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Functionally distinct tendons have different biomechanical, biochemical and histological responses to *in vitro* unloading

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ABSTRACT

Tendons with different *in vivo* functions are known to have different baseline biomechanics, biochemistry and ultrastructure, and these can be affected by changes in loading. However it is not know whether different tendon types respond in the same, or different ways, to changes in loading.

This study performed *in vitro* un-loading (stress deprivation) in culture on ovine medial extensor tendons (MET, a positional tendon), and superficial and deep digital flexor tendons (SDFTs and DDFTs, with energy-storing and intermediate functions respectively), for 21 days (n = 14 each). Tensile strength and elastic modulus were then measured, followed by biochemical assays for sulphated glycosaminoglycan (sGAG) and hydroxyproline content. Histological inspection for cell morphology, cell density and collagen alignment was also performed.

The positional tendon (MET) had a significant reduction (~50%) in modulus and strength ($P < 0.001$) after *in vitro* stress-deprivation, however there were no significant effects on the energy-storing tendons (SDFT and DDFT). In contrast, sGAG was not affected in the MET, but was reduced in the SDFT and DDFT ($P < 0.001$). All tendons lost compactness and collagen organisation, and had reduced cell density, but these were more rapid in the MET than the SDFT and DDFT.

These results suggest that different tendon types respond to identical stimuli in different ways, thus; (i) the results from an experiment in one tendon type may not be as applicable to other tendon types as previously thought, (ii) positional tendons may be particularly vulnerable to clinical stress-deprivation, and (iii) graft tendon source may affect the biological response to loading in ligament and tendon reconstruction.

1. Introduction

Disorders of tendons, including tendinopathy and rupture, have a major and growing socio-economic impact (Hopkins et al., 2016). The prevalence of disease varies from 1 to 80% depending on the specific tendon and the age, sex and activity of the patient population being studied (Hopkins et al., 2016). Treatment and rehabilitation of tendon injuries require significant time and professional guidance, yet patients are seldom able to achieve previous activity levels. Our limited understanding of the disease mechanisms has beleaguered attempts to reduce the pain, rupture risk, and immobility amongst previously active individuals.

Tendons with different functions have different injury incidences. In the ankle the incidence of Achilles tendon disorder is 8% annually in elite runners (Lysholm and Wiklander, 1987), while the tibialis anterior tendon is rarely affected. This pattern of an injury-prone energy-storing tendon and unaffected positional tendon occurs frequently, and while it may simply reflect relative exposure to injurious loading conditions, it may also be a manifestation of deeper differences in these "functionally distinct" tendons. It may be possible to improve treatment of injury-prone energy-storing tendons through improved understanding of mechanobiological differences from positional tendons.

One well-investigated injury-prone tendon is the equine superficial digital flexor tendon (SDFT), and like the human Achilles, this experiences more frequent injury than the positional counterpart, the common digital extensor tendon (CDET) (Pinchbeck et al., 2004). The elastic modulus (614 vs 1012 MPa) and ultimate tensile strength (UTS; 115 vs 157 MPa) (Thorpe et al., 2012a) are consis-

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tently lower in SDFTs, however the fascicles and the interfascicular matrix of SDFT are more fatigue resistant than CDET (Thorpe et al., 2016; Thorpe et al., 2017). Underlying these differences in functional properties are distinct ultrastructure, biochemistry (Birch et al., 2013) and metabolic (Birch, 2007) profiles. These energy-storing SDFTs of the horse are characterised by smaller diameters of collagen fascicles (Thorpe et al., 2012a), greater non-collagenous material (Batson et al., 2003) and increased turnover of this non-collagenous material (Thorpe et al., 2010)), distinct to their positional counterparts, the CDETs. The SDFT and CDET also have vastly different collagen half-lives (Thorpe et al., 2010), which may indicate different remodelling potentials. Similar mechanical and ultrastructural differences have been observed in the bovine counterparts (Shepherd et al., 2014; Herod et al., 2016), even down to the level of single fibrils (Quigley et al., 2018).

Although mechanically and biochemically distinct, the question remains: do baseline differences in properties of functionally distinct tendons also give rise to a different biological response, or adaptation, to changes in mechanical loading? In particular, we are interested in the consequences of completely removing mechanical loading, since clinical observations indicate this is detrimental to the mechanical properties of muscle and tendon. For example, bedrest and cast-immobilisation of the Achilles tendon reduce its stiffness (Reeves, 2005) and alter its metabolic profile (decreased collagen synthesis, increased levels of the collagenase MMP-2 (Dideriksen et al., 2016)).

Further, pre-clinical studies in various species have unloaded tendons by suspending the hind limbs, unloading tendons directly either by physically severing the tendon or implanting an apparatus, or by paralysing the muscle responsible for loading. Together, these studies show reductions in modulus and UTS with limb-suspension in rats (Almeida-Silveira et al., 2000), reduced modulus and UTS with *in situ* tendon suspension (Sun et al., 2010), increased expression of key proteoglycans causing widespread reductions in modulus and UTS in transected equine SDFT (Jacobsen et al., 2015; Choi et al., 2016), increased expression of TGF-β1 and scleraxis in rat muscle-paralysing models (Killian et al., 2013), and increased expression of fibrosis-associated genes as well as differentially expressed proteins, including fibromodulin, in studies involving early mobilisation or cast-immobilisation in rabbit tendon transection models (Jielile et al., 2016).

In vitro loading studies enable more precise application and removal of loads, including at the level of the fascicle and cell. They have been instrumental in revealing that stress-deprivation causes the strain-sensing cilia on tendon cells to become elongated (Gardner et al., 2011) and that cells in stress-deprived constructs alter their gene expression compared to stressed constructs (Bayer et al., 2014). Changes include upregulation of inflammation-associated genes, and downregulation of some matrix and tendon-associated genes.

