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Effect of fatigue loading on structure and functional behaviour of fascicles from energy-storing tendons

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Abstract

Tendons can broadly be categorised according to their function; those that act purely to position the limb and those that have an additional function as energy stores. Energy-storing tendons undergo many cycles of large deformations during locomotion, and so must be able to extend and recoil efficiently, rapidly and repeatedly. Our previous work has shown rotation in response to applied strain in fascicles from energy-storing tendons, indicating the presence of helical substructures which may provide greater elasticity and recovery. In the current study, we assessed how preconditioning and fatigue loading affects the ability of fascicles from the energy-storing equine superficial digital flexor tendon to extend and recoil. We hypothesised that preconditioned samples would exhibit changes in microstructural strain response, but would retain their ability to recover. We further hypothesised that fatigue loading would result in sample damage, causing further alterations in extension mechanisms and a significant reduction in sample recovery. The results broadly support these hypotheses, preconditioned samples showed some alterations in microstructural strain response, but were able to recover following the removal of load. However, fatigue loaded samples showed visual evidence of damage and exhibited further alterations in extension mechanisms, characterised by decreased rotation in response to applied strain. This was accompanied by increased hysteresis and decreased recovery. These results suggest that fatigue loading results in a compromised helix substructure, reducing the ability of energy-storing tendons to recoil. A decreased ability to recoil may lead to an impaired response to further loading, potentially increasing the likelihood of injury.

Keywords: Mechanical testing, fatigue damage, micromechanics, confocal microscopy, hysteresis.

1. Introduction

Tendons provide the attachment from muscle to bone, facilitating movement of the limbs during locomotion. Specific tendons also act as energy stores, stretching and recoiling by up to 16 % with each stride to decrease the energetic cost of locomotion [1, 2]. To store and release sufficient energy in a useable form, these tendons need to be more elastic than tendons with a purely positional function [3, 4]. Such differences in mechanical properties between tendon types must be conferred by differences in structural organisation and composition. All tendons can be considered as hierarchical fibre-composite materials, in which type I collagen molecules are grouped together in a highly ordered fashion, forming subunits of increasing diameter [5], the largest of which is the fascicle. At the larger hierarchical levels, the collagenous units are interspersed with a predominantly non-collagenous matrix [6]. While the basic structure of all tendons is similar, numerous studies have documented structural and compositional differences between energy-storing and positional tendons [7-12].

We have previously observed rotation in response to applied strain within fascicles from energy-storing tendons, which suggests the presence of helical substructures [9]. We have previously proposed that this helical formation may provide a more elastic mechanism for extension and recoil than the viscous fibre sliding that governs extension in positional tendons [9]. Despite this specialisation, energy-storing tendons such as the human Achilles and equine superficial digital flexor tendon (SDFT) are highly prone to injury [13-16]. Injury is thought to occur due to accumulation of microdamage over the course of many loading cycles, rather than as a sudden rupture [17]. In support of this, our recent work has demonstrated that cyclic fatigue loading results in alterations to the fascicle microstructure and response to applied strain [18]. We have shown that fatigued fascicles rotate less on extension suggesting loss of the helix structure.

1 However, if the helix structure is utilised by energy storing fascicles to provide better recoil than

2 fibre sliding, fatigued fascicles may have reduced ability to recover following extension. In our

3 previous work we assessed the effect of fatigue on fascicle extension mechanisms. In this study

4 we now investigate how repetitive loading affects recoil mechanisms within tendon fascicles.

5 Furthermore, our previous studies compared the microstructural strain response of fatigue loaded

6 fascicles with fascicles that had experienced no prior loading (controls) [9, 19]. However, when

7 assessing the loading response of soft tissues such as tendon, it is common practice to apply a

8 few loading cycles (typically between 10 and 30 [4, 20, 21]) prior to testing to precondition the

9 sample so it reaches a steady state [22]. Therefore previous studies could not distinguish between

10 the effects of preconditioning and fatigue loading. Indeed, while preconditioning is universally

11 accepted as a part of any mechanical testing protocol [23], the processes that occur within the

12 tissue during preconditioning are not well understood. A few recent studies have demonstrated

13 that during the first few cycles of loading there is a considerable degree of collagen fibre

14 realignment and recruitment [24, 25]. However, to the authors' knowledge, no previous studies

15 have determined how both preconditioning and fatigue loading affect the microstructural strain

16 response and recoil capacity of soft tissues.

17 The aim of this study was therefore to assess the effects of preconditioning and fatigue loading

18 on extension and recoil mechanisms within fascicles from energy-storing tendons. This provides

19 a greater understanding of the mechanisms occurring during preconditioning and allows

20 comparison of fatigue loaded samples to samples which have been preconditioned to reach a

21 steady state. We have used equine tissue for our studies as the human Achilles and equine SDFT

22 show remarkable similarities in terms of healthy function and injury risk [26, 27].

