Some tendons, such as the human Achilles and equine superficial digital flexor tendon (SDFT), act as energy stores, stretching and recoiling to increase efficiency during locomotion. Our previous observations of rotation in response to applied strain in SDFT fascicles suggest a helical structure, which may provide energy-storing tendons with a greater ability to extend and recoil efficiently. Despite this specialization, energy-storing tendons are prone to age-related tendinopathy. The aim of this study was to assess the effect of cyclic fatigue loading (FL) on the microstructural strain response of SDFT fascicles from young and old horses. The data demonstrate two independent age-related mechanisms of fatigue failure; in young horses, FL caused low levels of matrix damage and decreased rotation. This suggests that loading causes alterations to the helix substructure, which may reduce their ability to recoil and recover. By contrast, fascicles from old horses, in which the helix is already compromised, showed greater evidence of matrix damage and suffer increased fibre sliding after FL, which may partially explain the age-related increase in tendinopathy. Elucidation of helix structure and the precise alterations occurring owing to both ageing and FL will help to develop appropriate preventative and repair strategies for tendinopathy.

1. Introduction

Tendons provide an attachment from muscle to bone, transferring force generated by muscle contraction to the skeleton and facilitating movement. The ability to withstand large unidirectional forces is provided by their structure; tendons are hierarchical fibre-composite materials, in which type I collagen molecules are grouped together, forming subunits of increasing diameter, the largest of which is the fascicle [1]. At the larger hierarchical levels, the collagen is interspersed with a predominantly non-collagenous matrix [2]. Historically, many assumed that the structure of all tendons was very similar, but there is recent evidence demonstrating numerous compositional, structural and organizational specializations according to tendon function [3–9].

Tendons can broadly be divided into two categories depending on their function, those that act purely to position the limb for locomotion and those that act as elastic springs during locomotion, storing energy and thus reducing the energetic cost of locomotion [10,11]. Energy-storing tendons are often subjected to high forces and are more compliant than positional tendons to allow the elongation required for maximal energy storage and return [3,6,12]. Therefore, energy-storing tendons must withstand large, repetitive stresses and strains during exercise. In the Achilles tendon, which is the predominant energy store in humans, strains in excess of 10% have been recorded during hopping exercise [13]. The energy-storing equine superficial digital flexor
tendon (SDFT) has a similar function to the human Achilles [3,7,14,15] and experiences similar high strains, which can reach 16% during galloping [16]. However, the mechanisms that enable these high levels of extension in energy-storing tendons are yet to be determined.

Previous analysis of the microstructural strain response in tendon fascicles has demonstrated that, in general, fascicle extension is dominated by sliding between the collagen fibre components rather than fibre extension [17–20]. However, the majority of these studies were performed on fascicles from rat tail tendon, which has a purely positional function. Our recent work comparing the microstructural strain response of fascicles from the energy-storing SDFT and positional common digital extensor tendon (CDET) has demonstrated a novel mechanism for elongation involving rotation of the fascicle; this rotation occurs to a much greater extent in the SDFT fascicles than in CDET fascicles in response to applied strain [7]. This rotation suggests the presence of helical substructures within energy-storing tendons. Further, our previous results demonstrate that SDFT fascicles are better able to recoil and recover following loading than CDET fascicles, suggesting that these helical substructures may provide a mechanism for efficient extension and recoil [7].