Although unloading appears to reduce the load-bearing capacity of energy-storing tendons, evidence is lacking to demonstrate that positional tendons are affected in the same way. If different functions and properties exist between different tendon types, the biological response to loading may also differ by tendon type. Sheep have been a commonly used species to study tendon injury and repair, including the positional infraspinatus to model human rotator cuff tendinopathies (Melrose et al., 2013; Smith et al., 2008), quadriceps and patella tendons mimicking similar human knee structures (Bertollo et al., 2013; Petri et al., 2018), and Achilles (Huri et al., 2013; Lopez-Najera et al., 2016; Sarrafian et al., 2010; Virchenko et al., 2008), DDFT (Khan et al., 2018; Peltz et al., 2017) and SDFT (Biasutti et al., 2017) for studying load-bearing/energy-storing tendon injuries and disease. While the sheep provides an excellent pre-clinical model in which to study tendon injury, repair and pathophysiology (Hast et al., 2014), to date no direct comparison has been made in this species of the response to altered biomechanical loading of functionally distinct tendons. The ovine SDFT is a bona-fide energy-storing tendon, and the medial extensor tendon (MET) is a bona-fide positional tendon. The deep digital flexor tendon (DDFT) has both positional and, to a small extent, energy storing roles, so this tendon was included to determine whether its response to stress deprivation was aligned with, or intermediate between, the energy storing SDFT and positional MET. In this study, we aim to use the well-documented ovine model (i) to learn if immobilisation/stress-deprivation of different tendon types affects biomechanics differentially, and (ii) learn if biochemical or ultrastructure changes might underlie these effects.

2. Methods

2.1. Tissue source, dissection and allocation

2.1.1. The methods will be described briefly here. Further detail is found in the appendix.

Sheep forelimbs (n = 14 legs, merino first cross, 6-11mo (adolescent), wethers and ewes) were sourced as unwanted tissue from the food processing industry at a local abattoir by disarticulating the forelimb at the carpometacarpal joint within 30 min of death. Left and right legs were randomly assigned to outcome streams of i) histology, or ii) biomechanics and biochemistry (Fig. 1). The SDFT, deep digital flexor tendon (DDFT) and medial extensor tendon (MET) (Fig. 1A) were explanted from limbs under sterile conditions and the central 30 mm length was dissected from each tendon (Fig. 1B). Further dissection with a blunt blade technique minimised the crosscutting of fibres.

While biomechanics-allocated tendon portions were randomised to a) *ex vivo* control or b) *in vitro* stress-deprivation treatment (21-days) groups (Fig. 1B), histology-allocated tendons had additional portions allocated to stress-deprivation for intermediate time-points of one or 7-days. *Ex vivo* strips for biomechanics were wrapped in saline-soaked gauze, sealed in tubes and stored at −20 °C for subsequent biomechanical testing.

The stress-deprivation "treatment" groups were cultured for zero (*ex vivo*), 1-, 7-, or 21-days at 37 °C. Tendons were laid flat, floating free in media. Media was refreshed twice weekly. At two weeks, tendons were transferred to new petri dishes, to isolate samples from outgrowth fibroblasts adhering to the petri dishes. At the end of the period of stress-deprivation, tendons were rinsed using phosphate-buffered saline pH7.2 and stored (wrapped in saline-soaked gauze) at −20 °C (Fig. 1C).

2.2. Biomechanical testing

Ex vivo and stress-deprived strips were thawed at room temperature and carefully dissected to produce consistent widths for testing (mean ± SD: 2.79 ± 0.98 mm (ex vivo samples); 2.41 ± 0.88 mm (stress-deprived samples)). The exact cross-sectional area was measured using a custom micrometer device (to measure specimen thickness) and optical imaging (to measure specimen width) (Choi et al., 2016). Specimen ends were blotted to remove excess saline, double-folded into a paper strip (Probst et al., 2015) and gripped in sandpaper-lined pneumatic clamps at 100psi, with a grip-to-grip spacing of 10 mm (Fig. 1D). The specimen was elongated to failure at 0.5 mm/s (strain rate ~ 5%/s). Tested specimens were immediately removed from paper and stored at −20 °C for subsequent biochemical analysis (see below). Force was measured using a 250 N load cell (Instron Dynacell 2527–131) at 100 Hz, the crosshead displacement was recorded (100 Hz) by the Instron Biaxial Mechanical Testing Machine (Instron 8874) and images of

