (b,d,f) are shown for each sample.

correlation with horse age (p = 0.011), increasing from 0.36 ± 0.21 N/mm in the young age group to 0.46 ± 0.30 N/mm in the middle aged group, and further increasing to 0.53 ± 0.30 N/mm in the old age group. The mechanical properties of the IFM showed no correlation with horse age in samples from the CDET, with a mean stiffness of 0.54 ± 0.30 N/mm at an extension of 1 mm across all age groups.

Representative SEM images of transverse sections of the SDFT and CDET from young and old horses are shown in Fig. 7. The percentage area occupied by the IFM was significantly greater in young SDFTs than in old SDFTs (15.13 ± 1.30 % *vs.* 9.00 ± 3.48 %; p = 0.024).

Discussion

The results of this study support the hypothesis, showing a significant decrease in the capacity for sliding between SDFT fascicles at physiological loads in aged individuals, with no age-related alterations occurring in the CDET. As with previous studies on equine tissue (Batson *et al*., 2003), our data showed a significant difference between the material properties of the SDFT and CDET at the whole tendon level but did not demonstrate a difference in mechanical properties of whole tendons with ageing. However, there were large variations in tendon mechanical properties within each age group. It was not possible to

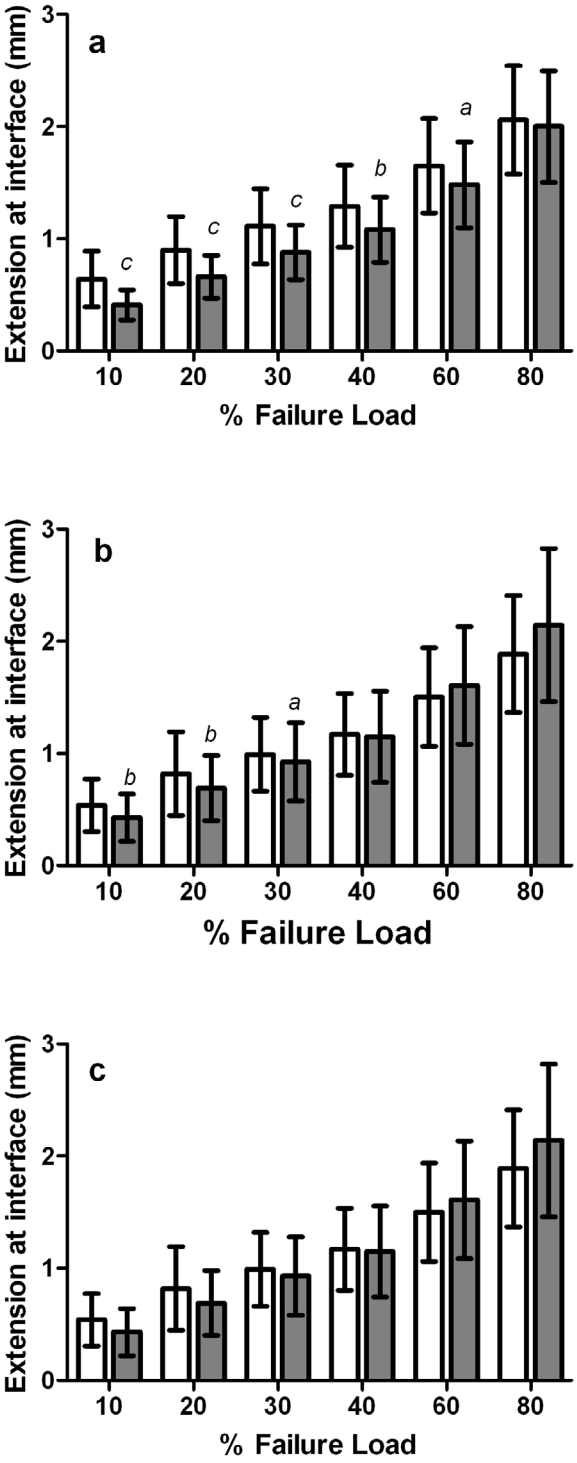

Fig. 5. The amount of extension at the fascicular interface was calculated at varying percentages of failure load (as shown in Fig. 3d). The amount of IFM extension was significantly greater in the SDFT (□) than in the CDET (■) at and below 60 % of failure load in horses aged 3 to 8 years **(a)**, and at and below 30 % of failure load in horses aged 9 to 14 years **(b)**. There was no significant difference in the amount of IFM extension at any percentage of failure load in the SDFT and CDET from horses aged 15 to 20 years **(c)**. Significance between tendon types is indicated by: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$. Data are displayed as mean ± SD.

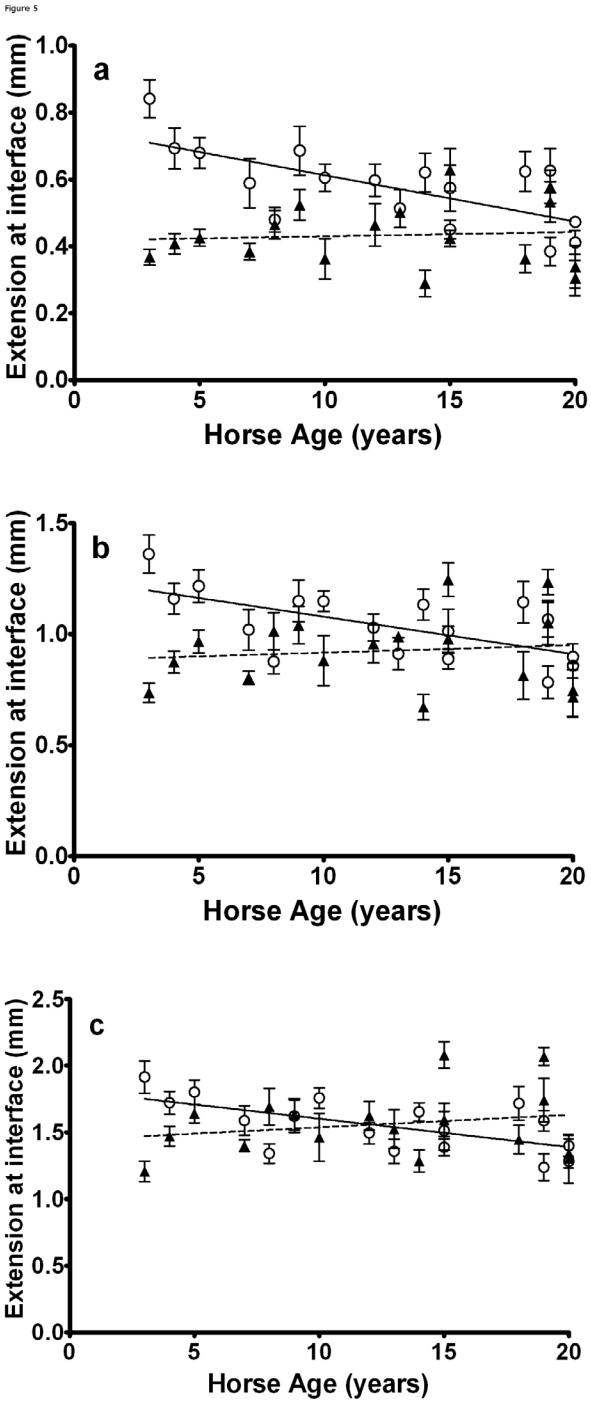

Fig. 6. The amount of extension at the interface in the SDFT (○) and CDET (▲) at 10 % **(a)**, 30 % **(b)** and 60 % **(c)** of failure load. Extension at the interface showed a significant negative correlation with horse age in the SDFT at 10 % ($r = 0.68$, $p = 0.02$), 30 % ($r = 0.63$, $p = 0.04$) and 60 % ($r = 0.62$, $p = 0.04$) of failure load. There was no correlation between horse age and interface extension in the CDET. Data are displayed as mean ± SEM.