Despite these adaptations, energy-storing tendons are particularly prone to injury, which is thought to occur as a result of accumulation of microdamage within the tendon matrix rather than acute injury [21]. In humans, the prevalence of Achilles injury is reported as 3% in the general population under 45 years of age [22], increasing to 15–56% in elite athletes, depending on the type of sport undertaken [22,23]. Injury to the SDFT in athletic horses is also common, with SDFT pathology reported in 11–24% of thoroughbred racehorses [24,25] and shows a similar initiation and progression to human Achilles tendinopathy [26,27]. Further, tendons become more prone to injury with increasing age, with regards to both the human Achilles [22,28] and equine SDFT [29,30]. This age-related predisposition to injury is not well understood. However, our previous work has demonstrated that ageing results in decreased rotation in SDFT fascicles under monotonic loading and that this is accompanied by decreased recovery and increased hysteresis [7]. These findings suggest that ageing results in alterations to the helix substructure, resulting in a decreased ability to recoil and recover, which may predispose aged tendons to injury. Therefore, the aim of this study was to assess how cyclic fatigue loading (FL) affects the extracellular matrix microstructural response in SDFT fascicles from young and old horses. We hypothesized that FL would result in alterations in tendon fascicle extension mechanisms and that these alterations would show age-specific differences, with greater changes in samples from aged individuals.

2. Material and methods

2.1. Sample collection and preparation

Forelimbs distal to the carpus were collected from half- to full-thoroughbred horses aged 3–6 years (n = 10, young (skeletally mature) group) and 17–20 years (n = 10, old (geriatric) group [31]), euthanized at a commercial equine abattoir. Only tendons that had no evidence of previous tendon injury at post-mortem examination were included in the study. The SDFT was dissected free from the limbs from the level of the carpus to the metacarpophalangeal joint, wrapped in tissue paper dampened with phosphate-buffered saline and stored frozen at −20°C wrapped in aluminium foil within 24 h of animal’s death. It has previously been shown that one freeze–thaw cycle does not affect tendon mechanical properties [32]. On the day of testing, the tendons were allowed to thaw at room temperature and fascicles (8–12 fascicles per tendon, approx. 25 mm in length, diameter of 0.2–0.4 mm) were isolated from the mid-metacarpal region of the tendon by cutting with a scalpel longitudinally through the tendon using previously established protocols [33,34]. Fascicle hydration was maintained by storing the fascicles on tissue paper dampened with Dulbecco’s modified eagles medium (DMEM) prior to testing. Fascicle diameter was measured continuously at a single angle along a 10 mm region in the mid-portion of the fascicle using a laser micrometer [6]. The smallest diameter recorded was used to estimate fascicle cross-sectional area, assuming a circular cross section. All experiments were performed at room temperature. Fascicles were observed carefully during each experiment to ensure that only one fascicle was being tested.

2.2. Fascicle creep tests to failure

Preliminary tests were carried out to assess the cyclic creep response of fascicles from young and old SDFTs (n = 4 tendons per age group, replicates = 10–12 fascicles per tendon). Each fascicle was secured in a custom-made chamber system [34] at a resting grip-to-grip length of 10 mm. Fascicles were maintained in DMEM for the duration of the experiment. A displacement of 0.2 mm was applied to remove any slack from the samples prior to testing, and reported strains were established relative to this consistent starting condition. Half of the fascicles were tested to failure at a speed of 1 mm s\(^{-1}\), and force–displacement data were recorded at a frequency of 100 Hz (WINTEST software, Bose ElectroForce 5500 Biodynamic test instrument, Gillingham, UK). The average failure stress was calculated for the fascicles from each tendon. The remainder of the fascicles were subjected to cyclic FL to 50% of their predicted failure stress, with a median stress of 25% and a minimum force of 0.2 N using a sinusoidal waveform at a frequency of 1 Hz until failure. Force–displacement data were recorded at a frequency of 100 Hz, recording data from 30 consecutive cycles in every 300 cycles. In addition, every 10th maximum and minimum reading was recorded. Per cent strain was calculated from maximum displacement data and the resultant data were used to plot creep curves to failure.

2.3. Effect of fatigue loading on fascicle extension mechanisms

2.3.1. Fatigue loading

Fascicles from a subset of young and old SDFTs were analysed to assess the effect of FL on fascicle extension mechanisms (n = 6 tendons per age group, replicates = 6–8 fascicles per tendon). Half of the fascicles acted as unFatigue-loaded controls, whereas the remaining fascicles were subjected to cyclic FL for 1800 cycles at a frequency of 1 Hz using the cyclic loading protocol described above. After 1800 cycles had been completed, fascicles were removed from the chambers and the fibre-level response to applied strain was assessed.

