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Cyclical strain improves artificial equine tendon constructs *in vitro*.

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Running Title:

Straining artificial tendon constructs

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Summary

Tendon injuries are a common cause of morbidity in humans. They also occur frequently in horses and the horse provides a relevant, large animal model in which to test novel therapies. To develop novel cell therapies that can aid tendon regeneration and reduce subsequent re-injury rates, the mechanisms that control tendon tissue regeneration and matrix remodelling need to be better understood. Whilst a range of chemical cues have been explored (growth factors, media etc.), the influence of the mechanical environment on tendon cell culture has yet to be fully elucidated. To mimic the *in vivo* environment, in this study we have utilised a novel and affordable, custom-made bioreactor to apply a cyclical strain to tendon-like constructs generated in 3-dimensional (3D) culture by equine tenocytes. Dynamic shear analysis (DSA), dynamic scanning calorimetry (DSC) and Fourier Transform Infrared (FTIR) spectroscopy were used to determine the mechanical and chemical properties of the resulting tendon-like constructs. Our results demonstrate that equine tenocytes exposed to a 10% cyclical strain have an increased amount of collagen gel contraction after 7 and 8 days of culture compared to cells cultured in 3D in the absence of external strain. While all the tendon-like constructs have a very similar chemical composition to native tendon, the application of strain improves their mechanical properties. We envisage these results will contribute towards the development of improved biomimetic artificial tendon models for the development of novel strategies for equine regenerative therapies.

Key words

3D culture; tendon cells; dynamic shear analysis; mechanical properties; equine

Introduction

Tendon injuries are a leading source of morbidity in human athletes(Kannus, 1997). Likewise, injuries to the superficial digital flexor tendon (SDFT) commonly occur in horses(Williams, Harkins, & Wood, 2001) and the horse provides a relevant large animal model in which to study injury pathogenesis and test novel therapies to improve tendon regeneration(Patterson-Kane, Becker, & Rich, 2012). Following a tendon injury the reparative process produces biomechanically inferior scar tissue which leads to high rates of re-injury (up to 67% in horses(Dyson, 2004)). To develop successful new therapies a better understanding of the molecular pathways involved in tendon regeneration and modulation is required. Tendon cells constantly adapt to their environment through anabolic and catabolic processes(Birch, 2007). Therefore *in vitro* studies to develop cell therapies must attempt to mimic the *in vivo* tendon environment as closely as possible.

Reorganisation of the collagen matrix to generate tendon-like constructs is an important property of tenocytes and collagen gel contraction is commonly used as a measure of cell-mediated matrix reorganisation(Bell, Ivarsson, & Merrill, 1979; Montesano & Orci, 1988; Ngo, Ramalingam, Phillips, & Furuta, 2006; T. H. Yang et al., 2015). We have previously utilised 3-dimensional (3D) culture in a collagen gel to drive tendon differentiation by equine pluripotent stem cells(Barsby, Bavin, & Guest, 2014; Bavin, Smith, Baird, Smith, & Guest, 2015) and determine the implications of gene knock-down(Bavin, Atkinson, Barsby, & Guest, 2017) and inflammation(Alyce McClellan et al., 2019) on tendon cell function. However, our previous model did not account for the significant mechanical forces that tendon cells are exposed to *in vivo*. In horses, the strain placed upon the SDFT (which is most at risk of injury) can be up to 16%(Stephens, Nuamaker, & Butterweck, 1989). Although the cells themselves will experience a lower strain than the tendon fascicles(Screen, Lee, Bader,

& Shelton, 2004), previous studies have indicated that tendon cells can change their gene expression and function in response to strain(Thorpe, Clegg, & Birch, 2010). As a gradual return to exercise plays an essential part of the rehabilitation programme in both horses and humans(Frankewycz et al., 2017; Ortved, 2018), it is critical to understand the effects of force on both the endogenous adult tendon cells and any cells that may be applied therapeutically to improve regeneration.

There are commercially available systems to apply external strain to tendon-like constructs *in vitro(Garvin, Qi, Maloney, & Banes, 2003)*. However, these systems are expensive. The aim of this study was to develop a cost-effective, 3D *in vitro* system in house to mimic the mechanical environment equine tenocytes are exposed to *in vivo* and to determine the effects of strain on collagen gel contraction and the chemical and material properties of the resulting artificial tendons. To do this, a custom made bioreactor was created in house to match the conditions of our 3D static culture set up(Barsby et al., 2014) but apply a uniaxial cyclic strain. We hypothesised that the application of an external cyclic strain would increase the rate of collagen gel contraction by equine tenocytes.

Throughout the manuscript we refer to the 3D cultures grown in the absence of additional strain as "static" cultures, as has been defined by others(Kuo & Tuan, 2008). These constructs are attached to fixed anchors and as the cells contract the collagen gel a uniaxial tension is applied to the cells(Kapacee et al., 2010). We realise that the actual force applied to the cells will be changeable as the matrix undergoes re-organisation(Barsby et al., 2014). However the term static has been used to clarify that in these cultures no external force is applied.

Our work has demonstrated that the application of an external strain does increase the rate of collagen gel contraction and we use a variety of techniques to determine the material and chemical properties of the resulting tendon-like constructs. The culture system and analysis methods employed will enable further studies on the effect of the mechanical environment on cell fate and function of both endogenous adult tendon cells and other cell types which may be applied clinically.

Materials and Methods

Bioreactor design

A Polytetrafluoroethylene (PTFE) base was designed to hold two stainless steel bars each containing 10 stainless steel pins 0.6 mm in diameter. 316 grade stainless steel was used due to its resistance to oxidation. One bar is fixed to the base and one is fixed to a programmable stepper motor that controls the frequency, speed, distance and duration of movement. In the closed position, the pins are 15 mm apart. The stepper motor is controlled using an ARM Mbed microcontroller and a software application written in ARM Mbed OS (https://developer.mbed.org). The PTFE chamber and stainless steel components are fully autoclavable and the chamber is designed to fit a standard 6 well plate tissue culture lid (Figure 1). Computer aided design (CAD) was performed using Solid Edge to generate 3D models of the tendon-like constructs, when under a 10% strain, at different time points during culture. Images from a minimum of three tendon-like constructs were used at each time point and the dimensions calculated using ImageJ (National Institutes of Health). Finite element analysis was run using Solid Edge to simulate the effect of applying a 10% strain to the tendons and produce a stress heat map.