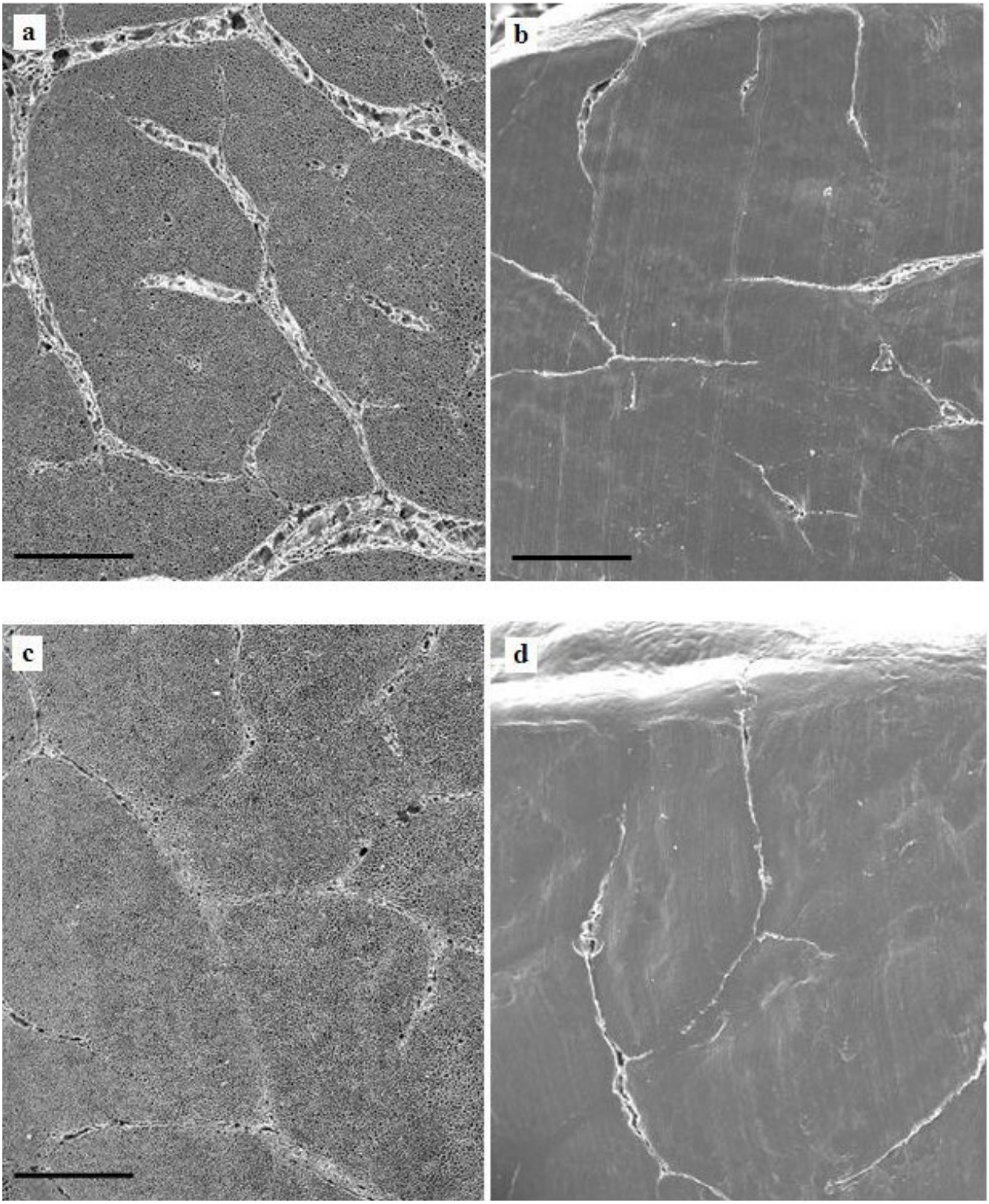

Fig. 7. Transverse SEM images of SDFT **(a)** and CDET **(b)** from 5 year old horses, and SDFT **(c)** and CDET **(d)** from 20 year old horses. Scale bar represents 500 μm.

