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Collagen Fibril Diameter and Leather Strength

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ABSTRACT: The main structural component of leather and skin is type I collagen in the form of strong fibrils. Strength is an important property of leather, and the way in which collagen contributes to the strength is not fully understood. Synchrotron-based small angle X-ray scattering (SAXS) is used to measure the collagen fibril diameter of leather from a range of animals, including sheep and cattle, that had a range of tear strengths. SAXS data were fit to a cylinder model. The collagen fibril diameter and tear strength were found to be correlated in bovine leather ($r^2 = 0.59$; $P = 0.009$), with stronger leather having thicker fibrils. There was no correlation between orientation index, i.e., fibril alignment, and fibril diameter for this data set. Ovine leather showed no correlation between tear strength and fibril diameter, nor was there a correlation across a selection of other animal leathers. The findings presented here suggest that there may be a different structural motif in skin compared with tendon, particularly ovine skin or leather, in which the diameter of the individual fibrils contributes less to strength than fibril alignment does.

KEYWORDS: *collagen, leather, small angle X-ray scattering, fibril diameter, orientation*

INTRODUCTION

The fundamental structural motifs that result in strong leather and skin are complex and not yet fully understood. For many high-value commercial applications, strong leather is necessary. The skins from some animals such as cattle, kangaroo, and goat generally produce strong leather, while other animals, including sheep, produce weaker leather.[1] To develop methods for increasing the strength of leather, especially that produced from the inherently weaker animal skins, it is necessary to understand the skin and leather structures that are characteristic of high-strength material.

The main structural component of leather is type I collagen, and this is primarily responsible for skin and leather strength.[2] Of the many factors contributing to the strength of natural skin and finished leather, the three most important have been proposed to be the type and nature of cross-linking between the collagen fibrils, the orientation of the collagen fibrils, and the fibril diameter. Skin and leather also contain keratins, type III collagen, and elastin, and these may contribute to strength to a lesser degree.

Natural cross-linking of collagen is present in living skin and is also achieved in processed leather using chromium, tannins, or glutaraldehyde. Cross-linking would be expected to mechanically couple the collagen fibrils and therefore enhance their ability to transmit force[3−8] and also increase toughness by absorbing energy through enthalpic changes.[9,10] While some researchers found that the tensile elastic modulus of tendon is reduced when the content of the natural cross-linking component glycosaminoglycan (GAG) is lowered,[11] others have found no altered mechanical properties in tendon from the removal of GAGs.[12,13] However, with synthetic cross-linking of bovine pericardium by glutaraldehyde, the material becomes stronger than untreated tissue,[14,15] suggesting that cross-links are important for strength.

It has been widely reported that when fibrils are more uniformly aligned in the plane of the leather, the material is stronger than otherwise.[1,16] The way in which collagen fibril alignment would increase strength in collagen tissues has been accurately modeled in two dimensions.[17,18] It has been shown that in two dimensions this model accurately predicts the observed behavior with fibrils that are more uniformly aligned in the plane of the leather, resulting in stronger material.[1,16] However, one study has shown that, when tear strength is used as a measure of strength, in the third dimension, the model does not give a complete picture as less alignment could be preferable for stronger material.[1]

These two factors that affect material strength, collagen fibril orientation and cross-linking, have been shown to be interdependent because cross-linking influences fibril alignment.[19]

The contribution of collagen fibril diameter to strength is the subject of this work. Fibril diameter varies with strength, with several studies finding larger diameter collagen fibrils present in stronger tissue. In human aortic valves, the collagen fibril diameter depends on whether the fibrils are from regions of high stress or low stress: larger diameter fibrils (in areas of lower fibril density) result from high stress, suggesting that these larger diameter fibrils provide increased strength.[20] Similarly for mouse tendon, fibril diameters increase with loading.[21] Rats that exercised were active and lean and had

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larger diameter collagen fibrils with a bimodal diameter distribution, compared to those of inactive obese rats. The mechanism proposed for this observation is one in which the extra mechanical load placed on the tendons on the exercising rats (because of their higher activity levels) stimulated fibril thickening.[22]