Preparation of equine tenocytes

Two-dimensional cell culture was carried out as described previously(Barsby & Guest, 2013). Tendon cells derived from three different adult horse superficial digital tendons were used in this study. These were isolated from post-mortem equine tendon tissue with institutional ethical approval granted by the Animal Health Trust Research Ethics Committee (02_2012). The horses were all skeletally mature, young Thoroughbreds (2-10 years old) with no macroscopic signs of tendon injury. A small piece of tendon tissue (approximately 1 cm^3) was taken from the central (core) part of the tendon body, cut into small pieces using a scalpel and forceps and digested overnight in a solution of 1 mg/mL type 1 collagenase produced by Clostridium histolyticum (Sigma) in culture media (DMEM high glucose containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 ug/mL streptomycin (all from Invitrogen)). Cells were pelleted and washed three times before culture. Tenocytes were passaged every 3-4 days when nearing confluency using 0.25% trypsin-EDTA (Sigma) and were used between passages 4 and 10 in the below experiments.

Three-dimensional cell culture

Three-dimensional culture of collagen gel seeded constructs was carried out both in static and cyclic conditions. Static cultures were performed as described previously(Barsby et al., 2014). Briefly, 0.2 mm diameter minutien pins were fixed into silicone-coated six-well plates (Sylgard 184 Silicone elastomer; Dow Corning) in pairs 15 mm apart. Tenocytes were suspended in a solution of two parts culture medium to eight parts PureCol (Bovine collagen type I; Advanced Biomatrix) with the pH adjusted to 7.2 to 7.6 to give a final suspension of $4x10^5$ cells/ml (based on work done by (Nirmalanandhan, Levy, Huth, & Butler, 2006) and optimised in (Barsby et al., 2014) for adult equine tenocytes). 200 μL of collagen-tenocyte suspension was pipetted between each pair of pins and the plates incubated at 37°C for 60-90

minutes to allow setting. Finally, 3 mL of culture medium was added to each of the wells. Cultures were maintained until the 14 day end point and media was changed every 3-4 days during this time.

Bioreactor cultures were prepared directly in the bioreactor, in an identical manner to that described above, and 25 mL of culture medium used to fill the PTFE base to fully submerge the tendon-like constructs. The bioreactor was programmed to run for 20 minutes every 24 hours from day one to day 14 of culture. It applied a uniaxial strain of 10% (1.5 mm displacement) at a frequency of 0.67 Hz. The strain is based on that which is reported to occur in horses at a canter(Stephens et al., 1989) and the frequency is similar to those that have been used in other *in vitro* studies(Chen et al., 2012; Garvin et al., 2003; Tsuzaki et al., 2003; Wang et al., 2003).

Contraction analysis

This was performed as described previously(Barsby et al., 2014). Briefly, the tendon-like constructs were photographed daily and their diameters calculated using ImageJ. Contraction data are calculated as a percentage of the starting Day 0 value. To determine statistically significant differences in the degree of contraction between the two conditions (cyclical versus static) for each time point, an unpaired Student's t-test was used. Three biological repeats using three independent lines of tenocytes (i.e. derived from three different horses) were performed in static and cyclic conditions for this analysis. The contraction for each biological replicate was carried out on 3-18 constructs per time point.

Cell survival

This was performed as described previously(Barsby et al., 2014). Cell survivals were performed on days 3, 7 and 14. Tendon-like constructs were digested in a solution of 1 mL culture medium with 1 mg/mL type 1 collagenase produced by Clostridium histolyticum (Sigma) at 37°C for 1-2 hours. Live cell counts were performed using trypan blue exclusion staining and cell counting on a haemocytometer and the survival data calculated as a percentage of the number of cells originally seeded. To determine statistically significant differences in cell survival between the two conditions (cyclical versus static) for each time point, an unpaired Student's t-test was used. Three biological repeats using three independent lines of tenocytes were performed, with each biological replicate carried out on 3-6 constructs per time point.

Tensile Testing of artificial and native tendons

Dissected tendon tissue, approximately cylindrical with a 1 mm diameter, and tendon-like constructs (14 days of culture) were secured using Aluminium oxide 180 grit sandpaper along with a drop of Loctite® Super Glue (ethyl cyanoacrylate) 98. They were loaded into a custom immersion bath on a tensile test machine (Zwick, Herefordshire, UK), and the clamps were manually moved apart such that 0.01 N of force was placed on the sample (using a 50 N load-cell). The samples were then subjected to a tensile load at a rate of 10% strain per second, until catastrophic failure. Once loaded, samples were imaged and dimensions measured using Image J to calculate the circular cross section. Gauge length was adjusted to account for the length of grip linings, and stress-strain curves were obtained. Replicates were performed on 3 samples of tendon tissue, and 12 samples of artificial tendons. The samples were derived from cells/tissue taken from three different animals (i.e. 1 technical replicate for each of 3

biological replicates of tendon tissue and 4 technical replicates for each of 3 biological replicates of artificial tendons).

Dynamic Shear Analysis

Dynamic shear analysis was performed as described previously(Chaudhury, Holland, Vollrath, & Carr, 2011; Alyce McClellan et al., 2019) using an AR 2000 rheometer (TA Instruments, New Castle, USA). Entire tendon-like constructs were used (after 14 days of culture) and loaded in a coiled shape in order to create an area under the geometry that was filled with material. As the native equine tissue proved too stiff to coil sections 1 mm thick were taken and positioned to entirely fill the geometry. Samples were then compressed to a minimum force of 0.02 N between two parallel plates.

During testing samples were kept in PBS at 37°C to prevent water loss whilst an oscillatory frequency sweep was performed (from 1 to 0.1 Hz at 1% strain). Measures of G' over this frequency range were averaged and compared. Sample size was determined by an area measurement of a post-test photograph of the sample in Image J. Data was normalised to reduce sample size as a variable, as not all samples entirely filled the geometry and unpaired Student's t-tests were used to compare sample means. Replicates were performed on 5 samples derived from cells/tissue taken from three different animals (i.e. 1 or 2 technical replicates for each of three biological replicates).

Fourier Transform Infrared (FTIR) Spectroscopy of tendon-like constructs

Equine tendon tissue and tendon-like constructs (14 days of culture) were dried at room temperature for a minimum of two hours and placed on a Nicolet 380 spectrometer (Thermo Scientific, Waltham, USA) with a Golden Gate ATR single-bounce diamond accessory with

ZnSe focussing optics (Specac, Orpington, UK), and purged with dry air (Parker Balston FTIR purge gas generator) to remove spectra caused by atmospheric CO_2 or H_2O. Spectral information was collected using OMNIC 7.6 software (Thermo Fisher Scientific, Worcester, USA). Each spectrum represents 32 scans at a resolution of 4 cm^{-1}. Background spectra were collected before each sample spectra, and spectra were collected at four locations along each sample. These spectra were averaged and normalised to the amide I peak at ~ 1630 cm^{-1}(Barth, 2007).