obtain a full exercise history for all horses, and differences in exercise levels between individuals may explain some of the variability observed. Previous studies have investigated the effect of increasing age on tendon material properties in a variety of species, but results are inconclusive. Some findings have shown that ageing does not result in changes in the mechanical properties of human tendon (Carroll *et al*., 2008). However, some *in vivo* studies have reported that tendon stiffness decreases with increasing subject age (Kubo *et al*., 2003; Reeves *et al*., 2003), whereas others have found an increase in tendon stiffness with ageing, both *in vivo* (Faria *et al*., 2011) and *in vitro* (Gillis *et al*., 1995; Wood *et al*., 2011).

The majority of tendon ageing studies have focussed on alterations in matrix composition and organisation rather than mechanical properties. It is well established

that the advanced glycation end-product pentosidine accumulates with age in tendons from several different species (Avery and Bailey, 2005; Bank *et al*., 1999; Thorpe *et al*., 2010b). It has also been shown that partially degraded collagen accumulates with age in the equine SDFT, but not in the CDET (Thorpe *et al*., 2010b), while levels of glycosaminoglycans have been reported to decrease in human supraspinatus tendon (Riley *et al*., 1994). It has not been established how these changes in matrix composition impact on tendon ultimate properties, but it seems likely that compositional or mechanical responses to ageing may be tendon specific. Furthermore, it is possible that compositional variations due to ageing result in alterations in the mechanisms by which load is distributed throughout the different levels of the tendon hierarchy. Therefore, even small alterations may make aged tendons less fatigue resistant and more susceptible to overload injury. The impact is also likely to differ between tendon types; for example; the material properties of the SDFT need to be maintained within the narrow limits required for efficient energy storage (Smith *et al*., 2002).

Our previous data show that the failure stress of fascicles is lower than that of the tendons they constitute; however, this is likely to be a result of increased fascicle CSA caused by swelling during the isolation procedure rather than an inherent property of the fascicles (Thorpe *et al*., 2012). When considering the effect of increasing age, the material properties of SDFT fascicles were maintained in aged tendon, whilst the strain at failure increased with increasing horse age in fascicles from the CDET. While changes in fascicle material properties have been assessed during maturation (Yamamoto *et al*., 2004), to the authors' knowledge no previous studies have investigated the effect of ageing after maturation on fascicle material properties. However, it has been reported that fascicle CSA decreases significantly in the core region of the SDFT with increasing age (Gillis *et al*., 1997). This is in contrast to the current study, which did not identify any alterations in core or peripheral fascicle CSA with age. Age-related alterations to the collagenous component of the SDFT, such as decreased crimp angle (Patterson-Kane *et al*., 1997), increased type III collagen content (Birch *et al*., 1999) and accumulation of advanced glycation end-products and partially degraded collagen (Thorpe *et al*., 2010b) might be expected to result in altered fascicle mechanical properties. While no age-related alterations in the failure properties of SDFT fascicles were identified in the current study, it is possible that compositional changes may alter other parameters, such as visco-elastic behaviour and fatigue resistance.