The size distribution of the fibril diameter may also change with age. Fetal tissue has been found to have a unimodal distribution with smaller collagen fibril diameters, whereas older tissue has larger fibrils and may have a unimodal or bimodal size distribution depending on the tissue type and animal.[23] Growth of collagen in tissue culture has shown that larger fibril diameters are associated with an increase in strength.[24] It has been proposed that with weak strain the smaller diameter fibrils prevent creep while with strong strain the larger diameter fibrils provide a higher tensile strength.[23,25] These ideas have been further developed with the concept of the total absorbed energy providing tendon resilience and resistance to rupture.[25]

In studies of equine digital flexor tendons, the fibril diameter decreases with exercise, suggesting weakening of tendon with exercise (i.e., a thicker fibril is stronger). Unusually, the fibril diameter in these tendons decreases with age, but this is associated with the decrease in strength.[26,27]

The collagen fibril diameter has been measured with a range of techniques, each having advantages and limitations. Transmission electron microscopy (TEM) has been used to determine fibril diameters.[28−30] However, this requires that a large number of individual fibrils be measured,[28] and the diameters obtained from the analysis depend on the processing methods and the type of fixation used in sample preparation;[31] the results of TEM analysis need to be interpreted with caution. Atomic force microscopy (AFM) is another microscopic method used for fibril diameter analysis.[32,33] It also requires a large number of measurements to test for statistical significance, but unlike that of TEM, the environment is relatively easy to control and does not require any fixing or chemical processing of the sample.

In this study, the fibril diameter was measured using small angle X-ray scattering (SAXS). This method has been used to measure collagen fibril diameters in tendons.[34] The key advantage of SAXS is that it gives a size distribution for a large number of fibrils (the whole analysis volume) with one measurement. This allows robust statistical analyses and minimizes the sampling error and experimenter bias that may be present with techniques such as TEM and AFM. Like in AFM, the samples require no fixing or specific chemical processing prior to analysis, and some degree of environmental control may be possible.

There is a general consensus that larger fibril diameters result in stronger tendons and other tissues. The purpose of this study is to investigate whether collagen fibril diameter is associated with strength in leather produced from bovine and ovine skins and to see if such a relationship might be generalized to other animals.

METHODS

Ovine pelts were from 5-month-old, early season lambs of breeds with "black face", which may include Suffolk, South Suffolk, and Dorset Down. The bovine hides were from 2−3-year-old cattle of a variety of breeds.

Skins were processed to produce leather by the following procedure. After mechanical removal of adhering fat and flesh, conventional lime sulfide paint, comprising 140 g/L sodium sulfide, 50 g/L hydrated lime, and 23 g/L pregelled starch thickener, was applied to the flesh side of the skin at a rate of 400 g/m^2. The skin was incubated at 20 °C for 16 h and the keratinaceous material manually removed. The skin was then washed to remove the lime, and the pH was lowered to 8 with ammonium sulfate, followed by the addition of 0.1% (w/v) Tanzyme (a commercial bate enzyme). After 75 min at 35 °C, the treated skin was washed and then pickled [20% (w/v) sodium chloride and 2% (w/v) sulfuric acid]. Pickled pelts were degreased [4% nonionic surfactant (Tetrapol LTN, Shamrock, New Zealand)] at 35 °C for 90 min and then washed. The skins were neutralized in 8% NaCl, a 1% disodium phthalate solution (40% active) (Feliderm DP, Clariant, U.K.), and 1% formic acid for 10 min. The running solution was then made up to 5% chrome sulfate (Chromosal B, Lanxess, Germany) and processed for 30 min followed by addition of 0.6% magnesium oxide, based on the weight of the skins, to fix the chrome, and processed overnight at 40 °C. These wet-blue pelts were neutralized in 1% sodium formate and 0.15% sodium bicarbonate for 1 h and then washed followed by retanning with 2% synthetic retanning agent (Tanicor PW, Clariant, Germany) and 3% vegetable tanning (mimosa) (Tanac, Montenegro, Brazil). Next, 6% mixed fatliquors were added to the leathers, which were then maintained at 50 °C for 45 min, fixed with 0.5% formic acid for 30 min, and finally washed in cold water.

In addition, a single leather sample each from crocodile, deer, elephant, goat, horse, pig, possum, seal, and water buffalo was similarly processed.