2.3.2. Assessment of fibre-level response to incrementally applied strain

Fascicles in both the control and FL groups were stained with the collagen stain 5-(4,6-dichlorotiazin-2-yl)amino)fluorescein hydrochloride (5-DTAF) at a concentration of 2 mg ml\(^{-1}\) in 0.1 M sodium bicarbonate buffer, pH 9 for 20 min. Following staining the fascicles were washed in two changes of DMEM...
for 20 min, and secured in a custom-made tensile testing rig [17], at a resting grip-to-grip length of 10 mm. Fascicles were maintained in DMEM for the duration of the experiment. Previous experiments have shown that 5-DTAF does not affect fascicle material properties [7].

Each fascicle was viewed under the laser scanning confocal microscope (TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany) using a ×20 objective (HC PL Fluotar, Nikon, Kingston-Upon-Thames, UK). Fascicle alignment and orientation was checked under brightfield settings to ensure that fibres were aligned with the loading direction and no gross twisting of the sample had occurred. The grips were slowly moved apart and the sample monitored visually until a small amount of tension was applied, which was signified by the fascicle slightly lifting off the base of the rig [35]. This corresponded to a load of approximately 0.1 N (range: 0.05–0.15 N). A grid was then photobleached at a depth of approximately 20–25 μm within the fascicle, using a 488 nm krypton–argon laser. The grid encompassing a series of 2 μm thick lines, bleached in the central region of the fascicle away from the gripping regions, to create a grid of four squares, each 50 × 50 μm² (figure 1a), covering approximately half of the fascicle width. The laser intensity was then reduced to the imaging range, and the sample imaged in the same focal plane with the same objective lens at a resolution of 2048 × 2048 pixels², with each pixel measuring 0.18 × 0.18 μm². A focal plane of 20–25 μm within the fascicle was chosen as images at this depth had the greatest clarity. The fascicle was then strained to 2% at a rate of 1% s⁻¹ and the grid was refocused, before imaging again (figure 1b). Incremental strains of 2% were applied in this manner up to a maximum of 10%, and the grid was imaged at each strain increment. Previous experiments have established that at macrolevels, applied strains are distributed evenly across the fascicle [7]. There was a hold period of approximately 1 min before imaging at each increment, while the focal plane was located; it has previously been shown that this is sufficient time for complete stress relaxation to occur [7,17].

2.3.3. Image analysis

Images from the confocal experiments were processed using the analysis software IMAGEJ (1.34s, National Institute of Health, USA) as described previously [7]. The grid deformation parameters calculated are shown in figure 1c. In order to calculate the local x- and y-strains, the points where the lines in the x- and y-planes crossed were marked, and the image was thresholded and skeletonized; with the resulting image consisting of nine pixels representing the grid corners. The coordinates of these pixels were exported to Microsoft Excel for data analysis. In order to quantify the local strains within the grid, the following parameters were calculated from the grid corner coordinates:

— local strain (%) in x-direction (100Δx/x): percentage strain in x-direction (with axis of loading); and
— local strain (%) in y-direction (100Δy/y): percentage strain in y-direction (perpendicular to loading axis).

Poisson’s ratios (v) were calculated at each strain increment using the following equation:

\[ v = \frac{\text{local y-strain}}{\text{applied strain}} \]

In order to calculate the maximum deviation of the vertical gridline (y-axis), images were thresholded, despeckled and

Figure 1. Images showing grid bleached onto fascicles stained with 5-DTAF at 0% applied strain, with loading direction indicated by arrows (a), and grid deformation at 4% applied strain in control samples (b). The pixel lines generated during image analysis have been superimposed on to image (b). Schematic (c) showing the four parameters used to quantify grid deformation: fibre extension (x-strain), transverse strains (y-strain), fibre sliding (d₁ + d₂) and grid rotation (θₓ).
skeletonized in ImageJ to generate a single pixel trace of the left most gridline in the y-plane. Any noise was removed from the image and the coordinates of the line exported to Origin (OriginLab, Northampton, USA). A straight line was fitted between the start and endpoints of this line. The maximum deviations of the gridline from the straight line in either direction were also calculated, and the sum of these deviations \( (d_1 + d_2) \), figure 1c) used to provide a measure of fibre sliding.