The current study has highlighted a significant stiffening of the SDFT IFM with ageing, leading to a decreased capacity for sliding between fascicles in the aged SDFT. While there are no age-related alterations in the failure properties of the IFM, the data show that there are significant alterations in the shape of the force extension curve in samples from the SDFT with increasing age. A large toe region in the IFM force-extension curve in the SDFT when young would enable large extensions of the IFM with minimal force. However, as the tendons age and the toe region reduces, greater forces are required to enable IFM extension. This may have important implications for the susceptibility of aged SDFTs to fatigue-induced injury. We have previously shown that sliding at the fascicular interface prior to fascicle extension may be key to facilitating the high working strains experienced by the SDFT (Thorpe *et al*., 2012). Therefore, increased IFM stiffness in the aged SDFT may result in fascicles within this tendon being loaded at an earlier point during tendon extension. If this occurs, fascicles within aged tendons will be more likely to be damaged during normal loading conditions than those within young tendons. In contrast, there is no alteration in the amount of sliding at the interface in samples from the CDET. This, combined with our previous data (Thorpe *et al*., 2012), suggests that the IFM does not contribute significantly to tendon material properties in the positional CDET.

The mechanisms that result in stiffening of the SDFT IFM with increasing age are yet to be determined. The data presented in this study show that the area occupied by IFM in the SDFT decreases significantly with increasing age, such that fascicles in this tendon are more tightly packed in aged individuals. Tighter packing of fascicles may well contribute to the decreased capacity for sliding between fascicles in aged tendons. This may be as a result of alterations in tendon water content. However, previous studies have shown that, while the water content is significantly greater in the SDFT than in the CDET, it does not alter with age in either tendon (Thorpe, 2010). Alternatively, there may be age-related alterations in the structural components of the IFM. While IFM components have been characterised (Dahlgren *et al*., 2005; Fallon *et al*., 2002; Kannus, 2000), the organisation, function, and age-related alterations of these minor collagens, proteoglycans and glycoproteins have yet to be determined at this structural level.

Previous work has assessed the role of proteoglycans at lower levels of the tendon hierarchy, with studies on mice knockout models showing that lubricin, decorin and biglycan can influence tail tendon fascicle viscoelastic properties (Elliott *et al*., 2003; Reuvers *et al*, 2011; Robinson *et al*., 2004). However, these models are limited, as the structure and function of tail tendon fascicles are very different from equine energy storing tendons, and therefore care must be taken when applying these findings to the IFM. Indeed, it has been shown that the influence of decorin and biglycan on mechanical properties differs between fascicles and tendons, and between tendons from different locations (Robinson *et al*., 2005), suggesting that the role of individual proteoglycans may be specific to tendon type as well as hierarchal level.

While no previous studies have investigated age-related alterations in IFM components, both lubricin and elastin have been shown to diminish with ageing in other tissue types; the elastin content of blood vessels decreases with increasing age (Ãstrand *et al*., 2011), and an age-related decrease in lubricin levels in articular cartilage has been implicated in development of osteoarthritis (Hills, 2000). In addition, improper repair of any micro-damage occurring within or between the fascicles may result in the formation of adhesions, limiting the amount of sliding that can occur. Interestingly, a previous study has reported that the non-collagenous matrix is turned over more rapidly

in the SDFT than in the CDET (Thorpe *et al*., 2010b), which may indicate an attempt to repair damage to the IFM in this tendon. Taken together, these data suggest that the increased risk of tendon injury in aged individuals is governed by age-related changes to the non-collagenous fraction of the tendon matrix. The IFM may therefore be a potential target for therapeutic, conditioning and rehabilitative interventions, but further work is required to determine the exact role it plays in governing gross tendon mechanical properties.

Conclusions

The data presented in this study illustrate that, while the material properties of the whole SDFT and its constituent fascicles are maintained with increasing subject age, age-related changes occur to the mechanical properties of the IFM. This matrix becomes stiffer in aged tendons, which is likely to result in the fascicles within the tendon being loaded at an earlier point during tendon extension. The subsequent higher loads experienced by the fascicles during use may predispose these fascicles to damage and subsequently lead to increased risk of fatigue-induced tendon injury. However, the molecular mechanisms that govern tendon extension and recoil are yet to be elucidated. Full understanding of these mechanisms will aid in the development of appropriate preventative measures and treatments for age-related tendinopathy.