Thickness-normalized tear strengths were measured for all samples using standard methods.[35] Samples were cut from the leather at the official sampling position (OSP)[36] except for the "other animals", where the leather was taken from near the center line. The samples were then conditioned at a constant temperature (20 °C) and relative humidity (65%) for 24 h and then tested on an Instron 4467 instrument. Two groups were selected from the ovine leather, with one group consisting of low-strength material and one group consisting of high-strength material. A range of samples with strengths between the limits were not analyzed further.

For scanning electron microscopy (SEM), samples were sputter coated with gold and imaged using an accelerating voltage of 20 kV. Images were recorded on a FEI Quanta 200 instrument (FEI, Eindhoven, The Netherlands).

Samples were prepared for SAXS analysis by cutting 1 mm × 30 mm strips of leather. To record the scattering patterns, each sample was mounted in the X-ray beam either with the face of the leather normal to the incoming X-rays or with the edge facing the X-ray beam. For the edge-on analyses, measurements were made every 0.25 mm, with the samples analyzed from the grain to the corium. For when the beam was directed normal to the surface of the leather, samples were cut parallel to the surface, producing a grain sample and a corium sample.[37] Diffraction patterns were recorded on the Australian Synchrotron SAXS/WAXS beamline, using a high-intensity undulator source. An energy resolution of 10^{-4} was obtained from a cryo-cooled Si(111) double-crystal monochromator, and the beam size (full width at half-maximum focused at the sample) was 250 μm × 80 μm, with a total photon flux of ~2 × 10^{12} photons/s. Diffraction patterns were recorded with an X-ray energy of 8 keV using a Pilatus 1M detector with an active area of 170 mm × 170 mm and a sample−detector distance of 3371 mm. The exposure time for the diffraction patterns was 1 s, and initial data processing was conducted using SAXS15ID.[38]

Fibril diameters were calculated from the SAXS data using Irena[39] running within Igor Pro. The data were fit at the wave vector, Q, in the range of 0.01−0.04 Å^{-1} and at an azimuthal angle that was 90° to the long axis of most of the collagen fibrils. This angle was selected by determining the average orientation of the collagen fibrils from the azimuthal angle for the maximal intensity of the D-spacing diffraction peaks. The "cylinderAR" shape model with an arbitrary aspect ratio of 30 was used for all fitting. We did not attempt to individually optimize this aspect ratio, and the unbranched length of collagen fibrils may in practice have a length that exceeds an aspect ratio of 30.

The orientation index, OI, is defined as (90° − OA)/90°, where OA is the minimal azimuthal angle range, centered at 180°, that contains

50% of the microfibrils.[37,40] An OI of 1 represents a perfect alignment, while an OI of 0 represents a perfect isotropy. We calculated the OI from the spread in the azimuthal angle of the D-spacing peak at 0.059−0.060 Å^{-1}. Each OI value presented here represents the average of 14−36 measurements of one sample.

RESULTS

SEM. Scanning electron microscopy images of ovine and bovine leather (Figure 1) show collagen fibrils aligned and organized into fibril bundles (approximately 5−10 μm in diameter).

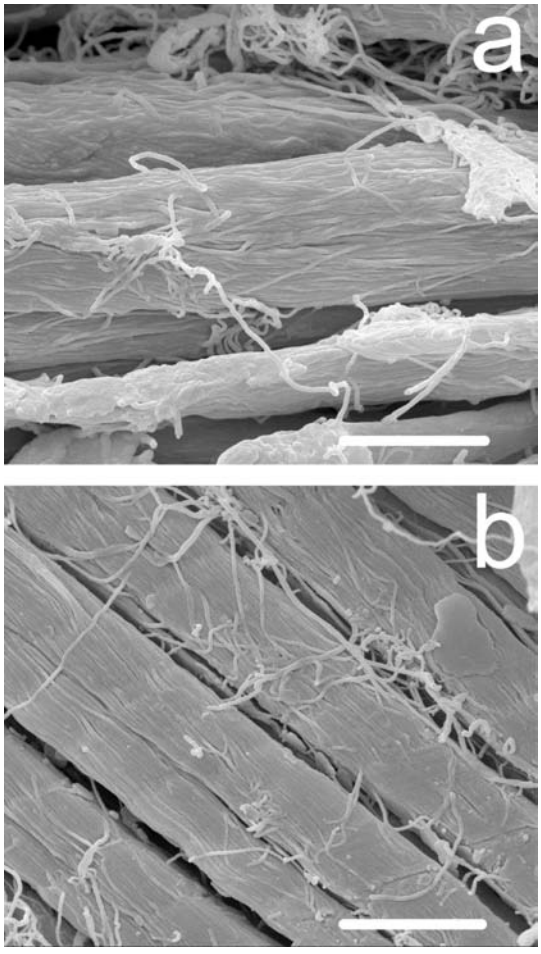

Figure 1. SEM images of (a) bovine and (b) ovine leather, with the aligned collagen fibrils visible and organized in bundles. Scale bars 10 μm. Panel (a) reproduced from ref 16. Copyright 2011 American Chemical Society.