In this study, we tested the hypotheses that: 1. Preconditioning will alter the microstructural

extension and recoil mechanisms in fascicles, but fascicles will retain their ability to recover

after the removal of load. 2. Cyclic fatigue loading will result in fascicle damage, causing further

alterations in microstructural extension and recoil mechanisms and reduced ability to recover.

2. Materials and Methods

2.1 Sample Collection and Preparation

Forelimbs distal to the carpus were collected from half- to full-bred, skeletally mature,

Thoroughbred horses (aged 3 to 6 years, n = 10), euthanased at a commercial equine abattoir. We

have previously shown an intact helical fascicle structure in tendons from this young age group

[9]. Only tendons which had no macroscopic evidence of previous tendon injury at post-mortem

examination were included in the study (approximately 1 in 20 SDFTs harvested show evidence

of injury). The SDFT was dissected free from the limbs from the level of the carpus to the

metacarpophalangeal joint, and wrapped in tissue paper dampened with phosphate buffered

saline, and then in aluminium foil. Samples were stored frozen at -20 °C in sealed bags within 24

hours of animal death for up to 6 months. It has previously been shown that one freeze-thaw

cycle does not affect tendon mechanical properties [28]. On the day of testing, the tendons were

allowed to thaw at room temperature and fascicles (8-12 fascicles per tendon, approximately 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 (Fig. S1). Fascicle hydration

was maintained by storing the fascicles on tissue paper dampened with Dulbecco's Modified

Eagles Medium (DMEM). Fascicle diameter was measured continuously along a 10 mm region

in the mid-portion of the fascicle using a laser micrometer, scanning perpendicular to the fascicle

1 [10]. The smallest diameter recorded was used to estimate fascicle cross sectional area, assuming

2 a circular cross section. While previous studies have demonstrated that fascicle cross section

3 within the equine SDFT may be irregular [29], we have previously demonstrated that assuming a

4 circular cross section to calculate cross sectional area results in an overestimation of 4 % [10].

5 All experiments were performed at room temperature. Fascicles were observed carefully during

6 each experiment to ensure that only one fascicle was being tested.

7 **2.2 Mechanical testing protocols**

8 Fascicles were stained with the collagen stain 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein

9 hydrochloride (5-DTAF) at a concentration of 2 mg/ml in 0.1M sodium bicarbonate buffer, pH 9

10 for 20 min. Following staining the fascicles were washed in 2 changes of DMEM for 20 min.

11 Fascicles from each tendon were then randomly assigned to 3 groups: control (n = 3 per tendon),

12 preconditioned (PC; n = 3-4 per tendon) and fatigue loaded (FL; n = 3-4 per tendon). Control

13 samples remained unloaded, while PC and FL samples were secured in custom made chambers

14 at a resting grip-to-grip distance of 10 mm [30]. Each chamber was placed in a materials testing

15 machine (Electropuls E1000, Instron) and a preload of 0.2 N was applied to remove any slack

16 from the sample and determine the resting length. We have previously shown that fascicle failure

17 strain is more consistent between samples than failure stress [10], and so to determine the

18 appropriate load to apply for the subsequent cyclic tests, one loading cycle to a displacement of 1

19 mm (10 % strain, equivalent to 50% of predicted fascicle failure strain) was applied, and the

20 maximum load reached at this displacement was recorded. A cyclic creep test was then

21 performed for either 30 cycles for PC samples or 1800 cycles for FL samples at 1 Hz, using the

22 load recorded at 10 % strain as the maximum load for each cycle, and 0.2 N as the minimum

23 load. During each test, force and displacement data were recorded at a frequency of 100 Hz. The

displacement at 0.2 N in the last cycle was used to calculate the increase in sample length in both

the PC and FL groups. We chose to apply 30 loading cycles to samples in the PC group, as we

have previously shown that this is within the primary phase of the creep curve, but provides a

relatively stable curve compared to the first few cycles of loading [18]. 1800 loading cycles was

applied to the FL group as this has previously been shown to be sufficient to induce mild damage

within SDFT fascicles, but is well below the average number of cycles to failure, which we have

shown to be in excess of 16,000 cycles in this tendon type [18], and so would be within the

secondary portion of the creep curve.

2.3 Calculation of Hysteresis

To determine the extent of damage with FL, the percent increase in hysteresis from cycle 30 (end

of PC) to cycle 1800 (end of FL) was calculated from the mechanical testing data (GraphPad

Prism).