Dynamic Scanning Calorimetry of tendon-like constructs

Equine tendon tissue and tendon-like constructs (14 days of culture) had excess water removed, were weighed and loaded in to hermetically sealed 50µl aluminium pans (PekinElmer Inc., Waltham, USA). A DSC 4000 with Autosampler (Perkin Elmer Inc., Waltham, USA) was used and samples were subjected to a temperature ramp between 10°C and 90°C at a rate of 10°C per minute. Three to five replicates were carried out for each sample type, with samples derived from cells/tissue taken from three different horses (i.e. 1 or 2 technical replicates per each biological replicate). An unpaired Student's t-test was used to compare the means of artificial tendons produced under static or cyclical loading conditions.

Results

The bioreactor can accurately apply a global strain to tendon-like constructs

The bioreactor was designed to apply an external uniaxial, cyclic strain to tendon-like constructs (Figure 1). CAD models were produced to determine the localisation of the global stress that was applied when under a 10% strain (Figure 2). The stress experienced in response to a global strain is not evenly distributed across the entire artificial tendon, with

uneven stress being exerted around the anchorage pins. However, the stress is evenly distributed across the main body of the tendon-like constructs. The bioreactor could be turned on from day 1 of culture, with the tendon-like constructs remaining firmly attached to the anchorage pins.

Application of an external strain increases the rate of collagen gel contraction

The tenocytes used in this paper had been characterised previously for their gene expression and ability to contract a collagen gel when used at a similar range of passages(Barsby et al., 2014; Barsby & Guest, 2013; Bavin et al., 2017; Alyce McClellan et al., 2019). When cultured in the bioreactor the tenocytes are able to contract a collagen gel in a similar manner to static conditions, with both cyclic and static cultures producing tendon-like constructs that have reached their maximum contraction of less than 20% the original starting size by day 14 of culture (Figure 3A). As reported previously(Barsby et al., 2014), for both static and cyclic cultures, after 14 days the tendon-like constructs contract to such a degree that they detach from the pins. We therefore only analysed artificial tendons that had been cultured for 14 days and any tendon-like constructs that detached prior to this were not analysed (there was no significant effect of static or cyclic culture on construct detachment). The application of a 10% cyclic strain increased the rate of contraction and after 7 and 8 days of culture there was a statistically significantly increase in the amount of contraction compared to static cultures. The increased rate of contraction was not due to differences in overall cell survival as there were no statistically significant differences in cell survival between static and cyclic cultures at 3, 7 or 14 days of culture (Figure 3 B). The cell survivals ranged from 40-70% (i.e. $3.2x10^4$ to $5.6x10^4$ live cells per construct compared to the $8x10^4$ cells per construct that were seeded). This cell survival range is similar to that which we have reported previously for static cultures(Barsby et al., 2014; Alyce McClellan et al., 2019).

Dynamic shear analysis (DSA) can be used to evaluate the mechanical properties of tendon-like constructs

Traditional tensile testing with axial strain was successfully performed on all native tendon tissue samples tested and produced stress-strain curves typical for tendon (Supplementary Figure 1). However, tensile testing of the tendon-like constructs proved difficult due to their fragile nature, small size and difficulty in maintaining grip. 75% of artificial tendon samples tested were irrevocably damaged on loading in the tensile testing machine and of those successfully loaded, 67% failed at the interface of the sample and the grip rather than within the main body of the sample (as per native tendon) which prevented the calculation of their modulus.

In contrast to traditional tensile testing, rheology was successfully used on 100% of artificial tendon samples to calculate the storage modulus (G'). G' values were shown to be frequency independent in the 0.1 – 1 Hz region. Native equine tendon had a significantly higher G' value than any of the tendon-like constructs (Figure 4). The strained, cyclic produced, artificial tendon had a significantly higher G' value than the empty collagen gel containing no cells and this was also higher than the G' of the static, non-strained, artificial tendon but this was not significant.

The tendon-like constructs have a similar chemical composition to native tendon

Similar FTIR spectra were produced by all samples (Figure 5), with clear amide I (1630 cm^{-1}), amide II (1547 cm^{-1}) and amide III peaks (1237 cm^{-1}) suggesting that all samples have a similar chemical composition and structure. The empty gel also has a similar spectrum to the other samples with amide I, II and III peaks but also displays some peaks between 940-1170 cm^{-1} that are not present in the other samples.

Application of an external strain to tendon-like constructs may lead to greater structural integrity

Differential scanning calorimetry (DSC) demonstrated that native tendon has a denaturation enthalpy over ten times greater than the tendon-like constructs (Figure 6). Both static and cyclic produced tendon-like constructs have an approximately 10-fold higher denaturation enthalpy than the empty gels. This demonstrates that the presence of cells is able to increase the structural integrity of the collagen gel. Furthermore, the cyclic produced tendon-like constructs have a slightly higher denaturation enthalpy than the static tendon-like constructs although this difference is not significant.

Discussion

We have previously demonstrated that adult equine tenocytes can contract a collagen gel under static 3D culture. The resulting tendon-like constructs were characterised using gene expression, histology and immunohistochemistry to demonstrate the organisation of the collagen fibres, expression of tendon genes and distribution of extracellular tendon proteins within the matrix(Barsby et al., 2014). The static 3D culture system has further been used to determine the role of the tendon progenitor marker scleraxis at different stages of tendon development(Bavin et al., 2017) and monitor the functional effect of IL-1β on tendon cells(Alyce McClellan et al., 2019). However, this model did not account for the significant mechanical forces which the tendon cells are exposed to *in vivo* and we have not previously assessed the chemical composition and structural integrity of the resulting tendon constructs.