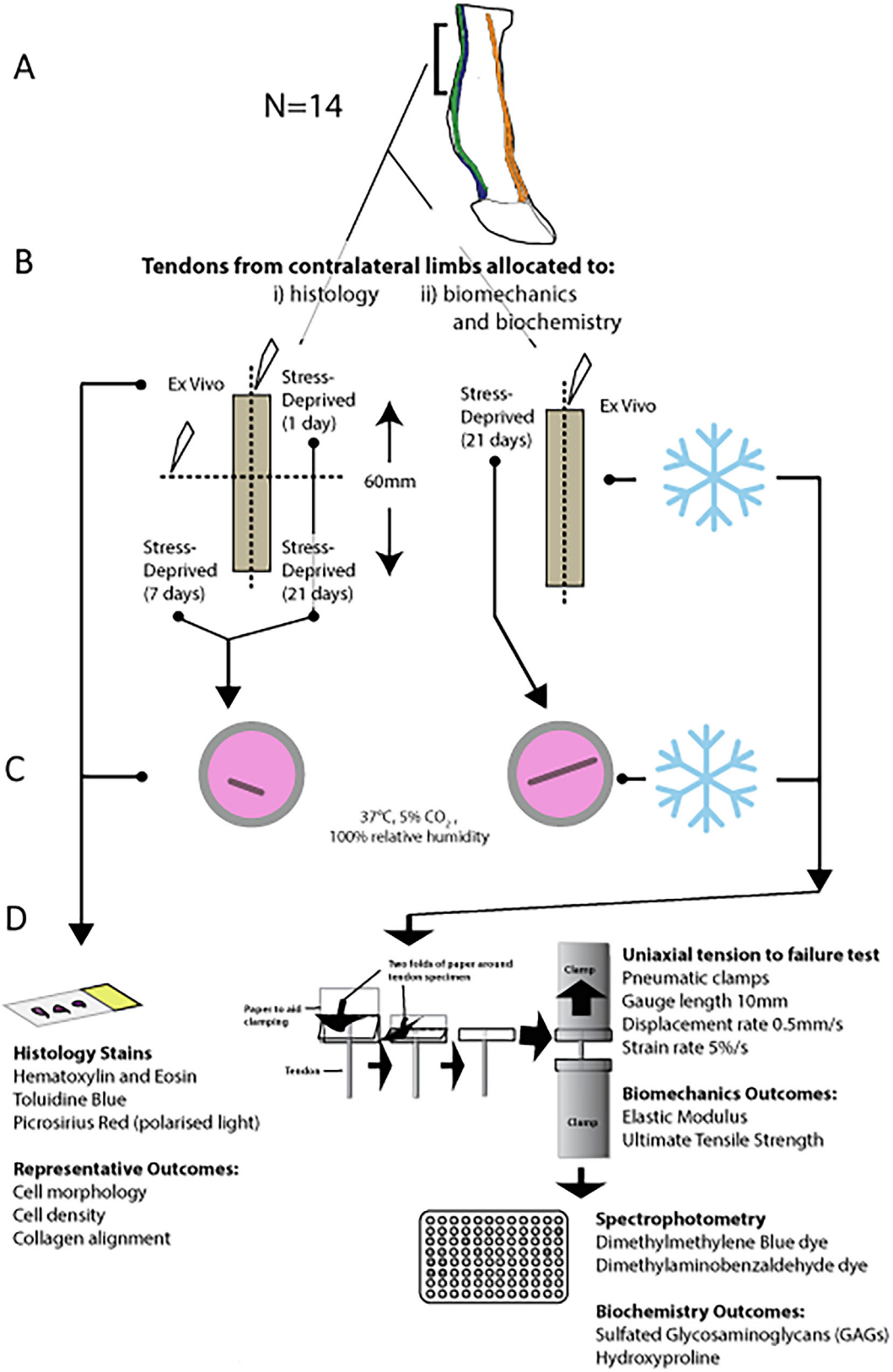

Fig. 1. Study design. (A) Superficial digital flexor tendons (SDFT in green), deep digital flexor tendons (DDFT in blue, close to SDFT) and medial extensor tendons (MET in orange on the frontal side of joint) were sterile-dissected from 20 abattoir-sourced limbs. (B) contralateral limbs, randomised left and right, were allocated to analysis by (i) histology and (ii) biomechanics and biochemistry. (C) *Ex vivo* histology specimens were fixed in neutral buffered formalin, while ex *vivo* biomechanics specimens were stored at −20 °C and stress-deprivation treatment specimens were incubated for 1, 7 or 21 days. (D) Final outcomes were histology, biomechanics and biochemistry. Biomechanics specimens had both ends double-folded into sheets of paper to help resist slipping from clamps during testing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the clamped specimen (2 Hz) were captured for later visual inspection for slip. One sample from each explant was biomechanically tested, however any specimens showing signs of slipping at the clamps were excluded and replaced with another specimen from the same tendon portion.

Post-hoc analysis of force data was used to determine when the load reached 0.25 N ("test start") and the actual gauge length was determined from the displacement data at this point in time. Subsequent data was used for analysis. Stress and strain were calculated, and smoothed using a custom Matlab program prior to calculating modulus and UTS.

2.3. Biochemistry

Papain-digested specimens were centrifuged and duplicate solution samples mixed with dimethylmethylene blue solution alongside chondroitin sulphate standards for spectrophotometry quantitation of 650 nm wavelength absorbance of sulphated glycosaminoglycans (sGAGs) in 96-well plates (Burkhardt et al., 2001).

Hydroxyproline content was quantified using the method of Stegemann and Stalder (1967) adapted for analysis in a microtitre plate. See appendix for details.

2.4. Histology

A small portion of tissue taken from the three tendons of two limbs had been allocated for histology and was immediately placed in 10%(v/v) neutral buffered formalin for 24 h, and prepared as in the appendix. Serial longitudinal sections were taken at five microns to be stained with hematoxylin and eosin (H&E), picrosirius red (PSR) or toluidine blue using standard methods (Smith et al., 2008). Four sections per tendon sample were scored for histopathology as previously described for ovine infraspinatus tendon (Smith et al., 2008), by one observer (CL) blinded to groupings (see Appendix for detail). Images of representative sections were taken with a light microscope (polarised for picrosirius red sections), digital camera and Image Manager software (all from Leica Microsystems).

2.5. Analysis

All statistical analyses were performed using Stata SE version 13 (Stata Corp, USA). Ten specimens (5 *ex vivo*, 5 stress-deprived) were removed from further analysis due to errors in cross-sectional area measurement and clamping. All remaining data were normally distributed. Mixed model regressions (clustered for individual sheep) were used to test for i) differences between *ex vivo* tendon types, ii) the effect of stress-deprivation on each tendon type and iii) differences between tendon types when stress-deprived, followed by Benjamini Hochberg correction for multiple comparisons between tendon types. Pairwise correlation was performed between each combination of biomechanics (modulus and UTS) and biochemistry (sGAG and hydroxyproline) outcomes followed by Benjamini Hochberg correction for multiple comparisons. No statistical analysis was performed on histology scores due to the small number of animals (four sections scored then averaged, for the three tendon types and four time-points for two animals).