SAXS Patterns. The well-defined rings that were observed are due to diffraction from the D-banding structure of the collagen fibrils and are most noticeable at high Q (Figure 2). Each ring can be seen to be of variable intensity around the azimuthal angle. This variation in intensity is due the alignment of the fibrils. The scattering at low Q, in the center of the pattern, provides the information from which the fibril diameter is determined. The diffraction from the D-banding and the scattering due to the fibril diameter are oriented at right angles to each other because the D-banding occurs along the length of a fibril and the diameter is at right angles to the length of the fibril. This can be seen to some extent in the raw diffraction images (Figure 2), with the central region elongated vertically

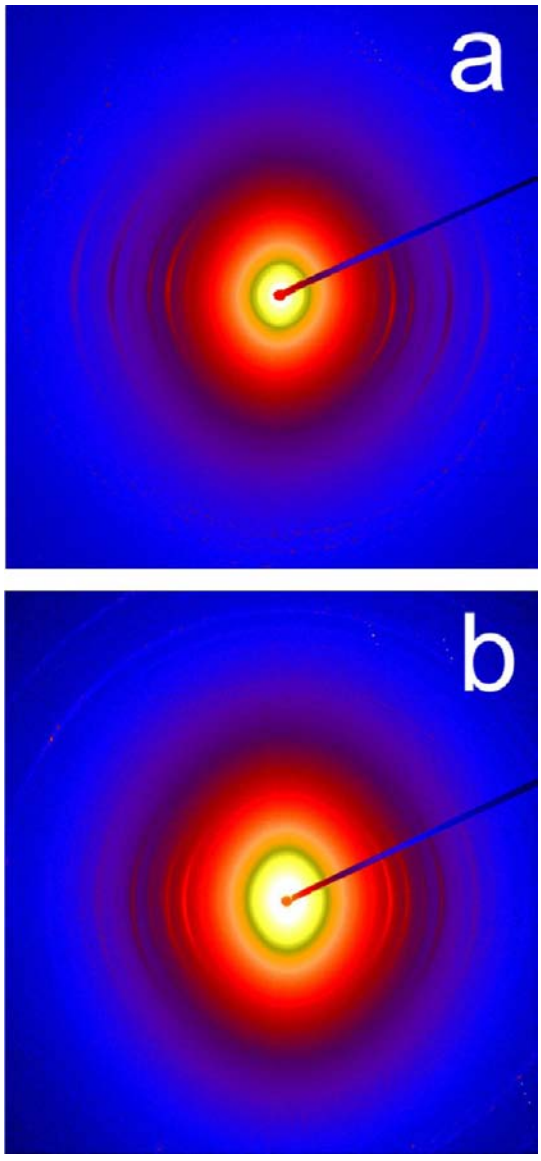

Figure 2. SAXS patterns for (a) ovine and (b) bovine leather.

and the D-banding diffraction bands aligned horizontally. Therefore, it is possible to partially separate these two components of the scattering pattern by using integrated scattering intensity versus Q information from azimuthal segments at right angles to each other (Figure 3). With the fibril diameter measurements, an azimuthal segment that was at right angles to the maximal D-banding diffraction peaks was therefore used so that there is minimal interference with the scattering due to fibril diameter by low-order D-band diffraction.

Fibril Diameter Measurement. A good fit is achieved using the cylinder mode with an aspect ratio of 30 for the bovine and ovine data. The ovine and bovine samples produce scattering profiles that are qualitatively different, with a straighter profile for bovine than for ovine. The average diameter (with the standard deviation) of collagen fibrils for ovine leather was 61.5 ± 48 nm and for bovine leather 59.8 ± 21 nm, and these are statistically different (t = 4.6; P < 0.0001).