The aim of this study was to determine the effects of applying an external strain on tenocyte-seeded collagen matrices to understand whether physical factors influence cell behaviour and the surrounding extracellular matrix and to establish a method to determine the mechanical

properties of the tendon-like constructs. The bioreactor was programmed to apply a cyclical 10% strain at 0.67 Hz for 20 min/day from days 1 to 14 of culture. This strain represents that experienced in tendons of horses at a canter(Stephens et al., 1989), a moderately fast exercise pace which is likely to be performed daily in horses in training for periods of around 20 minutes. However, the strain upon the tendon fascicles *in vivo* has been reported to be substantially higher than the strain upon the actual cells(Screen et al., 2004), so it is difficult to determine if a 10% strain used in this *in vitro* study truly reflects the strain experienced by tenocytes *in vivo*. The degree of collagen gel contraction also changes over time and matrix organisation has been shown to influence the actual degree of strain exerted on the tenocytes(Freedman et al., 2018). Strains ranging from 1% to 12% have been applied *in vitro* to non-equine tenocytes(Garvin et al., 2003; Tsuzaki et al., 2003; Wang et al., 2003) but more recent publications have used strains near the top of this range to promote tendon differentiation by MSCs(Chen et al., 2012; Nam, Pingguan-Murphy, Amir Abbas, Mahmood Merican, & Kamarul, 2015). As strain has been reported to increase the expression of inflammatory proteins by tendon cells(Tsuzaki et al., 2003; Wang et al., 2003) and we have demonstrated that inflammatory cytokines can inhibit collagen gel contraction(Alyce McClellan et al., 2019) we selected a high strain to use in this study to determine if it had positive or negative effects on collagen gel contraction by equine tenocytes.

The frequency selected is lower than the stride frequency at a canter, which is approximately 2 Hz(Ratzlaff, Grant, Rathgeber-Lawrence, & Kunka, 1995) but is similar to frequencies that have been used in other *in vitro* studies(Chen et al., 2012; Garvin et al., 2003; Tsuzaki et al., 2003; Wang et al., 2003). Future studies to determine the effects of applying different strains at different frequencies and durations are required. In this study we only applied the cyclical strain for 20 minutes per day. When high strains (14%) have been applied *in vitro* to tendon

fascicles, a significant reduction in the failure stress is observed in as little as 15 minutes(Legerlotz, Jones, Screen, & Riley, 2013), suggesting applying a strain for 20 minutes a day would be sufficient to observe an effect. However, performing a lower level of additional cyclical strain to mimic the amount of walk a horse may do in a day may be a future refinement.

CAD models of the tendon-like constructs after different time periods of culture demonstrated that although the resulting stress is not evenly distributed around the anchorage posts, it is evenly distributed across the main body of the artificial tendon. *In vivo*, tendon connects to muscle and bone and our bioreactor design does not model these tissue changes and interactions. Further studies are required to determine if the cells surrounding the anchorage posts exhibit different characteristics to cells in the main body of the artificial tendon.

Collagen gel contraction by cells exposed to cyclic external strain was significantly increased compared to those in static culture after 7 and 8 days of culture. The enhanced contraction was not due to differences in tenocyte survival which remained statistically insignificant at all time points studied throughout the 14 days. However, the rates of cell proliferation and death were not measured in this study and may be useful inclusions in future studies. We have previously demonstrated that exposure of tenocytes to IL-1β has no effect on cell survival, but has a negative influence on both tenocyte gene expression and ability to contract a collagen gel(Alyce McClellan et al., 2019). Future work will determine if the enhanced collagen gel contraction in response to cyclical strain correlates with changes in total collagen content, gene expression and/or protein localisation. Loading of equine induced pluripotent stem cell (iPSC) has recently been shown to promote tenogenic gene expression(F. Yang,

Zhang, & Richardson, 2019) but to our knowledge, gene expression changes in adult tenocytes in response to loading have not yet been reported. Scleraxis has recently been implicated in mechanosensing in tenocytes (Nichols, Settlage, Werre, & Dahlgren, 2018) and the mechanotransduction pathways which underpin our results should be explored in future work.

In order to ascertain the effects of tensile strain the material properties of the resulting tendon-like constructs were characterised. While tensile testing of tendon tissue was successfully performed, it proved impossible to use traditional tensile testing on tendon-like constructs. This was due to their small size and difficulty in gripping the samples. We therefore employed dynamic shear analysis (DSA) to calculate the storage modulus (G') of the tendon-like constructs. This technique was developed to enable the effective testing of small, wet, soft samples(Chaudhury, Holland, Thompson, Vollrath, & Carr, 2012; Chaudhury, Holland, Vollrath, et al., 2011) and has been used to measure the storage modulus of native tendons(Woods, Broomfield, Monk, Vollrath, & Glyn-Jones, 2018; Zellers, Cortes, Pohlig, & Silbernagel, 2018) and tendon-like constructs(Alyce McClellan et al., 2019) successfully.

All tendon-like constructs had G' values which were frequency independent in the 0.1-1 Hz region as previously found for human rotator cuff tendons(Chaudhury, Holland, Vollrath, et al., 2011). G' is primarily influenced by the solid fibril phase of the matrix(Kreger et al., 2010), rather than the hydrated surroundings, and both the stiffness of the fibrils themselves and inter-fibril bonding are responsible for different stiffness values (G')(Kreger et al., 2010). The cyclic straining produced tendon-like constructs had a significantly greater storage modulus than the empty gel containing no cells. Although not significant, the cyclic strain

produced tendon-like constructs have an approximately 30% greater storage modulus than the static produced tendon-like constructs after 14 days of culture. As traditional tensile testing could not be performed we could not determine the ultimate tensile strength (UTS) of the tendon-like constructs. However, UTS and elastic modulus are strongly correlated in tendon tissue(LaCroix, Duenwald-Kuehl, Lakes, & Vanderby, 2013) and the increased modulus of the tendon-like constructs in response to cyclical strain would likely result in an increased UTS and therefore a lower chance of rupture when the tendon is exposed to high forces.

Collagen fibres align *in vitro* with applied strain(Chandran & Barocas, 2004; Sellaro et al., 2007) and this results in increased mechanical strength(Pins, Christiansen, Patel, & Silver, 1997). The applied strain subjects the tendon fibres to straightening, realignment and sliding and tenocyte nuclei are also placed under strain which affects their mechanotransduction pathways and fibril alignment(Arnoczky, Lavagnino, Whallon, & Hoonjan, 2002; Screen et al., 2004). By day 14 of culture both the cyclic and static cultures had reached the same degree of contraction. It would therefore be useful to determine if the G' values of the cyclic and static cultures differ at day 7 or 8 when the cyclic cultures are significantly more contracted.

FTIR analysis of the tendon-like constructs demonstrated that they were very similar in their chemical composition to native tendon and all samples displayed typical amide I, II and III peaks. Empty gels containing no cells have similar spectra to the other samples but display additional peaks between 940 and 1170 cm^{-1}. These could be caused by the presence of the amino acids, for example L-Glutamine(La'Verne, Srivastava, Srivastava, & Gupta, 2009), in the media that is added during the gel setting process. These are likely to have been removed by 14 days from gels which are remodelled when the cells are present, but will still remain in

the empty gels. The FTIR analysis does not determine the structure and organisation of the matrix and future research to determine the effect of cyclical strain on these parameters is required. We have previously demonstrated that in static cultures, equine tenocytes produce well aligned collagen fibres and synthesise other tendon proteins(Barsby et al., 2014) and quantifying the effects of an external cyclical strain on these parameters would help to determine if improved matrix organisation and secretion of other matrix proteins contributes to the increased storage modulus of the artificial tendons exposed to an external strain.