In order to quantify grid rotation, images were processed in ImageJ (as described above) to generate a single pixel trace of the bottom horizontal gridline \((x\)-axis\) and the coordinates of this line were exported to Origin. A linear line of best fit was fitted to the coordinates, and the angle of this line relative to the \( x \)-axis \( (\theta, \text{ figure 1c}) \) was calculated.

2.4. Statistical analysis

The distribution of the data was tested using a D’Agostino–Pearson test for normality (GraphPad Prism). Data with a normal distribution were analysed using a two-way ANOVA followed by Bonferroni post hoc tests to assess the effect FL on microstructural strain response and determine whether this was altered with ageing. Data that were not normally distributed were analysed using Kruskal–Wallis tests followed by Dunn’s multiple comparison post hoc analysis (GraphPad Prism). Statistical significance was set at \( p < 0.05 \).

3. Results

3.1. Fascicle failure and creep properties

There was no difference in average fascicle diameter or failure stress in samples from young and old horses (diameter: 0.26 ± 0.03 mm versus 0.26 ± 0.04 mm; failure stress: 65.39 ± 27.84 MPa versus 61.80 ± 29.22 MPa). These values are likely to be lower than the true failure stress, owing to the slow test speed relative to the in vivo loading rate, and the presence of stress concentrations at the grips [36]. However, these values can be used to determine an appropriate stress for fatigue testing. Concerning fatigue testing, the number of cycles to failure was significantly lower in fascicles from aged horses, decreasing from 16 825 ± 927 cycles in young horses to 4062 ± 927 cycles in old horses \( (p = 0.02, \text{ figure 2}) \).

3.2. Effect of fatigue loading on response to incremental strains in young samples

FL caused mild damage to the tendon matrix in fascicles from young horses, characterized by the presence of mildly kinked fibres throughout the matrix (figure 3a), which were observed in the fluorescently stained fascicles under the scanning confocal microscope.

3.2.1. Microstructural strain response

In agreement with previous studies [7,17], local longitudinal strains, representing fibre extension, were consistently smaller than overall applied strain. The amount of fibre extension did not differ between FL and control samples (figure 4a). In control samples from young horses, large compressive strains were observed perpendicular to the axis of loading. There was a trend towards decreasing transverse strain with FL but this was not significant (figure 4b). Therefore, the resulting Poisson’s ratios were larger in control samples than that in FL samples, although this only reached significance at 6% applied strain \( (p < 0.05, \text{ figure 4c}) \). A small amount of between fibre sliding \( (d_1 + d_2) \) was seen in the majority of samples, but FL had no effect on the degree of fibre sliding measured in young samples (figure 4d). Levels of grid rotation were similar to those recorded previously in unloaded SDFT fascicles [7]. FL resulted in a significant decrease in rotation at all strain increments above 2% in samples from young horses \( (p < 0.05, \text{ figure 4e}) \).

3.3. Effect of fatigue loading on response to incremental strains in old samples

Damage to the fascicle matrix was more obvious after FL in old samples than in young (figure 3b), with a greater number of fibre kinks observed. In addition, the majority of FL samples from old animals exhibited widening of the interfibre space, and in a few cases fibre discontinuities were observed.