Fibril Diameter and Strength. The measured fibril diameters are plotted against strength for three data sets: ovine leather (Figure 4a), bovine leather (Figure 4b), and

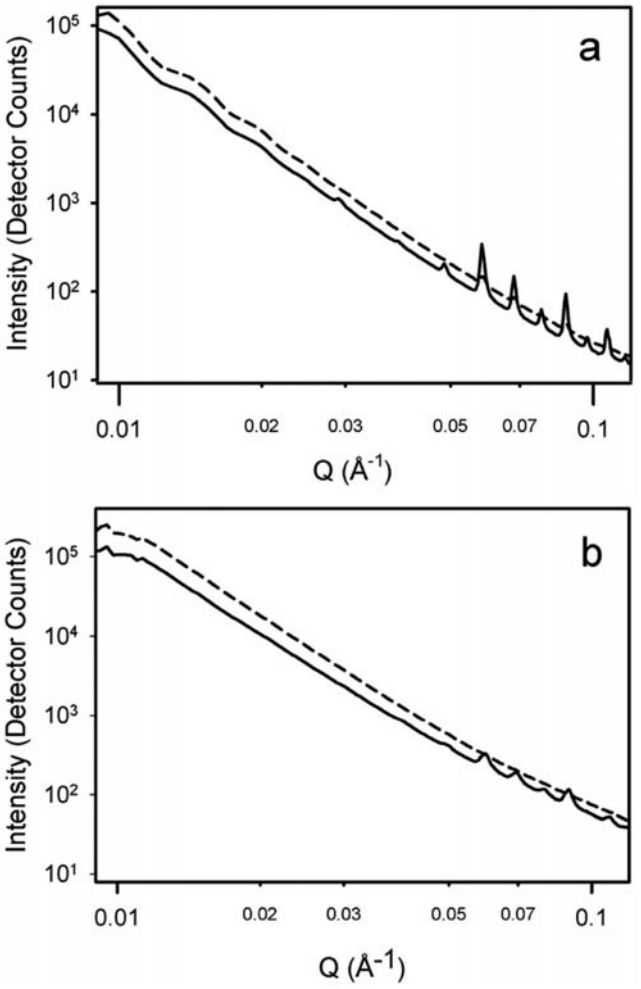

Figure 3. SAXS profiles of examples of (a) ovine and (b) bovine leather. The profiles at two azimuthal angle ranges are shown with the solid lines at the azimuthal angle range where the D-spacing diffraction peaks are maximal (typically a ψ range from −30° to 30°) and the dashed lines at right angles to this (typically ψ = 60−120°). The data shown as dashed lines represent those used for fibril diameter analysis.

leather of other animals, including crocodile, deer, elephant, goat, horse, pig, possum, seal, and water buffalo (Figure 4c). There is no correlation between strength and fibril diameter for the leather from sheep or the other animals. However, for bovine leather, there is a statistically significant correlation between fibril diameter and strength, with stronger leather containing thicker fibrils (r^2 = 0.59; P = 0.009).

Fibril Diameter and OI. A strong correlation between leather strength and fibril orientation (OI) has been reported;[1,16] therefore we wished to see whether fibril diameter is correlated with OI. Plots of fibril diameter versus OI for ovine and bovine leather (Figure 5) show no correlation between these two structural aspects of leather.

DISCUSSION

Fibril Diameter. The average diameters (with standard deviation) of collagen fibrils found here for ovine leather of 61.5 ± 48 nm and for bovine leather of 59.8 ± 21 nm are similar to those found in other studies. Fibril diameters reported for sheep included 65 nm[41] (skin, measured using TEM), 73 ± 20 nm (spine ligament, measured using SAXS), and 69 ± 14 nm (spine ligament, measured using TEM).[34]