DSC was used to calculate the energy required to fully denature the proteins present in the sample (Chaudhury, Holland, Porter, et al., 2011; McClain & Wiley, 1972; Samouillan et al., 2011). The native equine tendon has a higher peak temperature than the tendon like constructs and the highest denaturation enthalpy, which is fifteen times that of the tendon like constructs. This could indicate a larger amount of cross-linking within the collagen(Bailey, Sims, Avery, & Miles, 1993). However covalent cross-linking alone has only a small influence on thermal stability(McClain & Wiley, 1972) and it likely reflects the more complex hierarchical structure present in tendon tissue due to additional hydrogen bonding/degree of hydration in the collagen and a greater number and variety of intermolecular and interfibrillar bonding.

Empty, set collagen gels have approximately the same onset temperature as the tendon-like constructs, however the slope to the peak is much more gradual and the denaturation enthalpy is approximately ten fold lower. Interestingly, the empty gel has a higher peak temperature than the tendon-like constructs. This may be because it has an amorphous rather than crystalline structure. Crystalline structures, such as the collagen fibrils of cultured gels and native tendon, have been shown to conduct heat more rapidly, decreasing the time and

temperature (as the temperature increases at a constant rate) needed to denature the protein(Bozec & Odlyha, 2011). The cyclic generated tendon-like constructs had a slightly higher denaturation enthalpy than the static cultures but this was not significant. Additional work to measure the denaturation enthalpy of the static and cyclic tendons at earlier time points during their culture is required.

The tenocytes used in this study were at passages 4-10. Gene expression in tendon cells has been shown to change with culture(Shukunami et al., 2018; Yao, Bestwick, Bestwick, Maffulli, & Aspden, 2006) and can change rapidly from the expression levels found in the native tissue(Kuemmerle et al., 2016). However, we have previously demonstrated that *COL3A1* gene expression levels do not change between passage 0 and passage 10 during equine tenocyte culture(Liao et al., 2020). Additionally, although there is a significantly higher level of *COL1A1* in equine tenocytes at passage 0 than at passage 3 there are no further changes in *COL1A1* expression beyond passage 3(Liao et al., 2020). Furthermore, there is no significant difference in collagen gel contraction and response to inflammation in equine tenocytes used between passage 4 and 11(Alyce McClellan et al., 2019). However, adult tenocytes do have a limited self-renewal *in vitro,* which would limit their numbers for clinical application, and *in vivo* they generate reparative scar tissue rather than healthy tendon tissue(Tang et al., 2014). Therefore future work is required to examine the effects of force on cells which may aid tendon regeneration, for example mesenchymal stromal cells(Godwin, Young, Dudhia, Beamish, & Smith, 2012) and embryonic stem cells (Barsby et al., 2014; Alyce McClellan et al., 2019; McClellan, Paterson, Paillot, & Guest, 2019).

In conclusion, we have demonstrated that a novel, highly cost-effective bioreactor can be produced in house to reliably apply strain to tendon-like constructs. The application of strain increases the rate of collagen gel contraction by tenocytes and a number of techniques can be

utilised to determine the mechanical and chemical properties of the resulting tendon-like constructs. This system will allow future *in vitro* work to analyse the signalling mechanisms involved in matrix remodelling by tenocytes and determine if strain can enhance tenocyte differentiation by different types of stem cells. Performing this work on equine cells provides baseline data for a species in which it will be possible to perform future *in vivo* work using the most relevant model for human tendon injuries. Understanding the effects of exercise-induced force on both the endogenous cells and any clinically applied cells will enable the development of the most appropriate rehabilitation programmes following a tendon injury.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

Ethics

Equine superficial digital flexor tendons were recovered from horses that had been euthanised for reasons unrelated to this project with approval of the Animal Health Trust Ethical Review Committee (AHT_02_2012) and all experiments were performed in accordance with relevant guidelines and regulations.

Data Availability

All data generated or analysed during this study are included in this published article.

Author contributions

JG and DC designed and built the bioreactor with assistance from RE and EB. FA, RE and EB generated the static and cyclical cultures. FA performed the tensile testing, DSA, DSC, FTIR and helped draft the manuscript. RE performed the contraction and survival analyses, generated the CAD models and helped draft the manuscript. CH and DG conceived, designed and co-ordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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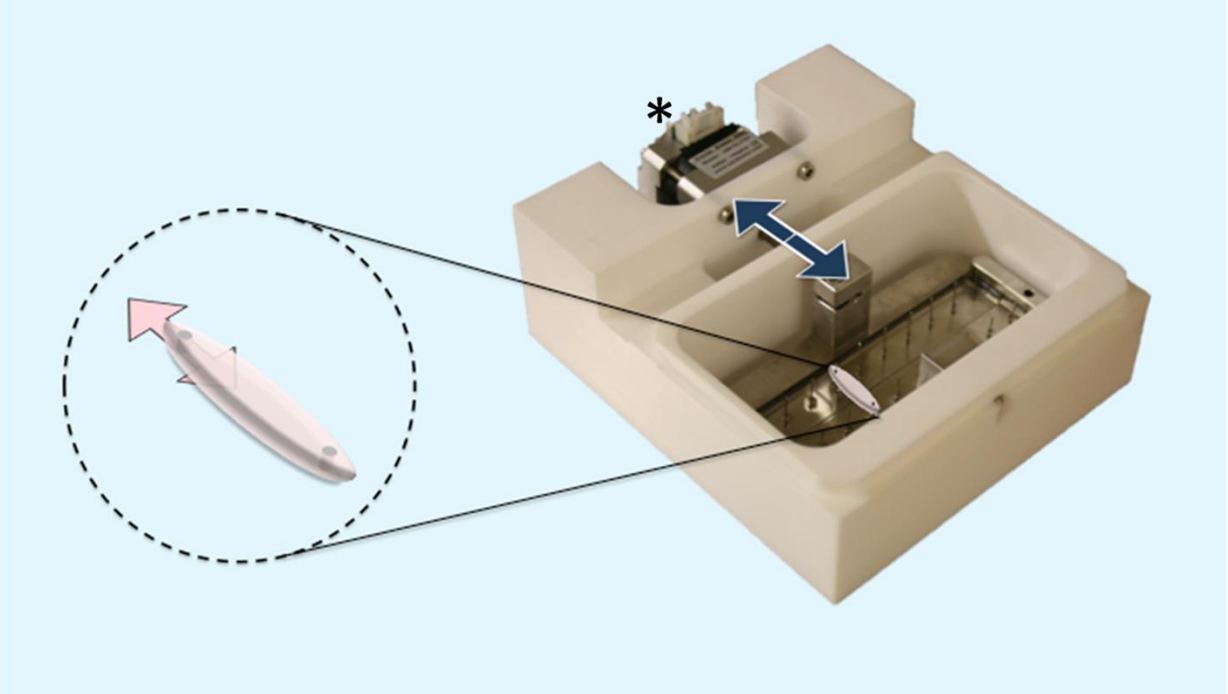