3.3.1. Microstructural strain response

The amount of fibre extension was similar to that seen in samples from young horses, and FL had no significant effect on the amount of fibre extension observed (figure 5a). In agreement with our previous findings [7], transverse strain was decreased in control samples from aged individuals compared with young \( (p < 0.02) \). FL appeared to result in a further reduction in transverse strain in aged samples, although this was not significant (figure 5b). Correspondingly, there was a trend towards decreased Poisson’s ratios in FL samples from aged individuals, but this was only significant at 2% applied strain (figure 5c, \( p < 0.05 \)). Levels of fibre sliding were similar between samples in the young and old control groups. Fibre sliding showed a significant increase after FL in old samples at all strain increments (figure 5d, \( p < 0.05 \)). In agreement with previous results, ageing resulted in decreased rotation in control samples [7]. However, there was no reduction in rotation after FL in these aged samples (figure 5e).

4. Discussion

The data support the hypothesis, showing that FL results in altered extension mechanisms in SDFT fascicles from both...
young and old horses, and that these alterations are age specific, with greater evidence of matrix damage after FL in old samples. While ageing did not cause alterations in observed tendon fascicle failure properties, it did result in a deterioration of fatigue resistance, suggesting that aged samples are less resilient to cyclic loading. This decrease in cycles to failure was accompanied by greater alterations in microstructural strain response after a bout of cyclic loading.

Several studies of the effect of fatigue on tendon properties have been carried out in a variety of species, from small to large animal models [34,37,38]. While species such as the mouse and rat provide a simple system to understand basic mechanisms of damage, it is not clear how data from these studies translate to human tissues, particularly in terms of energy-storing tendons. The horse is an excellent large animal in which to study tendon structure–function relationships, as the equine SDFT is an extreme example of an energy store, with a function very similar to that of the Achilles [15]. Indeed, the horse is one of a few species that suffers from naturally occurring tendon injuries, the initiation, progression and age-related susceptibility of which is comparable to that seen in Achilles tendinopathy [15,22,27,29]. Elucidation of structure–function relationships in the equine SDFT are therefore likely highly relevant to understanding human Achilles function in health, disease and ageing.

Figure 3. Typical confocal images of control and fatigue-loaded SDFT fascicles from young and old horses at (a) 0% and (b) 10% applied strain. Fatigue-induced damage is characterized by fibre kinks (indicated by arrows); these kinks were more prominent in aged samples. Widening of the interfibre space was also observed in samples from aged individuals (indicated by circles). Scale bar, 25 μm.
Previous studies have shown that cyclic loading causes damage to the tendon matrix, which becomes evident before any significant changes in tendon mechanical properties are observed [39]. Low-level fatigue damage is characterized by the presence of kinked fibre deformations [39–42]. As the level of fatigue damage increases, the number of fibre kinks also becomes larger; this is accompanied by widening of the interfibre space. High-level fatigue damage is characterized by severe matrix disruption, fibre thinning, angulations and fibre discontinuities [37,40,43]. In this study, similar changes were observed and it is evident that the fatigue damage generated by cyclic loading is more severe in SDFT fascicles from older horses. FL samples from young horses exhibited low-level fatigue damage, with the presence of a few kinked fibres. In aged samples, FL induced more notable damage, with evidence of disruption between fibres in the majority of samples, and severe matrix damage and fibre discontinuities in some samples.

While several studies have investigated the effect of FL on matrix appearance [39,40], alterations in mechanical properties [34,39,41] and cell response [34,38,44], to the authors’ knowledge, this study is the first to present data regarding the effect of FL on tendon fascicle microstructural strain response. Our results show that in samples from young horses, levels of fibre extension were not altered by FL, suggesting that fibre integrity was maintained throughout the test procedure. There were also no alterations in the amount of fibre sliding measured after FL. However, we have previously established that, while fibre sliding governs fascicle extension in tendons with a purely positional function [7], in the energy-storing SDFT, extension appears to be dominated by the ‘unwinding’ of helical substructures, resulting in grid rotation [7]. This rotation is associated with a greater ability to recoil and recover efficiently [7]. In this study, we observed a significant decrease in rotation after FL in samples from young horses, suggesting that FL causes alterations to the helix structure. This is somewhat supported by the trend for decreased Poisson’s ratios observed in samples from young horses; while the large Poisson’s ratios observed may be owing to exudation of fluid from the fascicles [17], finite-element modelling has shown that the presence of helical substructures within tendon predicts large Poisson’s ratios [45]. While the specific alterations that occur to the helix during FL are as yet unknown, it is possible that some of the characteristic sample lengthening observed during creep may occur as a result of straightening of the coil of the helix, such that helix pitch angle decreases. This has important implications for tendon fatigue resistance, as alterations to the helix may decrease the ability of SDFT fascicles to extend and recoil efficiently, which is likely to increase the risk of microdamage occurring to the tendon matrix.