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References

Alexander RM (1991) Energy-saving mechanisms in walking and running. J Exp Biol **160**: 55-69.

Ãstrand H, Stalhand J, Karlsson J, Karlsson M, Sonesson B, Lanne T (2011) *In vivo* estimation of the contribution of elastin and collagen to the mechanical properties in the human abdominal aorta: effect of age and sex. J Appl Physiol **110**: 176-187.

Avery NC, Bailey AJ (2005) Enzymic and non-enzymic cross-linking mechanisms in relation to turnover of collagen: relevance to aging and exercise. Scand J Med Sci Sports **15**: 231-240.

Bank RA, TeKoppele JM, Oostingh G, Hazleman BL, Riley GP (1999) Lysylhydroxylation and non-reducible crosslinking of human supraspinatus tendon collagen: changes with age and in chronic rotator cuff tendinitis. Ann Rheum Dis **58**: 35-41.

Batson EL, Paramour RJ, Smith TJ, Birch HL, Patterson-Kane JC, Goodship AE (2003) Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? Equine Vet J **35**: 314-318.

Biewener AA (1998) Muscle-tendon stresses and elastic energy storage during locomotion in the horse. Comp Biochem Physiol B Biochem Mol Biol **120**: 73-87.

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

Birch HL, Bailey JV, Bailey AJ, Goodship AE (1999) Age-related changes to the molecular and cellular components of equine flexor tendons. Equine Vet J **31**: 391-396.

Birch HL, Smith TJ, Poulton C, Peiffer D, Goodship AE (2002) Do regional variations in flexor tendons predispose to site-specific injuries? Equine Vet J Suppl **34**: 288-292.

Birch HL, Wilson AM, Goodship AE (2008) Physical activity: does long-term, high-intensity exercise in horses result in tendon degeneration? J Appl Physiol **105**: 1927-1933.

Carroll CC, Dickinson JM, Haus JM, Lee GA, Hollon CJ, Aagaard P, Magnusson SP, Trappe TA (2008) Influence of aging on the *in vivo* properties of human patellar tendon. J Appl Physiol **105**: 1907-1915.

Clayton RAE, Court-Brown CM (2008) The epidemiology of musculoskeletal tendinous and ligamentous injuries. Injury **39**: 1338-1344.

Dahlgren LA, Brower-Toland BD, Nixon AJ (2005) Cloning and expression of type III collagen in normal and injured tendons of horses. Am J Vet Res **66**: 266-270.

Dudhia J, Scott CM, Draper ERC, Heinegård D, Pitsillides AA, Smith RK (2007) Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. Aging Cell **6**: 547-556.

Elliott DM, Robinson PS, Gimbel JA, Sarver JJ, Abboud JA, Iozzo RV, Soslowsky LJ (2003) Effect of altered matrix proteins on quasilinear viscoelastic properties in transgenic mouse tail tendons. Ann Biomed Eng **31**: 599-605.

Fallon J, Blevins FT, Vogel K, Trotter J (2002) Functional morphology of the supraspinatus tendon. J Orthop Res **20**: 920-926.

Faria A, Gabriel R, Abrantes J, Bras R, Moreira H (2011) Biomechanical properties of the triceps surae muscle-tendon unit in young and postmenopausal women. Clin Biomech **26**: 523-528.

Funakoshi T, Schmid T, Hsu H, Spector M (2008) Lubricin Distribution in the Goat Infraspinatus Tendon: A Basis for Interfascicular Lubrication. J Bone Joint Surg Am. **90**: 803-814.

Gillis C, Sharkey N, Stover SM, Pool RR, Meagher DM, Willits N (1995) Effect of maturation and aging on material and ultrasonographic properties of equine superficial digital flexor tendon. Am J Vet Res **56**: 1345-1350.

Gillis C, Pool RR, Meagher DM, Stover SM, Reiser K, Willits N (1997) Effect of maturation and aging on the

histomorphometric and biochemical characteristics of equine superficial digital flexor tendon. Am J Vet Res **58**: 425-430.