2.4 Determination of extension and recoil mechanisms

The microstructural strain response of the control samples was assessed within 1 hour of

staining. The strain response of samples in the PC and FL groups was assessed immediately after

loading. Each fascicle was fixed into the tensile straining rig at a resting grip-to-grip length of 10

mm. Each fascicle was viewed under the laser scanning confocal microscope (TCS SP2, Leica

Microsystems GmbH, Wetzlar, Germany) using a x20 objective (HC PL Fluotar, Nikon,

Kingston-Upon-Thames, UK). Fascicle alignment and orientation was checked under brightfield

settings and 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 [18, 31]. This corresponded to a load of approximately 0.1 N (range: 0.05 - 0.15 N). A

1 grid of four squares, each 50 μm × 50 μm, was bleached onto the samples as described

2 previously [9]. The laser intensity was then reduced to the imaging range, and the sample imaged

3 in the same focal plane with the same objective lens at a resolution of 2048×2048 pixels^2, with

4 each pixel measuring 0.18 x 0.18 µm^2. A focal plane 20-25 µm within the fascicle was chosen as

5 images at this depth had the greatest clarity. A strain of 4 % was then applied to the fascicle at a

6 rate of 1 % sec^{-1}, and the grid was re-imaged. The sample was returned to the test start position

7 and the grid re-imaged. This process was repeated, straining to a value of 8 % before once again

8 returning to 0 % strain and re-imaging. There was a hold time of approximately 1 minute before

9 imaging at each increment whilst the focal plane was located; it has previously been shown that

10 this is sufficient time for the majority of stress relaxation to occur [9, 19].

11 **2.5 Image analysis**

12 Images from the confocal experiments were processed using the analysis software Image J

13 (1.34s, National Institute of Health, USA) as described previously to generate coordinates of the

14 grid corners and single pixel traces of the left-most *y* line and bottom *x* line [9, 18]. These data

15 were used to calculate a series of grid measures, representing local longitudinal strain, transverse

16 strain, fibre sliding and grid rotation, as described previously [9, 18].

17 To assess the ability of fascicles to recoil after the application of strain, the percent recovery of

18 fibre extension, transverse strains, fibre sliding and grid rotation was calculated following return

19 from a 4 % and 8 % applied strain, relative to the initial 0 % position.

20 **2.6 Statistical Analysis**

21 The distribution of the data was tested using a D'Agostino-Pearson test for normality (GraphPad

22 Prism). Data were non-normally distributed and were therefore subjected to Kruskal-Wallis tests

8

1   followed by Dunn's multiple comparison post-hoc analysis. Statistical significance was taken as

2   p<0.05. Data are displayed as mean±SEM.

3. Results

3.1 Effect of Preconditioning

5   Samples in the PC group increased in length after cyclic loading from a resting grip to grip

6   length of 10.43±0.32 mm to 10.63±0.19 mm, corresponding to an average length increase of 1.90

7   %. When visualising the samples under the confocal microscope, there were no discernible

8   differences in the appearance of the control and PC groups (Fig. 1a-f).

9   When considering the micromechanical response, there were no significant differences in any of

10   the measured extension mechanisms as a result of PC, with fibre extension, sliding and rotation

11   reaching similar values to controls (Fig. 2). However, PC caused a large and significant

12   reduction in the compressive strains that were measured perpendicular to the loading axis in

13   control samples (p<0.01; Fig. 2b). Correspondingly, Poisson's ratios were 0.91 ± 0.45 and 1.58 ±

14   0.32 in control samples at 4 % and 8 % strain respectively, decreasing to 0.46 ± 0.53 and 0.10 ±

15   0.24 after PC.

16   Recovery of fibre extension, transverse strains and rotation did not differ between control and PC

17   samples (Fig. 3). However, percent recovery of fibre sliding was significantly reduced as a result

18   of PC after both 4 % and 8 % applied strain (Fig. 3d).

3.2 Effect of Fatigue Loading

20   FL resulted in a more substantial increase in sample length with an average length, post-loading,

21   of 11.00±0.62 mm, which corresponds to an increase of 5.48 % compared to starting conditions.

9

1   Samples in the FL group exhibited low levels of fatigue damage, characterised by the appearance

2   of a small number of kinked fibres and widening of the inter-fibre space (Fig. 1g-i).

3   Differences in fascicle micromechanics post FL were more pronounced. As seen in PC samples,

4   levels of fibre extension were somewhat decreased compared to controls, but this was not

5   significant (Fig. 2a). The reduction in the large compressive transverse strain was similar to that

6   seen in PC samples, reaching significance at 8 % applied strain (p<0.01; Fig. 2b). Surprisingly,

7   Poisson's ratios were slightly negative in FL samples, with values of -0.08 ± 0.38 and -0.1 ± 0.41

8   at 4 % and 8 % applied strain respectively. Fibre sliding was slightly increased in the FL group

9   compared to PC and control samples, but this did not reach significance (Fig. 2c). However, in

10   agreement with previous results [18], FL resulted in decreased rotation, with significantly

11   reduced levels compared to PC and control groups at 8 % applied strain (p<0.05, Fig. 2d).

12   Percent hysteresis increased after 1800 cycles of fatigue loading compared to 30 preconditioning

13   cycles, increasing from an average of 10.9±1.7% to 16.8±1.4% (p<0.01, Fig. 3a). This reduction

14   in elasticity at the fascicle level was mirrored by reduced recovery of all microstructural

15   extension mechanisms. The percent recovery of fibre extension showed a significant reduction in

16   FL samples compared to both control and PC samples (p<0.05, Fig 3b) and recovery of fibre

17   sliding was significantly less compared to controls, at both 4 % and 8 % applied strain (p<0.01;

18   Fig. 3d). FL also resulted in a decreased recovery of rotation, which was significantly different to

19   the control group after 4 % strain, and significantly lower than both the control and PC groups

20   after 8 % applied strain (p<0.05, Fig. 3e). By contrast, there was no difference in the percent

21   recovery of transverse strains between groups (Fig. 3c).