Figure 1. The bioreactor. A polytetrafluoroethylene (PTFE) base supports one stationary and one mobile stainless steel plate, the latter is controlled by a hybrid stepping motor (indicated by *) that drives linear motion. The location of the artificial tendons is illustrated in white. The blue arrow highlights the direction of movement of the stainless steel chamber. The pink arrow highlights the direction of movement of the artificial tendon. The PTFE chamber is designed to fit a standard 6-well tissue culture plate lid.

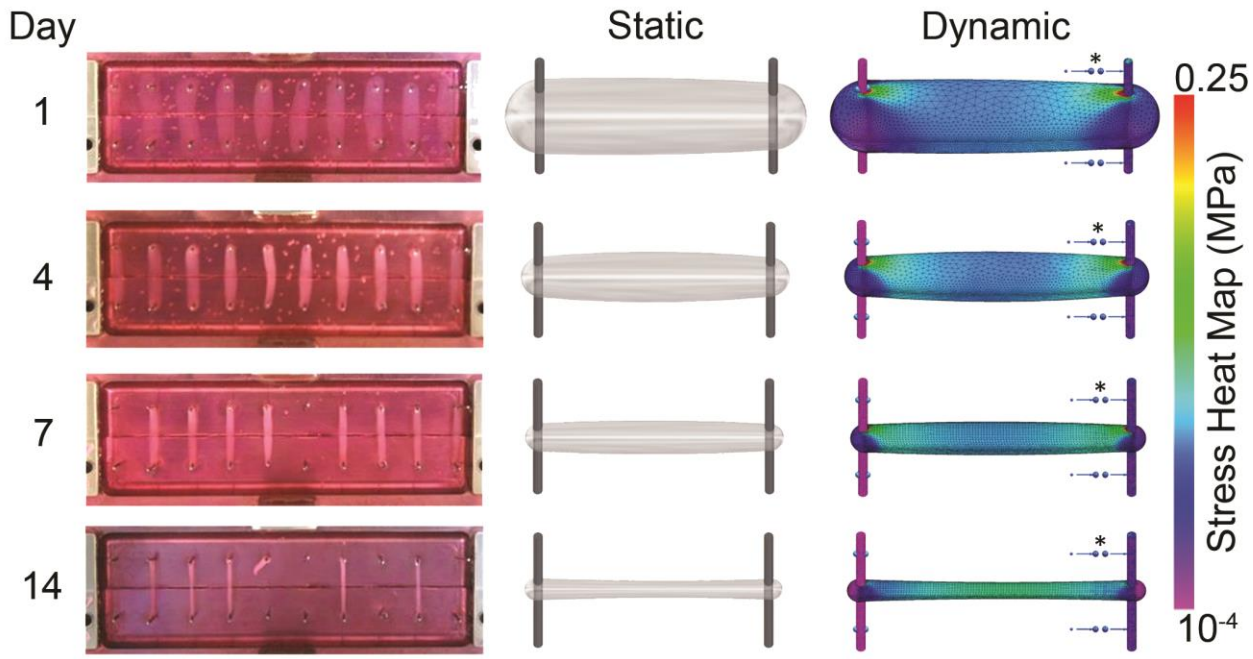

Figure 2. Images of artificial tendons during time in cyclical culture followed by their representative static and simulated CAD models. *Denotes the starting position of the artificial tendon before the strain is applied. The heat map demonstrates the localisation of the stress experienced following the application of a 10% global strain.