3. Results

Representative stress-strain curves from *ex vivo* and stress-deprived (SD) tendons, of the three tendon types are shown in Fig. 2. These graphs demonstrate qualitative reduction in modulus and UTS in all tendon types with 21-days SD.

Ex vivo, the positional MET had significantly higher modulus (Fig. 3A, $P < 0.001$) and UTS (Fig. 3B, $P < 0.001$) than the energy storing SDFTs and DDFTs which did not differ from each other. The sGAG content of the MET was the lowest of the tendon types, and all tendon types had significantly different levels of sGAGs to all others (Fig. 3C, $P < 0.001$). In contrast, the hydroxyproline content was only different in the SDFT, which was higher than both the DDFT and MET (Fig. 3D, $P < 0.05$).

Stress-deprivation treatment *in vitro* significantly reduced modulus (Fig. 3A, $P < 0.001$) and UTS (Fig. 3B, $P < 0.001$) in the MET, but not the energy-storing SDFT or DDFT. In contrast, sGAG was not affected in the MET, but was reduced in the SDFT and DDFT (Fig. 3C, $P < 0.001$). The same pattern of significant differences in sGAG levels remained between the tendon types, with levels highest in the SDFT and lowest in the MET, but the magnitudes were smaller after stress-deprivation. In contrast, hydroxyproline increased in the positional MET ($P < 0.001$), decreased in the SDFT ($P < 0.05$) and was unchanged in the DDFT after stress-deprivation (Fig. 3D).

Despite these findings, no significant associations were found between biomechanics and biochemistry, regardless of tendon type or stress-deprivation treatment. When pooled or evaluated by tendon type alone, none of the pairwise correlations were significant before correction for multiple comparisons. When evaluated by tendon type and treatment, one association was significant before, but not significant following correction for multiple comparisons (sGAG hydroxyproline, correlation coefficient −0.56, *P*-value < 0.05). Analyses were repeated for the percentage change in each outcome (e.g. percentage change in modulus following stress-deprivation), and no correlations were significant, whether pooled or not.

Contrasting with biomechanics and biochemical differences, in H&E-stained histology all *ex vivo* tendons had a remarkably similarly compact, organised structure, featuring high cell density in intra-fascicular regions, and a highly cellular interfascicular matrix region (Fig. 4, first row).

All tendons lost compactness and collagen organisation with stress-deprivation treatment (Fig. 4), however this was apparent earlier in the MET (7-days of stress-deprivation), compared to the SDFT and DDFT (visible at 21-days of stress-deprivation). Accompanying general loss of matrix integrity, the cell population became sparse, and this was again more rapid in the MET. Interestingly, cells with myofibroblastic morphology (enlarged, and much rounder cell nuclei) were observed to be clustered at the tissue edges at 7-days, particularly in the SDFT and DDFT.

As in H&E histology, all *ex vivo* tendon types showed similar appearances in PSR-stained histological appearance under polarised light (Fig. 5, first row). All *ex vivo* tendons showed predominantly green fibres, indicating highly aligned collagen I, with small regions of orange-red indicating less aligned collagen. Interestingly, the crimp period appeared longer in the MET, compared to the SDFT and DDFT.

Similar green-red ratios between tendons at 24 h (Fig. 5, row 2) indicated similar levels of collagen alignment, however this was less than *ex vivo* tendons and tissue disorganisation began appearing (less aligned to tendon loading axis). At 7 and 21-days, unaligned red fibres predominated in the MET's fragmented and unorganised tissue, while the SDFT and DDFT retained more of their organisation and majority-green appearance (Fig. 5, rows 3 and 4).

No notable differences were seen in toluidine histology; no proteoglycan accumulation, nor differences by tendon type or by time-points, as indicated by a consistent pale blue appearance which was observed through all slides (See Supplementary Material).

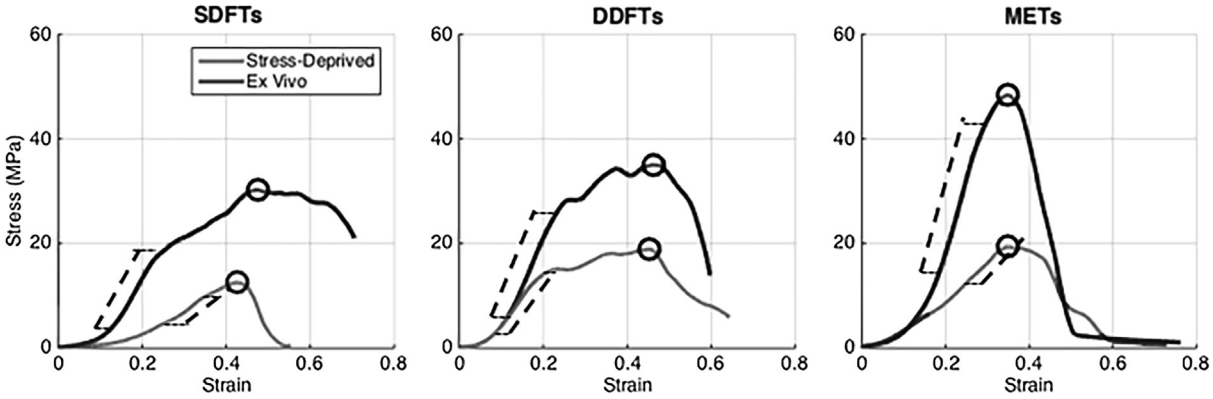

Fig. 2. Representative stress-strain curves acquired from mechanical testing of ovine tendons. *Ex vivo* (dark) and 21-day stress-deprived (light) groups both shown for each of the three tendon types: superficial digital flexor tendons (SDFTs), deep digital flexor tendons (DDFTs) and medial extensor tendons (METs). Dashed lines and circles indicate maximum modulus and ultimate tensile strength respectively of each stress-strain output.