22   **4. Discussion**

1 In support of the hypotheses, the data show that both preconditioning and fatigue loading result

2 in alterations to SDFT fascicle microstructural strain response with greater alterations observed

3 in fatigue loaded samples. Preconditioned samples retained most of their ability to recoil, while

4 fatigue loading was associated with increased hysteresis, visible regions of damage, and a

5 significantly reduced ability to recoil within the timeframe studied.

6 When interpreting the results, the level of stress applied and number of loading cycles needs to

7 be considered, as well as the time period between loading and imaging. Preconditioning is often

8 used to reach a steady state before performing further mechanical testing and to remove any

9 influence from prior loading [4, 24, 25]. Studies have indicated that it is important to

10 precondition samples to levels equivalent to the stresses and strains that will be applied during

11 the test procedure in order to elicit a consistent response [32, 33]. In the current study, the

12 preconditioning strain of 10 % exceeds the highest strain applied (8 %) during analysis of the

13 microstructural strain response. Whilst it has been shown that a steady state may not be reached

14 until in excess of one hundred cycles have been completed [34], a typical preconditioning step

15 usually consists of between 10 and 30 cycles [4, 20, 24].

16 The results are likely affected by the time between cyclic loading and imaging, and recovery

17 time. There was a period of approximately 15 minutes between removing the samples from the

18 loading chambers and straining under the microscope, and recoil capacity was assessed

19 approximately 1 minute after unloading. If the reductions in recovery are due to a decreased

20 recoil speed rather than absolute recoil ability, this time period may not have been sufficient to

21 allow full recovery of cyclically loaded samples. Indeed, it has previously been shown that, at

22 longer timescales, the effects of preconditioning are at least partially reversible, with

23 significantly reduced preconditioning effects after 30 minutes of recovery [35, 36]. It is therefore

11

1 possible that, had the preconditioned samples been left to recover for longer, they would have

2 exhibited a similar microstructural strain response to that seen in control samples. However, it is

3 unlikely that fatigue loaded samples would have been able to recover fully over a longer time

4 period due to the observed matrix damage, although some degree of recovery may have been

5 possible.

6 **4.1 Preconditioning effects**

7 The increased sample length measured during preconditioning is characteristic of the creep

8 response [37], and is thought to occur predominantly due to sliding between adjacent fibres and

9 fibrils within the fascicle [38]. However, the absence of any apparent damage in preconditioned

10 samples correlates with the gross mechanical data, all suggesting that the preconditioning

11 protocol loaded the fascicles within their elastic limit. Nevertheless, preconditioning does seem

12 to result in some alterations in the microstructural strain response, with a non-significant

13 decrease in fibre extension and significantly decreased transverse strains compared to unloaded

14 controls. These data support previous studies, which have reported reductions in fibre diameter

15 and fibre rearrangement during stress relaxation [39, 40]. It is possible that a small number of

16 loading cycles is sufficient to result in fibre extension which was not reversed during the short

17 time period between loading and visualisation on the confocal microscope (approximately 15

18 minutes). This may account for some of the increase in length observed as a result of

19 preconditioning, and leave no remaining capacity for further fibre extension.

20 The large compressive strains measured perpendicular to the loading axis in control samples are

21 similar to those reported previously [9, 18, 19]. These large reductions in diameter are thought to

22 be due to exudation of fluid from the matrix [19, 41]. The large decrease in these strains seen in

1   preconditioned samples suggests that only a relatively small number of loading cycles are

2   required to force fluid out of the matrix, and that this process is complete within 30 cycles of

3   loading. Further, fascicles do not seem able to imbibe fluid during the short period between

4   loading and imaging. Fluid movement within tendon has not been studied *in vivo*, but several

5   studies have reported significant extrusion of fluid from tendon as a result of both cyclic and

6   static loading [42-44], supporting the results of the current study.

7   While preconditioning did not result in alterations in the levels of fibre sliding, there was a

8   significant reduction in recovery of fibre sliding in preconditioned samples compared to controls.

9   This could be as a result of permanent deformation, which would be surprising after a small

10   number of loading cycles. However, it is likely that fibre sliding exhibits time-dependent

11   behaviour as this mechanism is modulated by the non-collagenous inter-fibre matrix, the

12   behaviour of which highly time-dependent [45]. A longer time period between cyclic loading and

13   analysis of recoil capacity in preconditioned samples may therefore have resulted in increased

14   recovery of fibre sliding.