One limitation of this study is the methods used to calculate fascicle cross-sectional area. Previous studies have demonstrated that fascicle cross section within the equine SDFT appears irregular [46] such that assuming a circular cross section may lead to errors when calculating the stress to apply during FL. This may partially account for the larger variation seen in the microstructural strain response in fatigue-loaded samples.

When assessing the effect of FL on fascicle microstructural strain response, it is also important to consider the gripping methods and sample length used, as well as the values of stress and strain that were applied. Gripping the samples during FL resulted in some damage and flaring at the fascicle ends, which may have contributed to the alterations seen in microstructural strain response. However, owing to the increase in sample length during FL, fascicle ends were gripped in a previously unclamped region when secured in the confocal rig for evaluation of strain response, minimizing any gripping artefact in FL samples. Furthermore, the large aspect ratio (approx. 33) of fascicles ensured that any flaring near the gripping sites did not persist into the central region.
of the fascicle where the grid was bleached, and so is unlikely to have had a significant effect on the results. However, the use of a control group that had been exposed to very low levels of cyclic loading would have ensured any observed differences were owing to FL rather than gripping of the samples during the loading procedure. The effect of sample length also needs to be taken into account. In this study, the grip-to-grip distance was 10 mm. Our previous work has shown that the end effects generated by such a grip-to-grip length artificially augment fascicle strains and should subsequently be corrected for [33]. Using our previous methods [6] and data from SDFT fascicle failure strains, the appropriate correction factor was calculated as 0.65. This results in a maximum applied strain of 6.5%, which is still more than three times the local longitudinal strain measured within the grids at this strain increment, showing that strain is distributed throughout the tendon matrix by mechanisms other than fibre extension.

It is also important to consider how the stress and strain applied during the fatigue test may relate to in vivo levels. In this study, FL samples were subjected to cyclic loading to 50% of their predicted failure stress. Data from previous studies suggest that the SDFT may experience in vivo stresses as high as 90% of their in vitro failure stress and strain during maximal exercise [3,16,47,48]. However, our previous results suggest that, in the SDFT as a whole, initial tendon extension is facilitated by sliding between adjacent fascicles [6], such that fascicles are likely to be exposed to lower stresses and strains than the whole tendon. Peak in vivo fascicle stresses are yet to be determined, so it is difficult to compare the stresses applied in this study with the in vivo stress environment. However, it is evident that the loads applied were high enough to induce mild to moderate damage within the samples.

Though several studies have investigated alterations in tendon mechanical properties as a function of ageing [14,49–52], few have assessed the effect of FL on aged tendon. Pike et al. [53] applied cyclic fatigue tests to ovine flexor and extensor tendons, and demonstrated that the time to rupture increased from birth to maturation. However, this study did not include tendons from aged individuals. A more recent study by Kietrys et al. [54], using an in vivo rat overuse model has shown that repetitive loading in aged individuals resulted in greater tendon inflammation and reduced limb agility compared with young tendons that had undergone the same loading regime [54]. These findings, combined with the data presented in this study, indicate that maturation results in an increased ability of tendons to resist repetitive loading, whereas ageing causes a decrease in fatigue resistance. The confocal images presented in figure 3 show evidence of more extensive damage within the matrix if FL samples are from old animals. While none of the tendons tested had gross evidence of pathology, it is possible that microdamage may have been present within the matrix of some aged samples prior to in vitro FL. Indeed, previous studies indicate that there are several mechanisms that predispose aged tendons to microdamage; it has been shown that partially degraded collagen accumulates in aged SDFTs, likely owing to an accumulation of non-enzymatic cross-linking rendering the collagen more resistant to proteolytic enzyme activity [5]. In addition, a decreased capacity for tendon extension through the interfascicular matrix may expose fascicles from aged tendons to higher levels of strain [14], increasing the risk of microdamage occurring to the fascicle matrix.