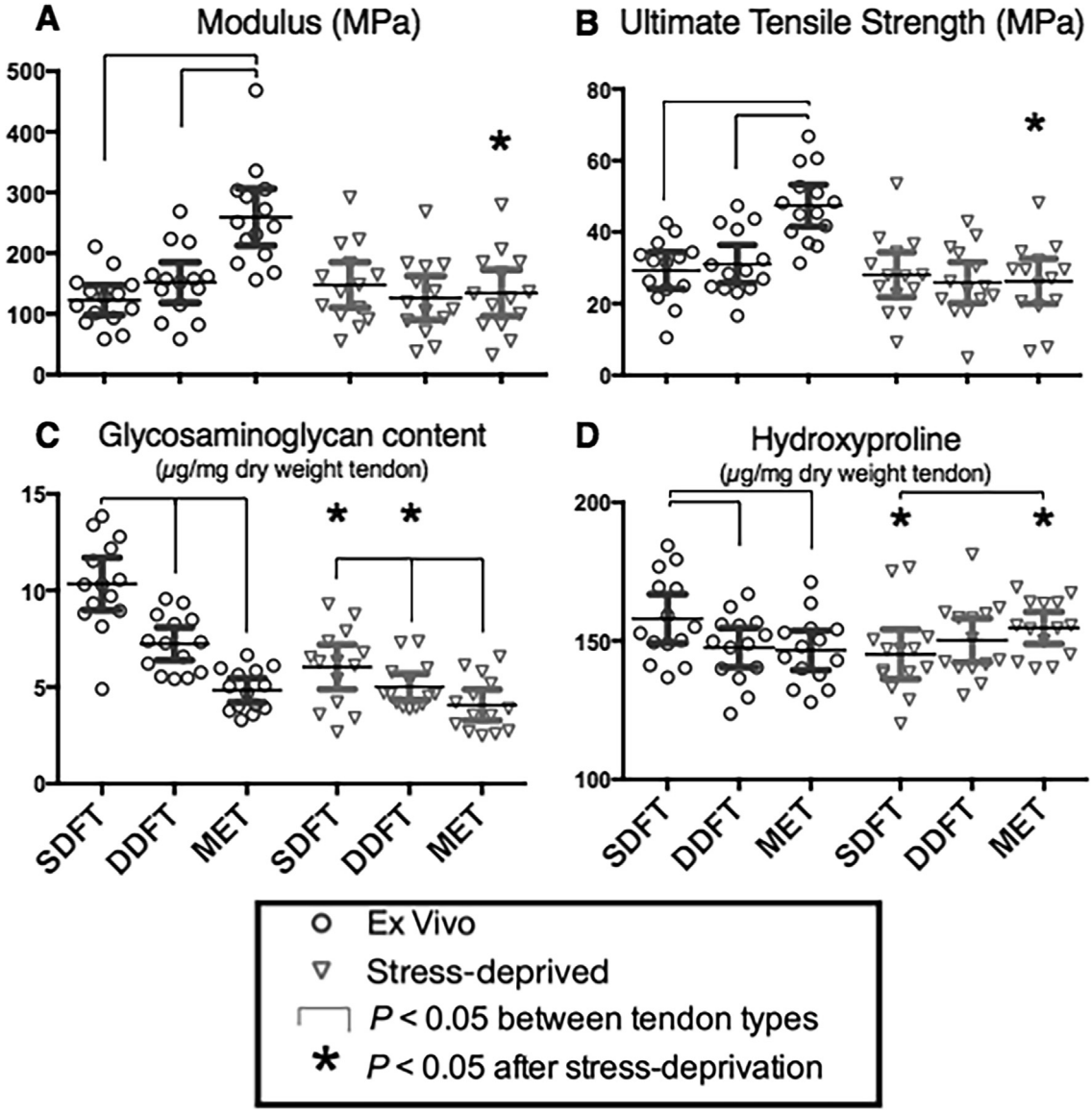

Fig. 3. Modulus (A), Ultimate tensile strength (B), glycosaminoglycan content (C), and hydroxyproline content (D), in *ex vivo* and 21-day stress-deprived tendons (Superficial digital flexor tendons (SDFT), deep digital flexor tendons (DDFT) and medial extensor tendons (MET)). Individual data are presented with overlying group mean (horizontal bar) with 95% confidence intervals. Brackets indicate significant differences between tendon types and asterisks (*) indicate significant differences between ex-vivo and stress-deprived groups within a tendon type. Significant differences indicated have been corrected for multiple comparisons (Benjamini-Hochberg).