15   It is well established that fibre sliding is the predominant mechanism for extension in tendons

16   with a purely positional function [9, 19, 46]. However, our previous work has demonstrated

17   relatively low levels of fibre sliding in the energy-storing SDFT; extension in this tendon type

18   appears to be governed by unwinding of helical substructures, indicated by sample rotation, a

19   mechanism that we propose provides greater elasticity [9]. There was a small reduction in

20   rotation in preconditioned samples, but this was not significant, and samples retained the

21   majority of their ability to recoil. These results suggest that the fascicular helix structure

22   maintains its integrity during preconditioning.

1 **4.2 Effect of Fatigue Loading**

2 Fatigue loading resulted in marked alterations in fascicle behaviour compared to both control and

3 preconditioned samples, with fatigue loaded samples exhibiting a further increase in length

4 compared to those in the preconditioned group. This was accompanied by alterations in fascicle

5 appearance, with mild to moderate damage evident in fatigue loaded samples. This damage was

6 consistent with that reported previously, with the presence of irregular fibre kinks and widening

7 of the interfibre space [47-50]. These visual differences were accompanied by some alterations in

8 extension mechanisms and a marked reduction in the fascicles' immediate ability to recoil and

9 recover, characterised by increased hysteresis and decreased percent recovery of grid

10 deformation parameters.

11 Levels of fibre extension were similar between preconditioned and fatigue loaded samples.

12 However, fatigue loading resulted in reduced recovery of fibre extension. This may be indicative

13 of permanent deformation within these samples, suggesting that the fibres have been stretched

14 beyond their elastic limit. This could be due to increased levels of fibril sliding, or alternatively

15 caused by unwinding of the helix substructures such that they are no longer able to recoil

16 efficiently.

17 Small transverse strains were measured in both preconditioned and fatigue loaded samples.

18 Surprisingly, positive transverse strains were measured in some fatigue loaded samples, leading

19 to average Poisson's ratios that were slightly negative. This may be because the fibres in some

20 samples were observed to pull apart during loading (see Fig. 1i), possibly due to reduced

21 integrity of the interfibre matrix. Recovery of transverse strains did not vary significantly in any

1   of the test groups. However, considering these data are calculations of a percentage of a very

2   small value, they are likely to be highly influenced by any variability or error in the data.

3   Levels of fibre sliding appeared to increase in fatigue loaded samples compared to

4   preconditioned samples and controls, although this was not significant. This apparent increase in

5   fibre sliding may be indicative of damage initiation within the matrix. Further, recovery of fibre

6   sliding was significantly reduced in fatigue loaded samples, suggesting that fibre sliding may

7   have reached irreversible levels. Previous studies have shown that fatigue damage is often

8   characterised by widening of the inter-fibre space [47, 49, 51], suggesting that damage has

9   occurred between the collagen fibres. Indeed, some fatigue loaded samples demonstrated

10   increased spacing between fibres, which was associated with greater levels of fibre sliding in

11   those particular samples.

12   In agreement with previous findings [52], fatigue loading caused a significant reduction in levels

13   of rotation compared to both control and preconditioned groups, indicating alterations to the

14   helix substructures as a result of repetitive loading. This was accompanied by decreased recovery

15   and increased hysteresis in these samples. The significant reduction in recovery of rotation

16   resulting from fatigue loading may indicate that the alterations in helix substructure caused by

17   repetitive loading decrease the ability of fascicles from energy-storing tendons to elastically

18   stretch and recoil. Interestingly, these results are similar to the decreased ability to recover

19   observed in fascicles from aged SDFTs [9]. Tendons from older individuals will have undergone

20   a larger number of loading cycles during the lifetime of the animal, and therefore there is more

21   likely to be microdamage present in aged tendons, resulting in a reduced ability to recover,

22   similar to that seen in fatigue loaded samples.

1   The results of this study show that preconditioning of soft tissues results in alterations in fascicle

2   microstructural strain response, with the largest alterations seen in the reduction of transverse

3   strains, likely due to fluid exudation and collagen fibre recruitment and realignment. This may

4   result in a more ordered structure more able to manage further applications of load. It also clear

5   that preconditioning has little effect on fascicle extension and recovery mechanisms, with the

6   lower recovery of fibre sliding in these samples possibly due to the viscous nature of the

7   interfibre matrix that governs this response. When comparing fatigue loaded with preconditioned

8   samples, it is evident that an extended period of loading results in alterations to the fascicle

9   extension mechanisms, characterised by a decrease in sample rotation. This decreased rotation is

10   accompanied by increased hysteresis, and a reduction in recovery speed once load has been

11   removed.

12   It is important to consider the physiological relevance of these findings. It is clear that fatigue

13   damage accumulates far more rapidly *in vitro* than *in vivo*, most likely due to a combination of

14   gripping effects, test parameters that may not entirely mimic the *in vivo* loading environment and

15   a lack of healing capacity [53, 54]. However, the damage observed in the fatigue loaded group is

16   similar to that seen in tendons which have been fatigue loaded *in vivo* [47-49, 55]. Further, we

17   observed that fatigue loading resulted in changes in microstructural strain response consistent

18   with those seen in *in vivo* aged tendon [9]. These findings suggest that, although the timescales

19   may differ, *in vitro* observations are representative of *in vivo* fatigue. Decreased recoil speed and

20   increased hysteresis as a result of fatigue loading are likely to reduce energy return during

21   locomotion, and increase the risk of microdamage occurring to the tissue. These changes may

22   reduce the mechanical competence of the tissue and also alter cell response to loading, which

23   may lead to clinical injury.