Goodship AE, Birch HL (2005) Cross sectional area measurement of tendon and ligament *in vitro*: a simple, rapid, non-destructive technique. J Biomech **38**: 605-608.

Gupta HS, Seto J, Krauss S, Boesecke P, Screen HRC (2010) *In situ* multi-level analysis of viscoelastic deformation mechanisms in tendon collagen. J Struct Biol **169**: 183-191.

Hess GW (2010) Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. Foot Ankle Spec **3**: 29-32.

Hills BA (2000) Boundary lubrication *in vivo*. Proc Inst Mech Eng H **214**: 83-94.

Innes JF, Clegg P (2010) Comparative rheumatology: what can be learnt from naturally occurring musculoskeletal disorders in domestic animals? Rheumatol **49**: 1030-1039.

Ireland JL, Clegg PD, McGowan CM, McKane SA, Chandler KJ, Pinchbeck GL (2012) Disease prevalence in geriatric horses in the United Kingdom: veterinary clinical assessment of 200 cases. Equine Vet J **44**: 101-106.

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

Kasashima Y, Takahashi T, Smith RK, Goodship AE, Kuwano A, Ueno T, Hirano S (2004) Prevalence of superficial digital flexor tendonitis and suspensory desmitis in Japanese Thoroughbred flat racehorses in 1999. Equine Vet J **36**: 346-350.

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

Knobloch K, Yoon U, Vogt PM (2008) Acute and overuse injuries correlated to hours of training in master running athletes. Foot Ankle Int **29**: 671-676.

Kohrs RT, Zhao C, Sun Y-L, Jay GD, Zhang L, Warman ML, An K-N, Amadio PC (2011) Tendon fascicle gliding in wild type, heterozygous, and lubricin knockout mice. J Orthop Res **29**: 384-389.

Kubo K, Kanehisa H, Miyatani M, Tachi M, Fukunaga T (2003) Effect of low-load resistance training on the tendon properties in middle-aged and elderly women. Acta Physiol Scand **178**: 25-32.

Legerlotz K, Riley GP, Screen HR (2010) Specimen dimensions influence the measurement of material properties in tendon fascicles. J Biomech **43**: 2274-2280.

Legerlotz K, Jones GC, Screen HR, Riley GP (2011) Cyclic loading of tendon fascicles using a novel fatigue loading system increases interleukin-6 expression by tenocytes. Scand J Med Sci Sports DOI: 10.1111/j.1600-0838.2011.01410.x.

Lichtwark GA, Wilson AM (2005) *In vivo* mechanical properties of the human Achilles tendon during one-legged hopping. J Exp Biol **208**: 4715-4725.

Lui PPY, Maffulli N, Rolf C, Smith RKW (2010) What are the validated animal models for tendinopathy? Scand J Med Sci Sports **21**: 3-17.

Maganaris CN, Paul JP (1999) *In vivo* human tendon mechanical properties. J Physiol **521**: 307-313.

Patterson-Kane JC, Firth EC, Goodship AE, Parry DA (1997) Age-related differences in collagen crimp patterns in the superficial digital flexor tendon core region of untrained horses. Aust Vet J **75**: 39-44.

Perkins NR, Reid SWJ, Morris RS (2005) Risk factors for injury to the superficial digital flexor tendon and suspensory apparatus in Thoroughbred racehorses in New Zealand. N Z Vet J **53**: 184-192.

Reeves ND, Maganaris CN, Narici MV (2003) Effect of strength training on human patella tendon mechanical properties of older individuals. J Physiol **548**: 971-981.

Reuvers J, Thoreson AR, Zhao C, Zhang L, Jay GD, An KN, Warman ML, Amadio PC (2011) The mechanical properties of tail tendon fascicles from lubricin knockout, wild type and heterozygous mice. J Struct Biol **176**: 41-45.