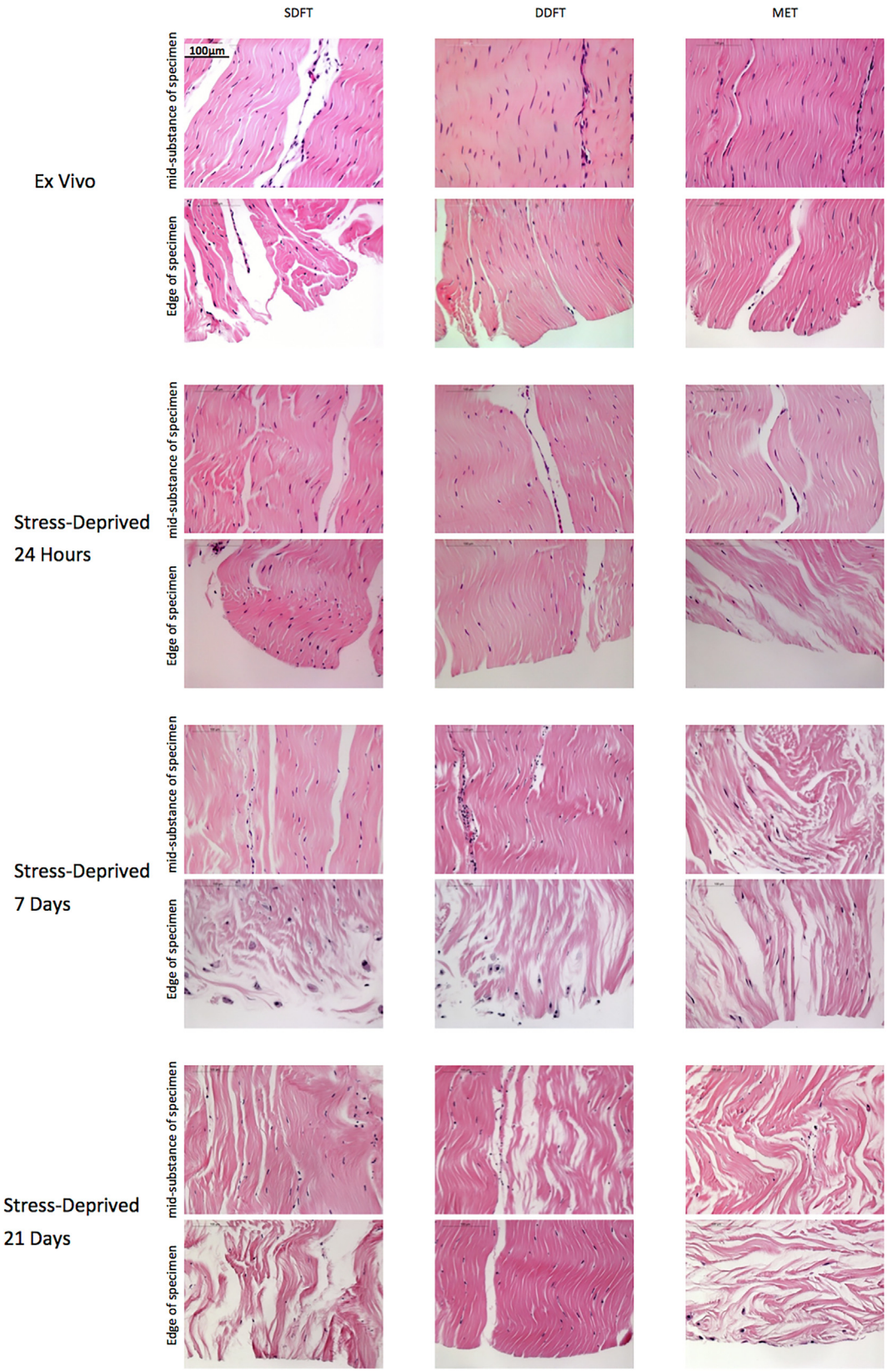

Fig. 4. Representative microscopic images (200x magnification) of haematoxylin and eosin-stained sections from ovine tendons. Sections are from *ex vivo* and 24-hour, 7-day and 21-day stress-deprived SDFTs, DDFTs and METs. Two images are shown from each section: one from a central "mid-substance" region of each section and one from an "edge" region of each section. Pink staining regions indicate tendon extra-cellular matrix and smaller deep blue staining regions indicate cell nuclei or dead cell debris. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