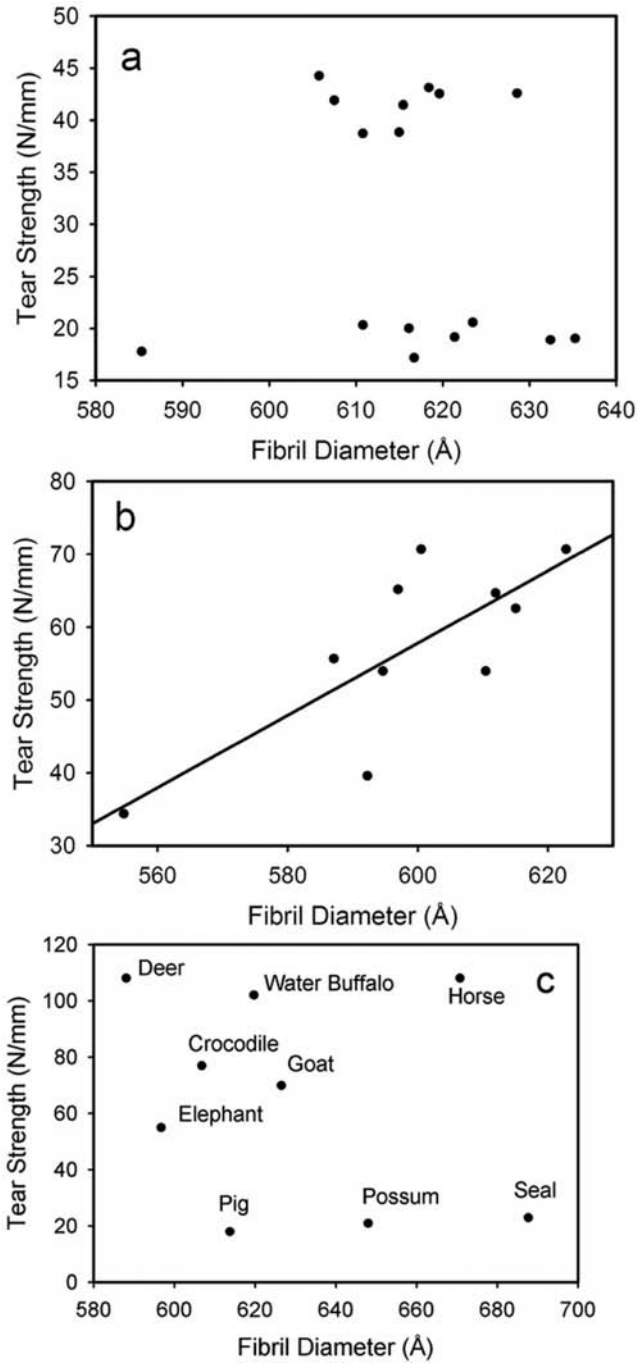

Figure 4. Collagen fibril diameter vs tear strength for (a) ovine leather, (b) bovine leather, and (c) a range of leathers from other animals. For bovine leather, r^2 = 0.59 and P = 0.009 (for slope). For ovine leather, r^2 = 0.0077 and P = 0.75 (for slope). For other animals, r^2 = 0.080 and P = 0.46 (for slope). Each point is the average value from 12−20 diffraction patterns.

Collagen fibrils produced *in vitro* from cow skin, measured with TEM, were found to have a diameter of 67 nm.[42] These studies also reported that collagen fibrils from sheep have a diameter slightly larger than that of collagen fibrils from cattle, which is consistent with our findings.

However, other reports give quite different values for fibril diameters in skin and tendon: 202−204 nm in diameter (ovine tendon, measured using TEM)[30] and 142−163 nm in diameter (bovine skin, measured using TEM).[43] As mentioned in the

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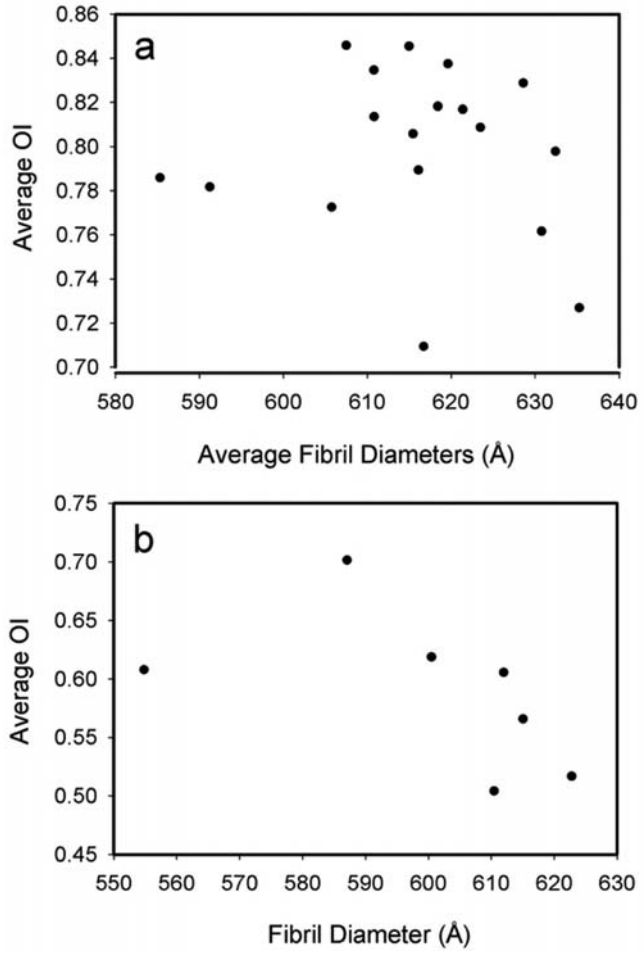