4.3 Conclusions

Previous work has indicated the presence of helical substructures within fascicles from energy storing tendons, which are associated with a greater ability for fascicles to elastically stretch and recover [9]. These structures appear to be compromised as a result of cyclic fatigue loading, indicated by a reduction in sample rotation. This is associated with increased hysteresis and incomplete recovery, suggesting that fatigue-induced alterations in the helix substructure in fascicles from energy-storing tendons reduce their ability to recoil. This may help to explain how fatigue damage affects tendon properties and injury risk *in vivo*. Elucidation of the effect of fatigue damage on fascicle substructure will aid in the development of novel treatment strategies and preventative measures.

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1 **Figure Legends**

2 Figure 1. Images at 0 %, 8 % applied strain and return to 0 % strain in control (a-c), PC (d-f) and

3 FL groups (h-i). Damage indicators are highlighted in images from FL samples, with dotted lines

4 showing widening of the inter-fibre space, and arrows indicating fibre kinking.

5 Figure 2. Local longitudinal strain (a), transverse strain (b), fibre sliding (c) and grid rotation (d)

6 at 4% and 8% applied strain in non-loaded (□), PC (▦) and FL (■) samples. Data are displayed

7 as Mean ± SEM. Significance is indicated by *: * p<0.05; **p<0.01.

8 Figure 3. Percent hysteresis at cycle 30 and cycle 1800 in fatigue loaded samples (a) and percent

9 recovery of grid deformation parameters: local longitudinal strain (b), transverse strain (c), fibre

10 sliding (d) and grid rotation (e) in non-loaded (□), PC (▦) and FL (■) samples. Data are

11 displayed as Mean ± SEM. Significance is indicated by *: * p<0.05; **p<0.01; ***p<0.001.