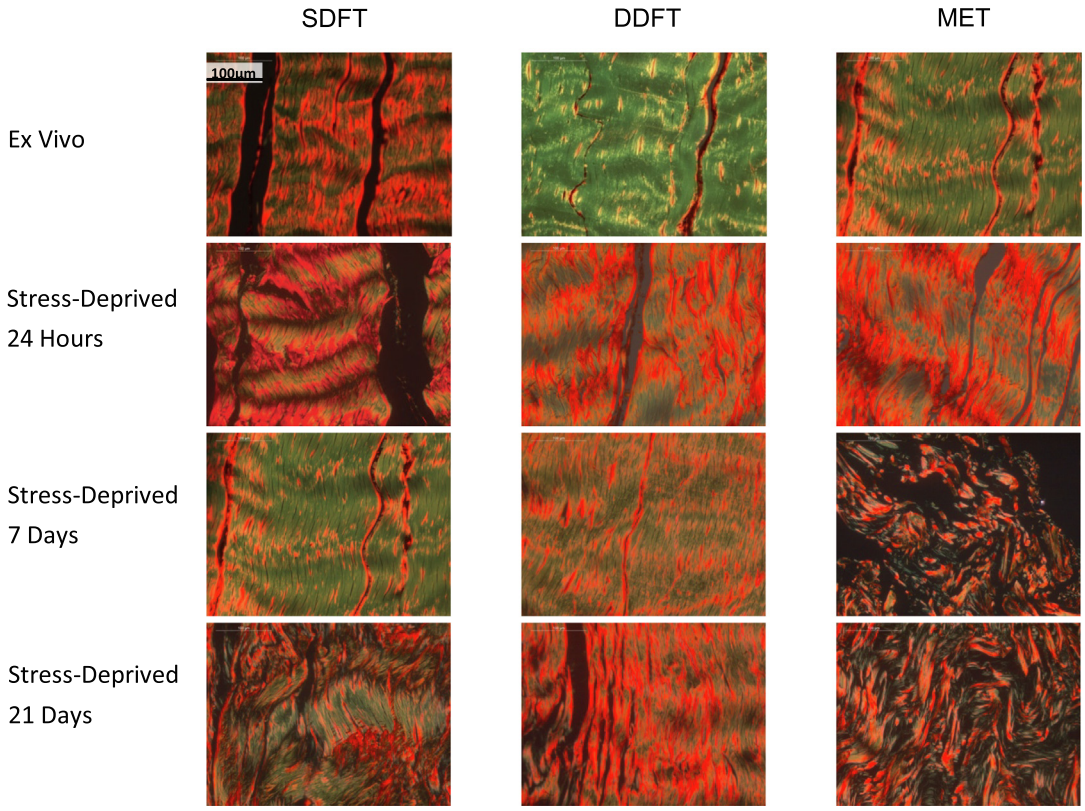

Fig. 5. Representative microscopic images (200x magnification) of picrosirius red-stained (polarised light) sections from ovine tendons. Sections are from *ex vivo* and 24-hour, 7-day and 21-day stress-deprived SDFTs, DDFTs and METs. Cell nuclei are not visible using this staining method. Deep green regions indicate highly aligned collagen fibres, as seen here in *ex vivo* sections. Yellow and red regions indicate decreasingly aligned collagen fibres, as seen here in stress-deprived sections. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Histology scoring indicated worsening tendon pathology with time in stress-deprivation culture (Fig. 6), and suggests some differences between tendon types. SDFT had the most rapid change in total histopathology score, but all three tendon types had marked pathology by 21-days in culture, with most of the pathology at this time-point indicating cell changes (number and morphology) and collagen fibre alignment changes.

4. Discussion

In agreement with previous studies of distal limb flexors and extensors in the horse (Thorpe et al., 2012a), we found the *ex vivo* sheep MET had higher elastic modulus and UTS, and lower sGAG than both the *ex vivo* SDFT and DDFT, and no consistent trend in hydroxyproline content between tendons of energy-storing and positional function. These results are consistent with literature comparisons, which show the same trends in equine (elastic modulus: 614 vs 1012 MPa, UTS: 115 vs 157 MPa (Thorpe et al., 2012a), sGAG content: 7.5 vs 1.3 μg/mg, hydroxyproline content: 75% vs 70% (Batson et al., 2003)) and bovine (elastic modulus: 102 vs 150.7 MPa, UTS: 23.1 vs 37.6 MPa (Herod et al., 2016)) forelimb flexor and extensor tendons.

Despite this knowledge, we have minimal mechanistic understanding of the differences between tendon types in biological response to altered load that may induce pathological change. Our results have identified distinct responses to stress-deprivation in the ovine SDFT, DDFT and MET, at both a biomechanical and biochemical level. The MET, which is predominantly positional in function, experienced a large drop in elastic modulus and UTS, which the SDFTs and DDFTs did not. Conversely, the SDFT and DDFT experienced a large drop in sGAG content, while the MET did not. Hydroxyproline content had small changes with stress-deprivation. The small but statistically significant increase in hyroxyproline detected with stress-deprivation culture in MET but decrease in SDFT may be associated with altered synthesis of the predominant "normal" tendon fibrillar collagen (Type I), and/or those associated with pathology/tendinopathy (Type II, III) in the different tendons. Additionally, there may be changes in turnover/proteolysis of existing and newly synthesized collagens. While beyond the scope of the current study it would be interesting to explore this further through evaluation of collagen and MMP gene expression and immunolocalization, enzyme activity assays, and release of collagen degradation products.

Upon stress-deprivation, the rapid rate of ultrastructural deterioration in the MET, from previously healthy collagen alignment, appeared to parallel its degeneration in biomechanical properties. Meanwhile, the relative inertness of the SDFT's and DDFT's collagen alignment was mirrored by unchanged biomechanical properties. Interestingly, these observations would suggest the changes to biomechanical properties of the tendon occurring with stress-deprivation are more closely related to ultrastructural collagen alignment properties than biochemical composition, including percentage collagen and sGAG content. However when including all histology outcomes in the total scores, albeit scored from only two animals, pathology developed quickest in the SDFT and appeared to be related mostly to cell changes.

There is little literature to provide directly comparable results, however previous reports of responses to loading and metabolic profiles differing between functionally distinct tendons would support our results. Further, differential responses to whole-body exercise between functionally distinct tendons can be inferred from previous *in vivo* investigations. For example, when young rats

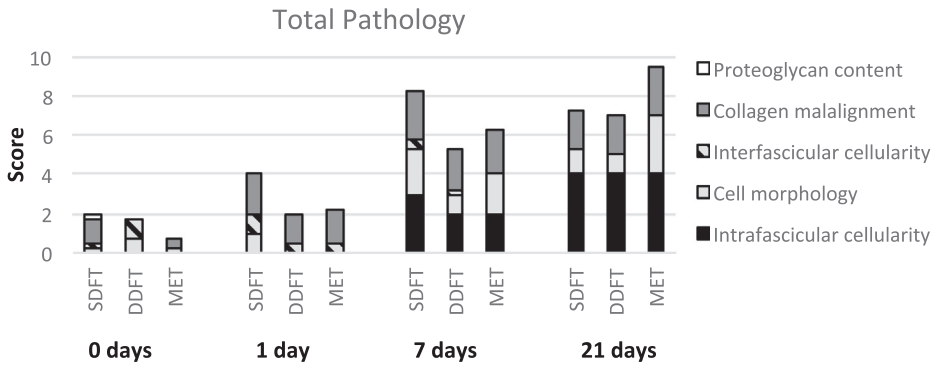

Fig. 6. Results from histology scoring showing total histopathology scores (bar height), as well as individual criteria (intrafasciclular cellularity, cell morphology, interfacsicular cell infiltration (cellularity), collagen fibre malalignment, and proteoglycan content). Scores are shown *ex vivo* (0 days), and 1-day, 7-day and 21-day stress-deprived SDFTs, DDFTs and METs. Scores were averaged from 4 sections per tendon at each time-point for two animals.

climbed with weights, the Achilles and SDFTs had a differential response in failure strain and elastic modulus, compared to DDFTs (de Cassia Marqueti et al., 2017). When jump-trained in a separate experiment, the tendon cross-section decreased in Achilles, SDFTs and DDFTs, whilst elastic modulus increased in only SDFTs.