Figure 5. Orientation index vs fibril diameter for (a) ovine and b) bovine leather, showing a lack of correlation between these properties. For ovine leather, r^2 = 0.011 and P = 0.6747 (for slope). For bovine leather, r^2 = 0.28 and P = 0.22 (for slope).

Introduction, diameters determined by TEM can vary greatly depending on the sample preparation procedures and fixation method.[31]

We find that in some parts of the cross sections (not shown) of some samples, particularly the ovine leather, there is a bimodal distribution of fibril diameter. In these cases, we took the modal size (which was the larger fibril diameter). The details of fibril diameter distribution through the cross sections and in different species are complex and could benefit from further study and analysis, particularly in light of the reported importance of a nonuniform fibril size with respect to strength in tendons.[23,25]

Fibril Diameter and Strength. For bovine leather, the collagen fibril diameter was correlated with strength (Figure 4b), and the correlation was statistically significant (P = 0.009). This provides robust and quantitative support for previous studies that relate fibril diameter to strength in a variety of tissues using various methods used to measure fibril diameter and inferring strength. For example, human aortic valves that, like skin, are composed largely of type I collagen show an increased fibril diameter when they have been exposed to high levels of stress.[20] In several studies of tendon, which also consists largely of type I collagen, mechanical loading, which is presumed to result in increased strength, is found to lead to larger diameter collagen fibrils.[21,22] The mechanism that yields larger fibril diameters providing higher strength has been proposed: tendons with larger diameter fibrils have a greater ability to absorb energy and thus are more resilient and resistant to rupture.[25]

By contrast, no correlation was found between fibril diameter and tear strength in ovine leather. It is known that the ovine leather has a less oriented structure than bovine leather (and much less oriented than tendon), and this contributes to its lower strength.[16,44] The lower OI suggests that any increase in the extent of alignment can have a significant effect on strength. This is in contrast to the more aligned structure of bovine leather, suggesting that OI as a determinant of strength is dominant in ovine leather. The difference in the spread of age of the ovine and bovine animals, as discussed later, could perhaps have contributed to these differences. Other factors that may contribute and were not explicitly considered in this study are the breed, the condition of the animal, and variations in processing conditions.

Comparison with Tendon. One of the important differences between skin and tendon in the arrangement of collagen is that the collagen fibrils are less aligned in skin than in tendon. The very strong relationship between fibril alignment and strength in skin, if extended to tendon, could to a large extent explain the high strength of tendon. In this study, the different degree of alignment between collagen in tendon and leather suggests that small increases in the extent of alignment in skin have significant consequences for strength but are less important in the already highly aligned tendon. Therefore, for tendon, where changes in fibril alignment are not significant, the effect of fibril diameter on strength becomes dominant. In contrast, for skin and leather, the extent of fibril alignment is much lower than in tendon, changes in alignment such as those found between different skins dominate, and changes in fibril diameter are less significant.

Interspecies Comparisons. While we see that there is a correlation with fibril diameter within the bovine group of samples, and there is not a correlation within the ovine group, we can also see that the relationship between fibril diameter and strength does not extend to interspecies comparison (Figure 4a–c). Bovine leather is stronger, yet ovine leather has the thicker fibrils. The strength differences have previously been shown to be related to fibril orientation, which is different in the two animals.[16] The lack of correlation between fibril diameter and strength across species leads us to conclude that comparisons of fibril diameters are valid only within a species and not between species.