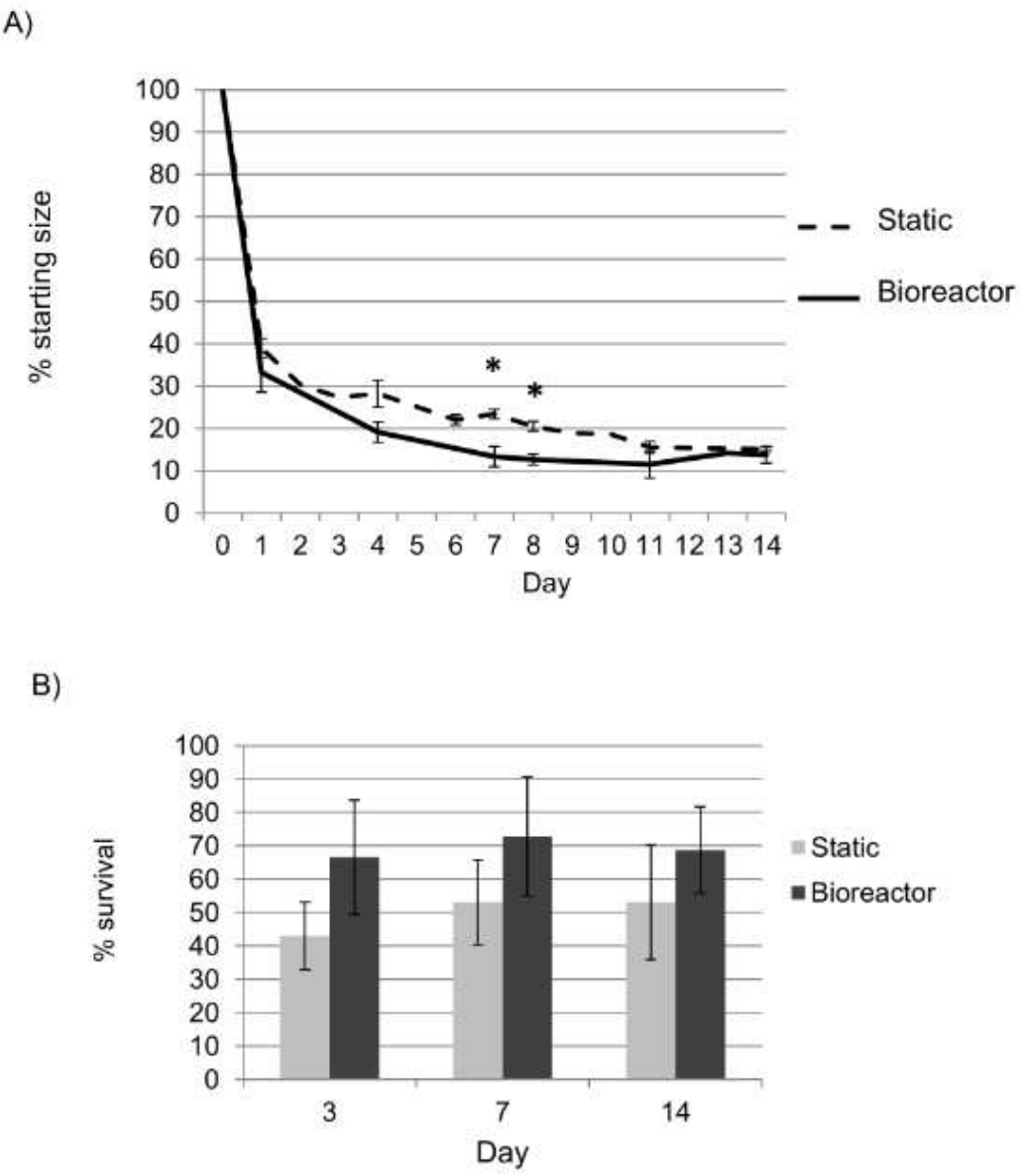

Figure 3. A) Contraction of the artificial tendons in static (dashed line) and cyclic (solid line) cultures. Although both cultures reach the same maximum contraction after 14 days in culture, the cyclic cultures contract more quickly, demonstrating a statistically significant difference to the static cultures at day 7 and day 8. *$p<0.05$ using a Student's t-test. B) The cell survival in the artificial tendons is not significantly different between the static (light grey) and cyclic (dark grey) cultures at any time point studied. Error bars represent the s.e.m. of three biological replicates (i.e. cells derived from three horses).

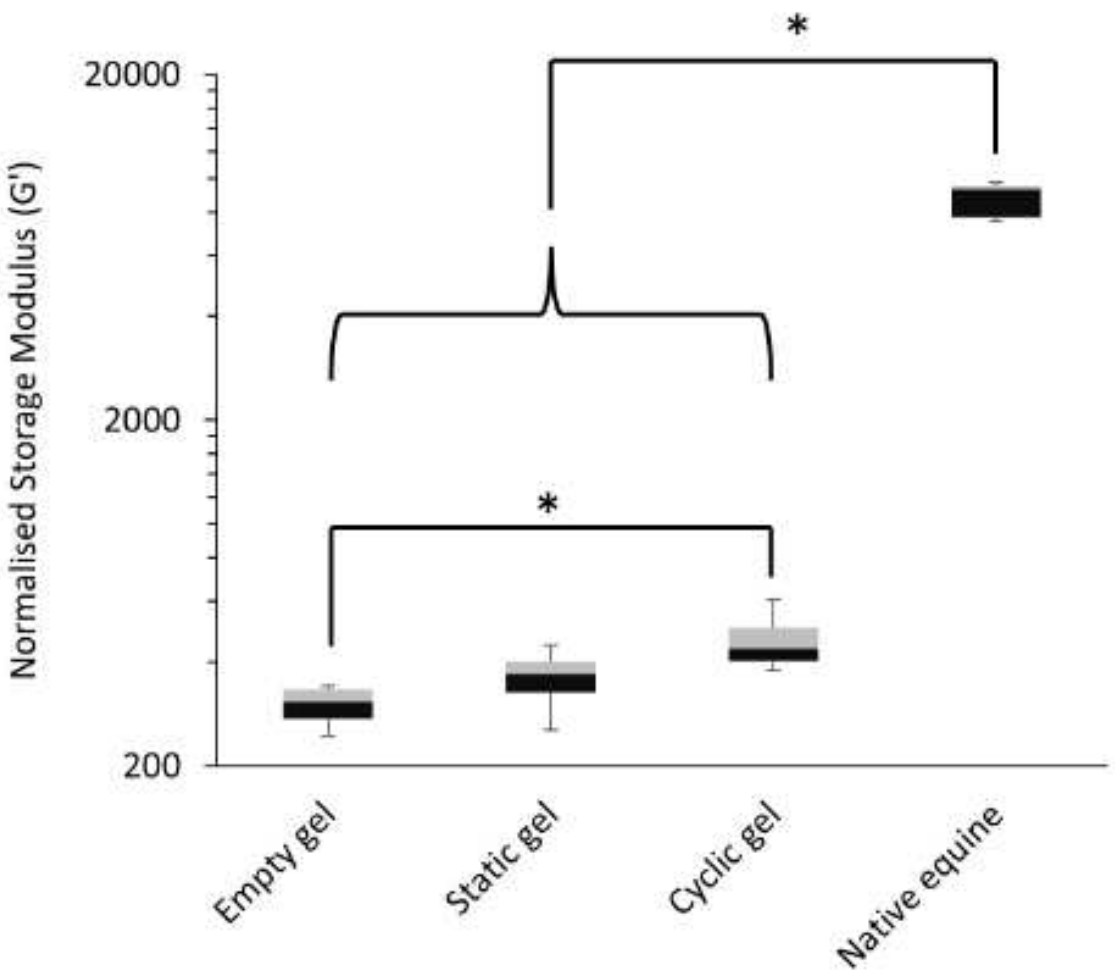

Figure 4. Dynamic shear analysis (DSA) was successfully used to calculate the storage modulus (G') of all tendon samples. Error bars represent the s.e.m. of three biological replicates (i.e. derived from three different hroses). Native equine tendon has a significantly higher G' than all artificial tendons or the empty collagen gel and the G' of cyclic gels is significantly different to that of the empty gel (*$p<0.05$).