To remove *in vivo* confounding effects, we turn to *in vitro* experiments, which show energy-storing tendons are less sensitive to the application and removal of load. Biaxial-strain loading of cells derived from equine SDFTs induced less proliferation than cells from CDETs (Goodman et al., 2004). In a bovine example, isolated CDET cells have larger upregulation of gene expression in response to stress-deprivation and hydrostatic compression than their load-bearing counterparts in the SDFT (Thornton et al., 2010). This may explain the lack of change in biomechanical properties in our energy-storing ovine SDFTs, compared to METs. Further, a study of matrix metabolism in equine SDFTs compared to CDETs, showed slower collagen turnover and faster turnover of sGAG in the SDFTs (Thorpe et al., 2010), potentially explaining the greater GAG reduction observed in the stress-deprived SDFTs of the current study. Taken together, the *in vivo* and *in vitro* literature support the current finding of a positional tendon's (MET) matrix being relatively resistant to sGAG degradation, whilst having biomechanical properties that are more rapidly degraded by stress-deprivation, perhaps due to faster collagen degradation.

Our observations of varied responses to stress-deprivation between SDFTs, DDFTs and METs, motivated an effort to correlate these differentially varying biomechanical and biochemical properties, but to no avail. Unlike previous models of pathology which involved sGAG-accumulation *in vivo* (Choi et al., 2016), our current toluidine blue histology and sGAG quantitation results showed that sGAG-accumulation had not occurred *ex vivo* and therefore had not been a driver of biomechanical deterioration of the MET. However, since small leucine-rich proteoglycans also have a further matrix regulatory role (reviewed by Chen and Birk, 2013), further investigation beyond correlations is needed to understand how specific proteoglycans may regulate tendon health during stress-deprivation. While there were differences between the tendon types for sGAG content ex vivo versus stress deprived, there was a lack of proteoglycan staining histologically in all specimens. It should be noted that the sensitivity of toluidine blue staining (as with all histochemical methods) to detect changes in sGAG is much less than the biochemical DMMB assay (Burkhardt et al, 2001). The biochemical assay is therefore considered the gold standard for quantitation of sGAG.

This highlights one of the limitations of this study: we have studied differential mechanical and biochemical effects between tendon types at a single time-point. Additionally, the use of an *in vitro* animal model and multiple freeze-thaw cycles (for biochemistry samples) is necessitated by the nature of the study. It was also necessary to divide the tendons longitudinally in this study, which we recognise could have caused cross-cutting of collagen fibrils, which may contribute to cross-sectional area measurements but not load-bearing ability. We minimised this through careful dissection techniques, however this was more difficult in SDFT and DDFT because they naturally twist. Further, the effect of cell death has not been quantified in this experiment. Given the reduction in cells seen in H&E histology in Fig. 4, it is possible that the observed changes are a result of cell migration out of the tissue or cell death, which may cause a skew in the population of remaining cells. Further investigation is needed to evaluate the underlying cell and molecular drivers of the differential response to stress deprivation that we have defined in the current study. It should also be noted that the histology scoring was only performed on a limited number of animals. With these limitations in mind, these results should be interpreted as patterns of differences between the tendon types and not raw values directly applicable to human tissue.

Since a large effect was noted with stress-deprivation in the extensor (MET), but not the flexors (SDFT and DDFT), we propose that further studies of this model investigate the mechanisms driving this differential response. In particular, this should identify the differences in molecular mechanisms which precede the divergence of mechanical and biochemical properties. We suggest the relative quantities of the regulatory proteoglycans may vary between energy-storing and positional tendons, and this may partly explain the differing biochemistry-biomechanics relationship observed. Therefore, future work to investigate mechanisms, such as the gene regulation for production and breakdown of these regulatory proteoglycans, is needed to understand the material changes we have observed.

To conclude, we have shown that tendons exposed to different *in vivo* loading environments are distinct in biomechanical and biochemical properties in the sheep. Despite the positional tendon (MET) showing ~ 50% reduction in modulus and UTS upon *in vitro* stress-deprivation treatment, there were no significant biomechanical effects in the energy-storing tendons (SDFT and DDFT). Correspondingly, deterioration of collagen alignment also progressed more rapidly in the MET. This has major implications for translating basic research to tendons in the clinic. Since the different tendon types respond to identical stimuli in different ways, the results from an experiment in positional tendons may not be as applicable to an energy-storing tendon as previously thought, and require validation in functionally distinct tendons. Furthermore, the current results suggest that clinical stress-deprivation situations, including routine post-surgical cast immobilisations, may render positional tendons particularly vulnerable to subsequent

damage. This also highlights a potential mismatch in tendon and ligament graft situations where a difference in biological response to loading depending on the graft tissue source may be as important as matching mechanical properties between hosts and grafts.

Declaration of competing Interest

The authors have no conflicts to disclose.

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Appendix A. Supplementary material

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