Age. The age of the animal may also contribute to skin strength, as it has been shown previously that tendon strength and collagen fibril diameter increase with age;[21,23,25] this may also apply to leather produced from skin. However, we did not explicitly factor age into the experimental design. The ovine leather was from young animals (5 months old) in a narrow age range (a few days), and the bovine leather was from older animals (2–3 years old) in a slighter broader age range (1 year). It is possible that the broader age range of animals supplying the bovine leather was a factor in the variation in the observed strength and the correlation with fibril diameter. It may therefore have contributed to the lack of correlation between fibril diameter and strength in ovine leather in contrast to the correlation observed for bovine leather. However, this is purely speculation, and the effect of age on fibril diameter and tear strength merits further investigation.

Cross-Linking. Cross-linking in tendon and pericardium has been shown to affect structure[19] and probably influence strength,[11,14,15] although this is not universally believed.[12,13] Therefore, the amount or nature of cross-linking could influence strength in leather. Because this cross-linking is between fibrils, the fibril diameter might influence the amount and effect of cross-linking and therefore the effect of cross-linking on strength. We did not explicitly consider variations in cross-linking in our experimental design.

Other Factors Affecting Fibril Diameter. It might be expected that genetic and environmental factors may also contribute to fibril diameter such as the breed of the animal and the condition of the animal (the amount of fat,[22] type of feed, and level of exercise).

Orientation Index and Fibril Diameter. It had previously been reported that there is a strong correlation between leather strength and fibril orientation.[1,16] It was therefore necessary to test whether the observed correlation between fibril diameter and strength for bovine leather was merely due to a correlation of fibril diameter with OI rather than a causal relationship between diameter and strength. We found that these two properties are not interdependent, with no correlation found between the OI and fibril diameter for bovine leather (Figure 5b). Therefore, we cannot dismiss the correlation we have observed between fibril diameter and strength in bovine leather as being simply a cross correlation with OI.

Bundle Size. Collagen fibrils form into bundles that include several tens or hundreds of fibrils (Figure 1). It would be interesting to see if there was a relationship between fibril bundle size and strength. Just as a highly braided, multistrand rope has a high strength compared with the strength of a loose collection of fibers, well-defined and thick fibril bundles, held together with covalent cross-links or multiple hydrogen bonds or hydrophobic interactions, could have increased strength. We did not collect data to sufficiently low Q to be able to confidently determine fibril bundle sizes, but we will be pursuing this in future studies.

Packing Density. It has been suggested that the fibril volume fraction could be a determinant of tissue strength.[45,46] Two factors that may influence this are fibril diameter and fibril size distribution. The claim that larger fibril diameters allow higher packing densities has been made,[45] with one experimental observation on age series for mouse tendon weakly supporting this.[46] However, geometric considerations indicate that the opposite should be true,[47] with the packing of circles in a circle resulting in a general increase in packing density as the number of circles is increased (equivalent to smaller fibril cross sections in a bundle). Fibril size alone leading to denser packing does not therefore provide an explanation for the experimental observations reported here. However, if the collagen fibrils are not of uniform diameter, then increased packing density is possible, and therefore, the volume fraction of collagen in a bundle increases,[45,46] which could be expected to influence strength. We have not investigated fibril packing density or different size distributions in detail, but the techniques reported here can be applied to study this further and form part of the ongoing research program.

Conclusions. We studied the relationship between tear strength and fibril diameter in ovine and bovine leather and leathers of a range of other animals. We found that there is a correlation between strength and fibril diameter in bovine leather. For ovine leather, however, we did not find a correlation of fibril diameter with strength. In bovine leather, the collagen fibrils are more aligned than in ovine skin, while tendon contains even more highly aligned fibrils. We conclude that where the tissue contains highly aligned fibrils, the fibril diameter becomes a significant determinant of strength. In tissues where the fibrils are not well aligned, the influence of fibril alignment on strength is greater than that of fibril diameter. Therefore, in leather and skin, larger fibrils may lead to stronger material, but for weaker leathers, fibril diameter is secondary to fibril alignment for strength. An interspecies assessment showed that it is not possible to make inferences about strength from interspecific comparisons of fibril diameter. Tissues composed of collagen have complex structures with many different aspects of the structure contributing to the mechanical properties. We have shown how one aspect of this structure may contribute in some types of leather and that these principles may be extended to other tissue types.

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