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Figure 1

Figure 2

Figure 3
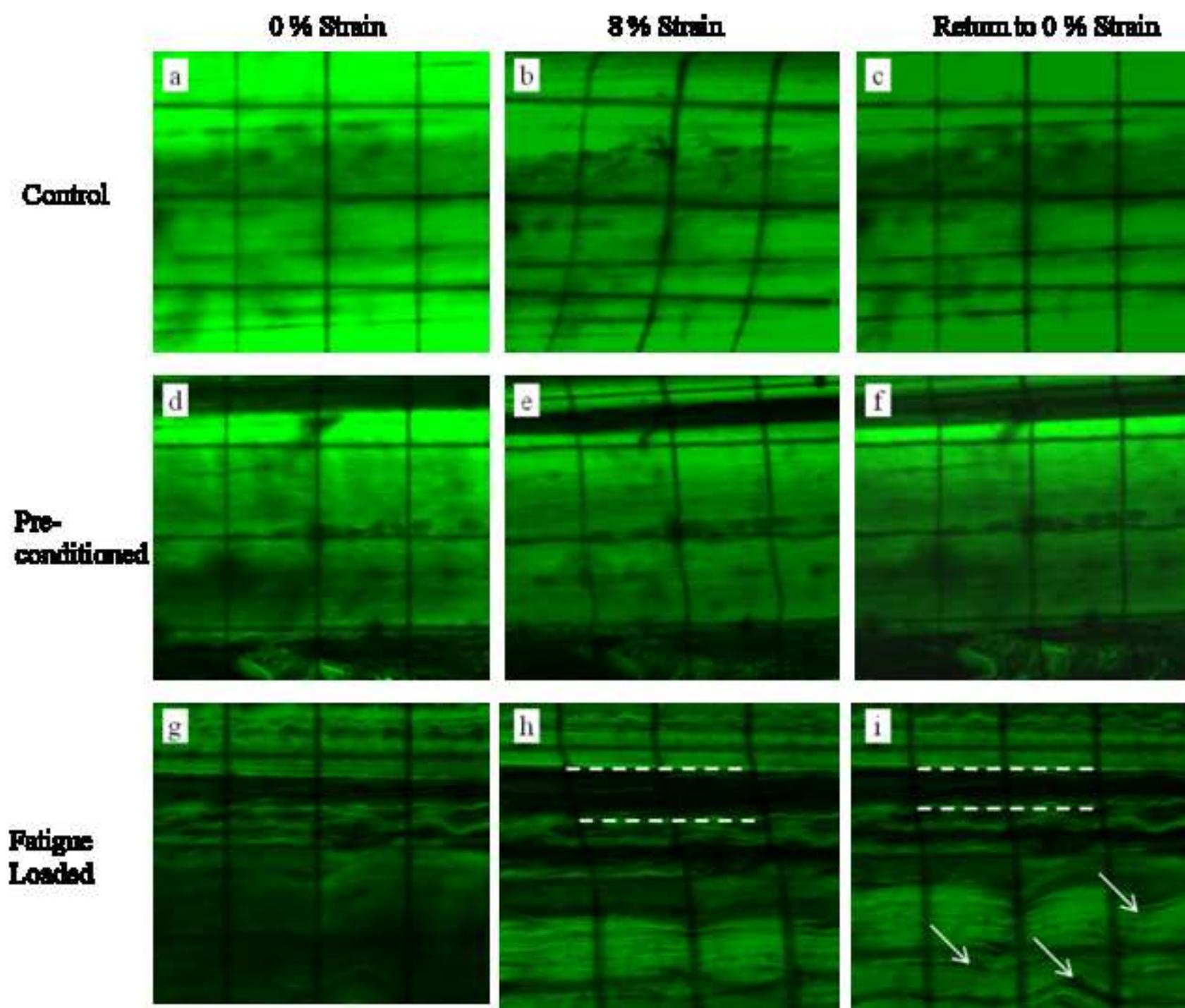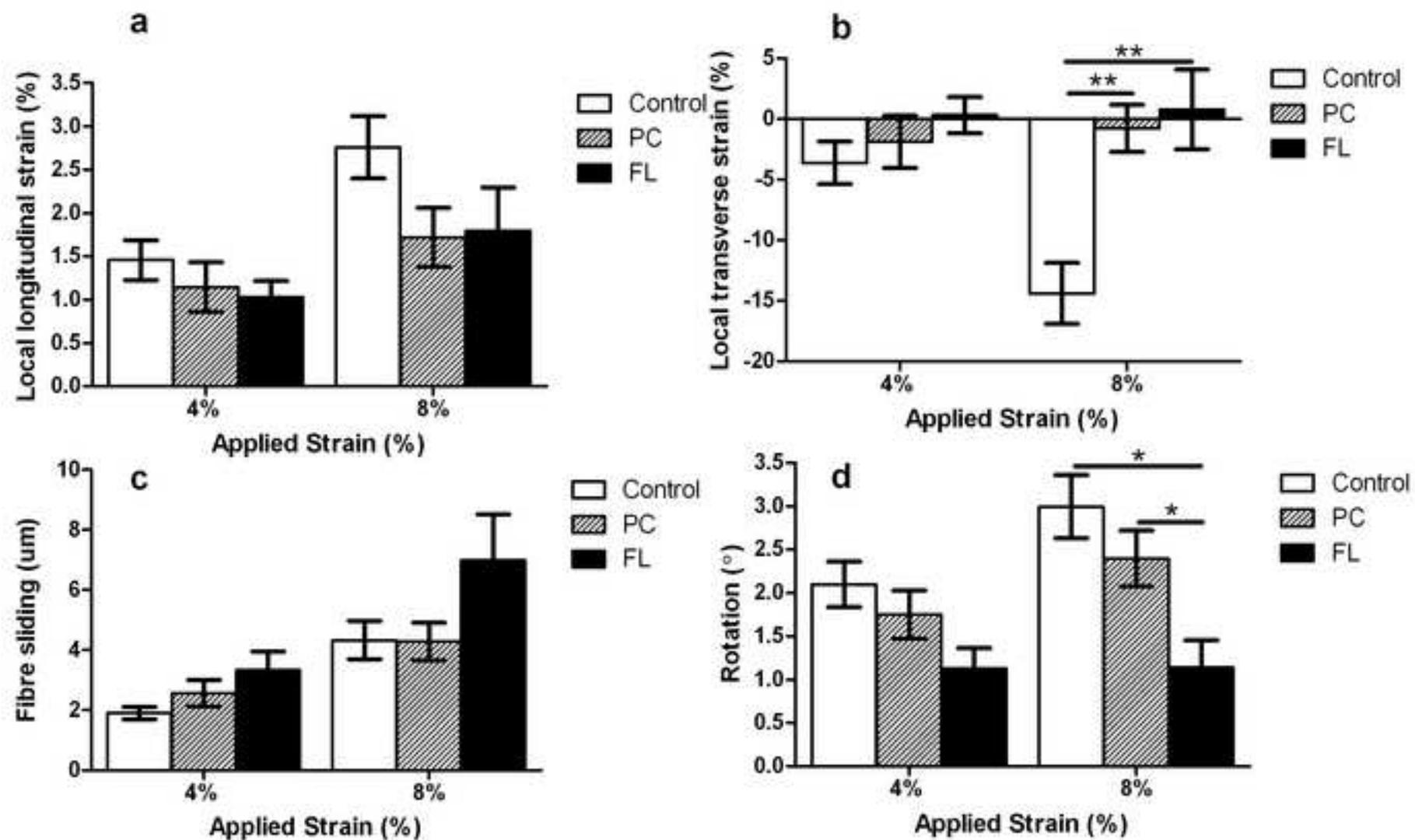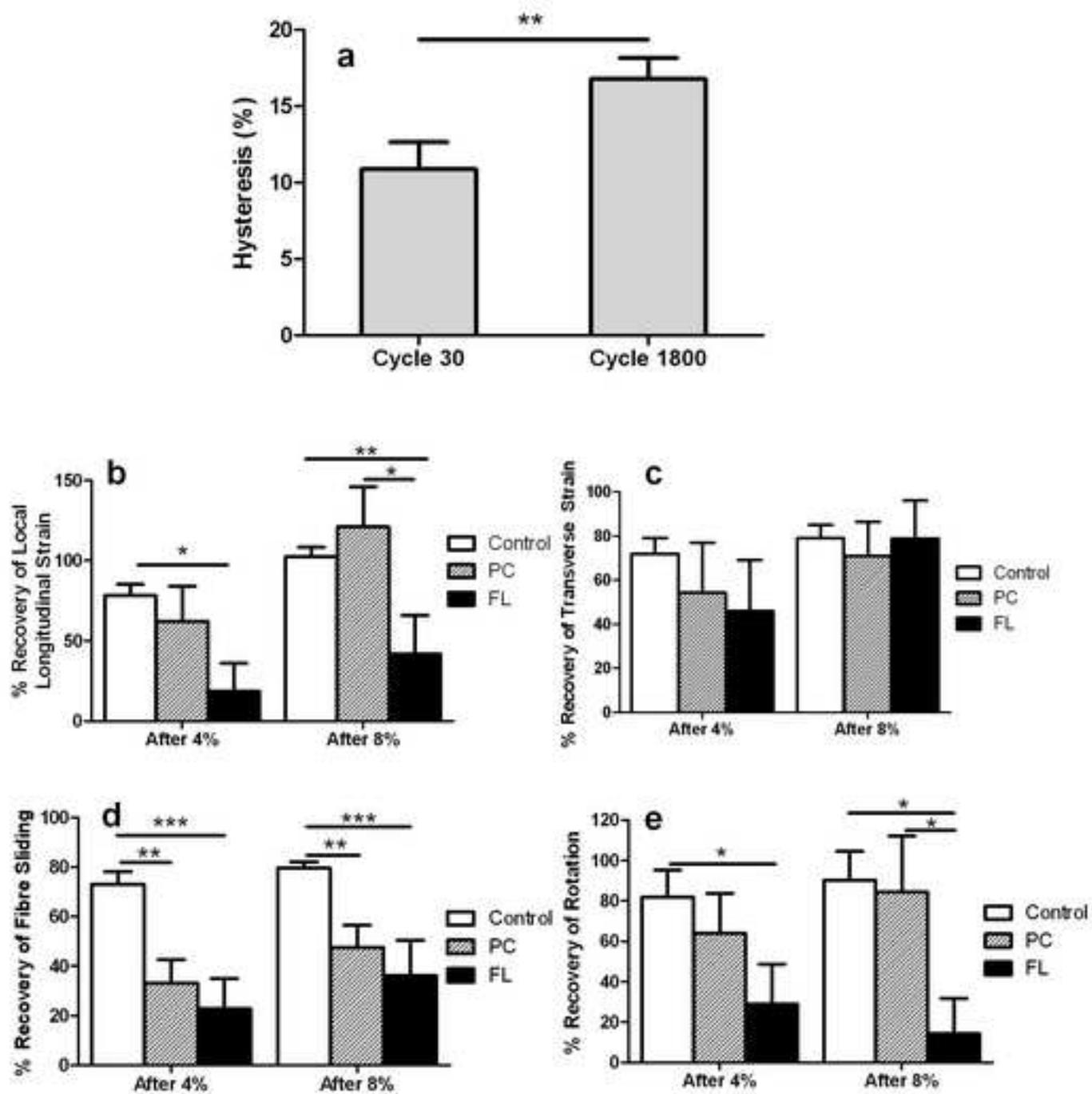
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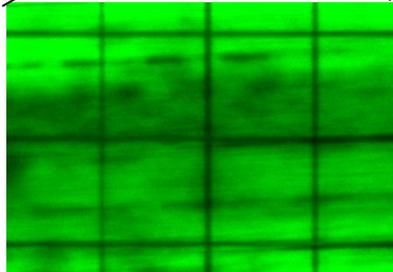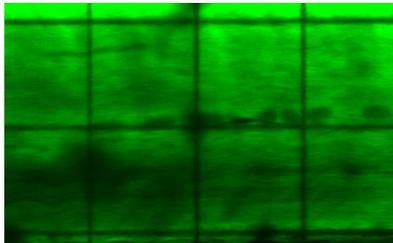
	0 % Strain	8 % Strain	Return to 0 % Strain
Control (unloaded)		Grid rotation	Good recovery
Pre-conditioned (30 cycles)			Good recovery
Fatigue Loaded (1800 cycles)	Matrix damage	Less rotation	Poor recovery