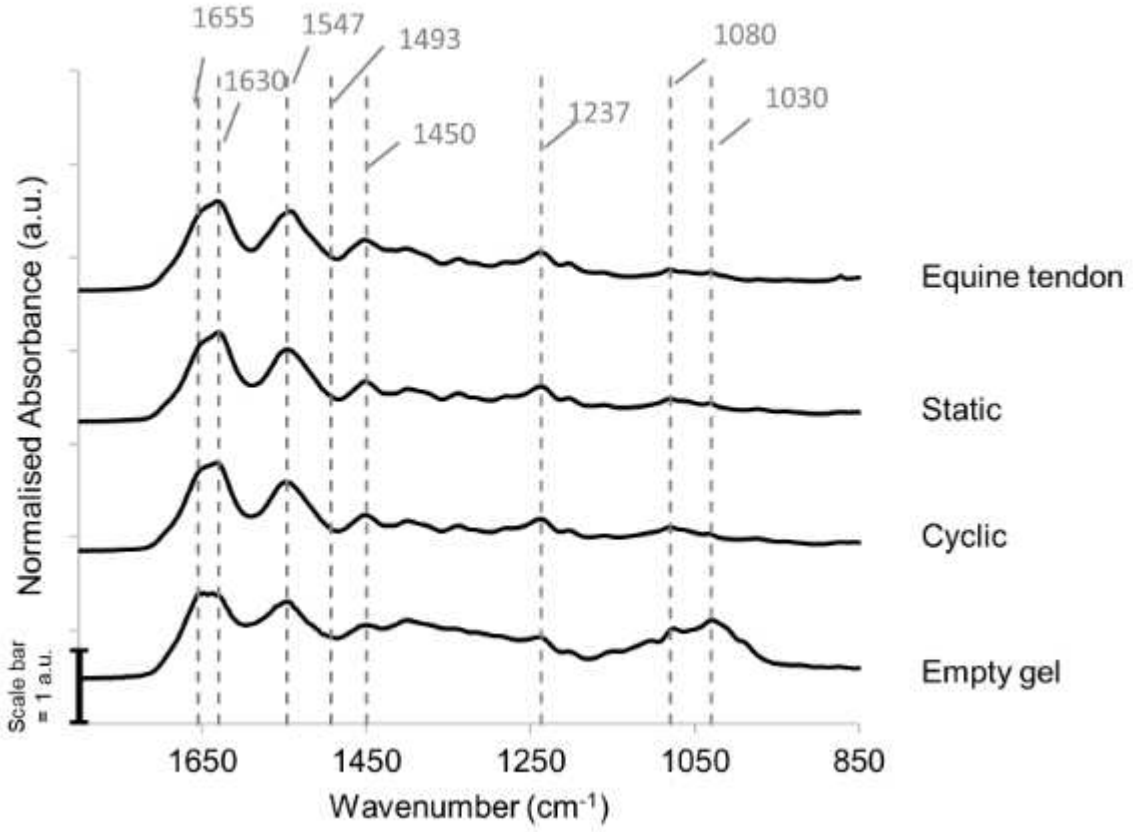

Figure 5. FTIR spectra of native equine tendon, empty set collagen gel without cells and artificial tendons produced with (cyclic) and without (static) the application of a 10% external strain. Spectra are normalised to the amide I band at ~1630 cm^{-1}. Scale bar = 1 arbitrary unit.

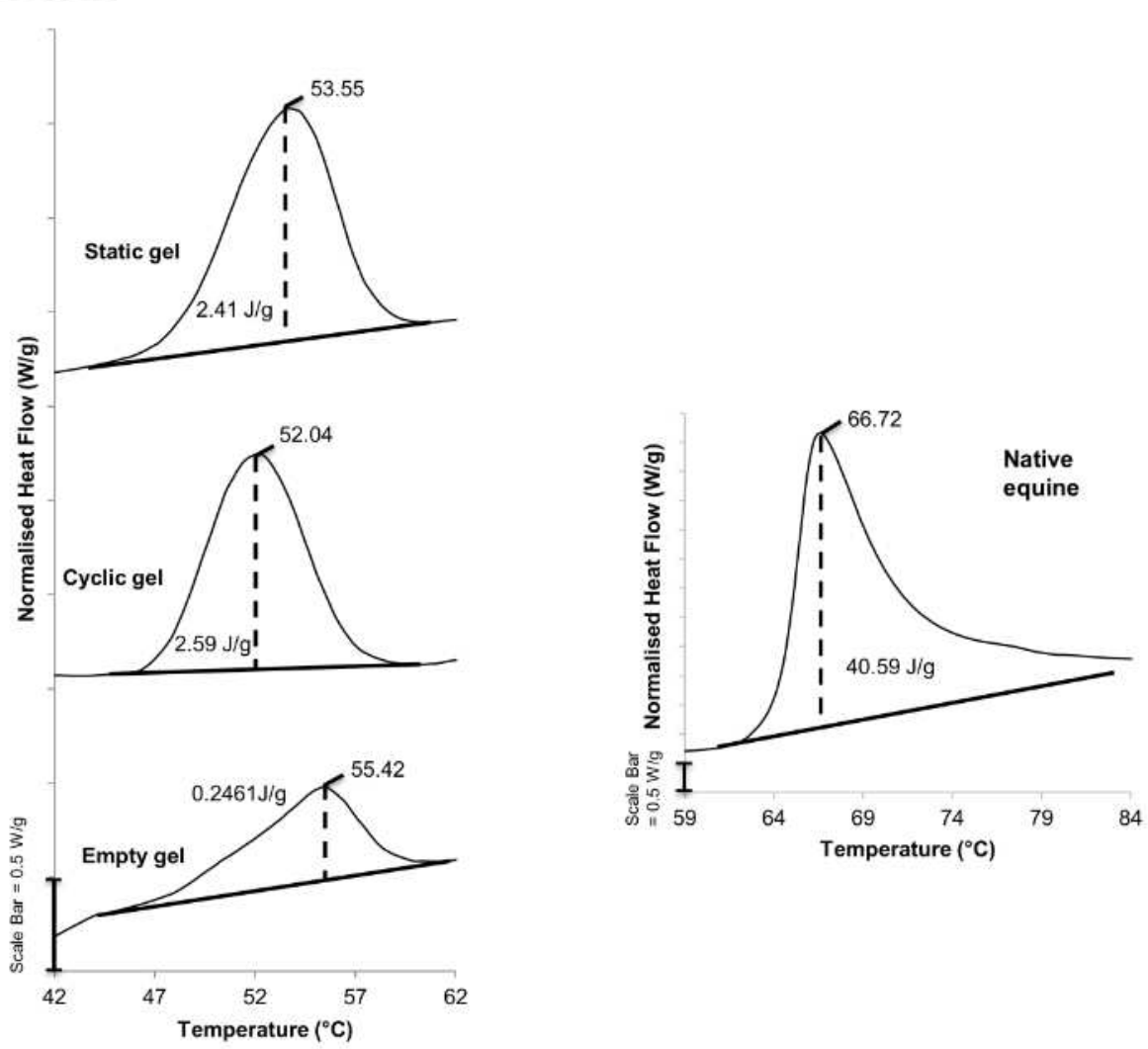

Figure 6. Differential scanning calorimetry (DSC) of the artificial tendons and native equine tendon to determine the energy required to for denaturation. Scale bars = 0.5 W/g. Representative graphs are shown from 3 biological replicates per sample.