Riemersa DJ, Schamhardt HC (1982) The cryo-jaw, a clamp designed for *in vitro* rheology studies of horse digital flexor tendons. J Biomech **15**: 619-620.

Riley G (2008) Tendinopathy: from basic science to treatment. Nat Clin Pract Rheum **4**: 82-89.

Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis. Ann Rheum Dis **53**: 367-376.

Robinson PS, Lin TW, Reynolds PR, Derwin KA, Iozzo RV, Soslowsky LJ (2004) Strain-rate sensitive mechanical properties of tendon fascicles from mice with genetically engineered alterations in collagen and decorin. J Biomech Eng **126**: 252-257.

Robinson PS, Huang TF, Kazam E, Iozzo RV, Birk DE, Soslowsky LJ (2005) Influence of decorin and biglycan on mechanical properties of multiple tendons in knockout mice. J Biomech Eng **127** :181-185.

Screen HRC (2008) Investigating load relaxation mechanics in tendon. J Mech Behav Biomed Mater **1** :51-58.

Smith RK, Birch HL, Goodman S, Heinegard D, Goodship AE (2002) The influence of ageing and exercise on tendon growth and degeneration--hypotheses for the initiation and prevention of strain-induced tendinopathies. Comp Biochem Physiol A Mol Integr Physiol **133**: 1039-1050.

Smith KD, Vaughan-Thomas A, Spiller DG, Innes JF, Clegg PD, Comerford EJ (2011) The organisation of elastin and fibrillins 1 and 2 in the cruciate ligament complex. J Anat **218**: 600-607.

Stephens PR, Nunamaker DM, Butterweck DM (1989) Application of a Hall-effect transducer for measurement of tendon strains in horses. Am J Vet Res **50**: 1089-1095.

Thorpe CT (2010) Extracellular matrix synthesis and degradation in functionally distinct tendons. PhD Thesis, University College London.

Thorpe CT, Clegg PD, Birch HL (2010a) A review of tendon injury: why is the equine superficial digital flexor tendon most at risk? Equine Vet J **42**: 174-180.

Thorpe CT, Streeter I, Pinchbeck GL, Goodship AE, Clegg PD, Birch HL (2010b) Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging. J Biol Chem **285**: 15674-15681.

Thorpe CT, Udeze CP, Birch HL, Clegg PD, Screen HRC (2012) Specialisation of tendon mechanical

properties results from inter-fascicular differences. J R Soc Interface **9**: 3108-3117.

Wood LK, Arruda EM, Brooks SV (2011) Regional stiffening with aging in tibialis anterior tendons of mice occurs independent of changes in collagen fibril morphology. J Appl Physiol **111**: 999-1006.

Wren TAL, Yerby SA, Beaupré GS, Carter DR (2001) Mechanical properties of the human achilles tendon. Clin Biomech **16**: 245-251.

Yamamoto E, Iwanaga W, Yamamoto N, Hayashi K (2004) Growth-related changes in the mechanical properties of collagen fascicles from rabbit patellar tendons. Biorheology **41**: 1-11.

Discussion with Reviewers

Reviewer III: Although it is acknowledged that mechanically testing tendon, especially at a small scale (e.g., fascicle, IFM), can be challenging, some of the methods used in this study require further validation (particularly with regard to measurement accuracy).

Authors: Mechanical testing at the small scales investigated in this study is indeed challenging, and is something we consider carefully when planning experiments so that we can be confident in the accuracy of our techniques. We validate all experimental techniques and ensure all the equipment used is regularly calibrated and suitable for each experiment. As detailed in the manuscript, the load cells and actuators used in this study have a resolution at least 1 order of magnitude lower than the smallest force or extension values recorded for any sample. Furthermore, previous work has established the accuracy of laser micrometer measures to assess sample diameter. We also assessed local sample strains by placing markers on the sample surface and videoing tests and comparing these with the grip-to-grip displacements, to show that grip-to-grip displacements did not differ from local strains and so could be used to calculate sample strain.