In addition to altering the microstructural strain response, FL and subsequent microdamage within the tendon matrix is also likely to result in alterations to cell behaviour. In this study, experiments were performed on tissues that had previously been frozen, and as such cell response could not be studied. However, it has previously been reported that cyclic loading of viable tendon explants in vitro results in increased cell death and production of prostaglandin-E2 and collagenases at high magnitudes of applied load [55].

![Figure 5](http://rsif.royalsocietypublishing.org/Downloaded from http://rsif.royalsocietypublishing.org/)

**Figure 5.** (a) Longitudinal strain, (b) transverse strain, (c) Poisson’s ratio, (d) fibre sliding and (e) grid rotation at increasing strain increments in control (filled square) and fatigue-loaded (unfilled square) SDFT fascicles from old horses. Data are displayed as mean ± s.e.m. Statistical significance: *p < 0.05; **p < 0.01.
It has been suggested that this degenerative cascade is owing to understimulation of the cells as a result of damage to the collagen fibres [56]. Further, injured tendons often exhibit cell rounding [21], which is likely to be the result of an altered mechanical environment. While any age-related alterations to cell behaviour as a result of FL are yet to be determined, it is likely that the greater levels of damage observed with FL in aged samples in this study would result in greater alterations in cell response, further increasing the risk of subsequent injury.
The age-specific alterations in microstructural strain response as a result of FL are highlighted in figure 6. In aged samples, there were no alterations in fibre extension as a result of FL. However, there was a trend towards decreased Poisson’s ratios, which were negative in some instances, suggesting that sample thickness was increasing as strain was applied. While this seems surprising, some samples appeared to pull apart as levels of strain increased, and widening of the interfibre space was observed (figure 3b), which may be indicative of the initiation of sample failure. In addition, the amount of fibre sliding increased significantly after FL, possibly owing to the disruption of the non-collagenous matrix observed between fibres.

In order to understand the interactions of ageing and FL, it is useful to review how fascicle micromechanics vary with both conditions (figure 7). In young SDFT fascicles, helical rotation appears to govern extension, and in agreement with our previous data [7], ageing resulted in decreased rotation in control fascicles from aged SDFTs, suggesting that ageing also results in alterations to the helical substructures in the SDFT. This loss of helix integrity may occur as a result of cumulative loading of the tendon during the lifetime of the animal, with the result that aged fascicles are less resistant to cyclic loading. When aged SDFT fascicles are then subjected to FL in vitro, there is subsequently little helical capacity to manage the loading response, which may explain the more severe response to FL in aged fascicles in terms of the greater variability seen in matrix appearance and the increased levels of fibre sliding.

5. Conclusion
Fascicles from the energy-storing equine SDFT show an age-related variation in response to cyclic loading. In samples from young horses, FL resulted in decreased fascicle rotation, indicating alterations to the helical substructures previously suggested within SDFT fascicles [7]. These alterations may decrease the ability of fascicles to recoil efficiently and increase the risk of damage occurring to the matrix. In aged samples, in which the helix structure may already be compromised, FL resulted in increased fibre sliding, which may be owing to accumulation of microdamage within the non-collagenous interfibre matrix and is likely to influence cell behaviour. This may explain the increased risk of injury to energy-storing tendons in aged individuals. It is therefore important to fully characterize this helix structure within energy-storing tendons, and determine how alterations in helix parameters affect tendon fatigue resistance to further understand the initiation and progression of tendinopathy.

Data accessibility. Mechanical test data and confocal images are available from the Dryad digital repository: doi:10.5061/dryad.8b35s.

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