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Collagen Fibril Orientation in Ovine and Bovine Leather Affects Strength: A Small Angle X-ray Scattering (SAXS) Study

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Supporting Information

ABSTRACT: There is a large difference in strength between ovine and bovine leather. The structure and arrangement of fibrous collagen in leather and the relationship between collagen structure and leather strength has until now been poorly understood. Synchrotron based SAXS is used to characterize the fibrous collagen structure in a series of ovine and bovine leathers and to relate it to tear strength. SAXS gives quantitative information on the amount of fibrous collagen, the orientation (direction and spread) of the collagen microfibrils, and the *d*-spacing of the collagen. The amount of collagen varies through the thickness of the leather from the grain to the corium, with a greater concentration of crystalline collagen measured toward the corium side. The orientation index (OI) is correlated strongly with strength in ovine leather and between ovine and bovine leathers. Stronger leather has the fibrils arranged mostly parallel to the plane of the leather surface (high OI), while weaker leather has more out-of-plane fibrils (low OI). With the measurement taken parallel to the animal's backbone, weak (19.9 N/mm) ovine leather has an OI of 0.422 (0.033), stronger (39.5 N/mm) ovine leather has an OI of 0.452 (0.033), and bovine leather with a strength of (61.5 N/mm) has an OI of 0.493 (0.016). The *d*-spacing profile through leather thickness also varies according to leather strength, with little variation being detected in weak ovine leather (average = 64.3 (0.5) nm), but with strong ovine leather and bovine leather (which is even stronger) exhibiting a dip in *d*-spacing (from 64.5 nm at the edges dropping to 62 nm in the center). This work provides a clear understanding of a nanostructural characteristic of ovine and bovine leather that leads to differences in strength.

KEYWORDS: leather, small angle X-ray scattering, SAXS, synchrotron, ovine, bovine, fiber structure, collagen, strength, orientation

INTRODUCTION

Ovine leather is traditionally a low-value product owing in part to its low strength relative to bovine leather. This low strength makes ovine leather unsuitable for footwear production, which is a major high-value use for leather. Whereas 4.6 billion pairs of footwear are made annually from bovine leather, no significant quantity of footwear is produced from sheep and goat leather.[1] Export trade in leather and footwear amounts to about $US 60 billion annually, with around 38% of bovine and 25% of ovine leather traded internationally. However, the raw material resource of ovine skins is large, with world annual production of sheep and goat leather being 5.3 billion square feet from 1.9 billion head of livestock (1.1 billion sheep, 0.8 billion goats), compared to 14 billion square feet of bovine leather from 1.5 billion head of livestock.[1] Clearly, the opportunity for shoe manufacture from ovine leather, were the leather sufficiently strong, is large.

It is not understood why ovine leather is weaker than bovine leather of the same thickness. However, it is clear that leather consists of a network of fibers based on collagens, a group of proteins that are responsible for the structure and physical properties of skin (and therefore leather) and several other animal tissues.[2] Leather structure is complex on several levels, including the microscopic scale as determined by the use of optical microscopy, scanning electron microscopy (SEM),[3−7] and atomic force microscopy (AFM).[6,8,9] These techniques generally provide qualitative information about fiber organization but may also give quantitative measurements.

The building blocks of collagen are pro-collagen molecules, each one a repeating sequence of $(\text{glycine-X-Y})_n$, where X and Y can be any amino acid, and n is the number of repeats (usually 100−400).[10] Three pro-collagen molecules that have combined in a coiled coil are a collagen molecule. Several collagen molecules aligned in a quarter-staggered array form a collagen microfibril, which has overlap and gap regions. Collections of microfibrils arranged in a parallel fashion, and connected by chemical cross-links, form collagen fibrils. These fibrils have characteristic banding patterns (observed with AFM or SEM) that result from the overlap and gap regions in the microfibrils comprising them.

Collagen fibrils can vary considerably among tissues. In skin, and therefore leather, the fibrils tend to vary only slightly in diameter and they form a somewhat random weblike structure.[11] The strength and softness of leather are believed to be related to a leather's internal structure[12,13] and its aesthetic properties to the internal fiber looseness.[14] In the leather-making process, it has been found that many collagens, in particular type I, III and VI collagens, are resistant to liming, bating and pickling[15] (skin/leather consists mostly of types 1 and III), and these collagen fibrils have a large inherent strength.

At a larger scale, fiber structure is responsible for the several distinct layers of leather. The outer "grain" layer and the "corium" layer found beneath it have visually different structures and impart specific properties to leather.[12,16,17] The cross-links (either natural

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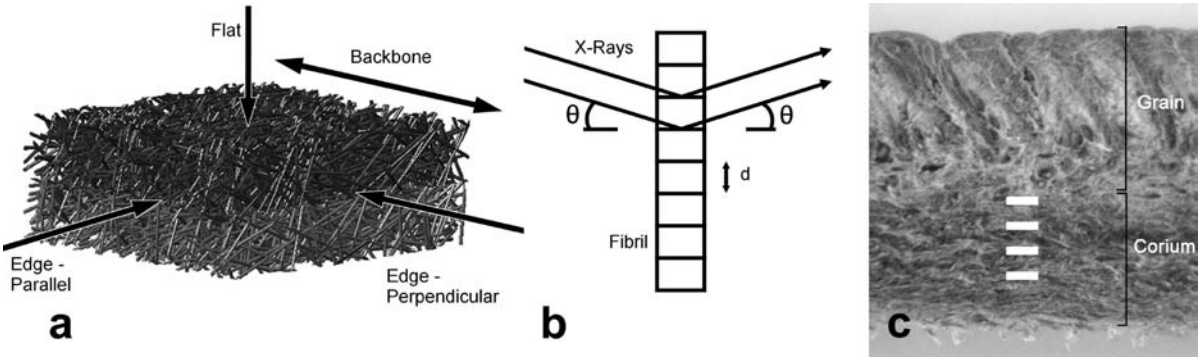

Figure 1. (a) Directions of X-ray beam with descriptors used in the text shown relative to the direction of the animal's backbone. (b) Interaction of X-rays with collagen fibril *d*-spacing. (c) Optical image of a cross section of ovine leather as seen by the SAXS X-ray beam. The rectangles indicate the size of the beam used to probe the sample and the approximate spacing.

or synthetic) between microfibrils also contribute to the differences observed between layers.[18−21]

In our earlier work, we showed how small-angle X-ray scattering (SAXS) investigations of leather can provide detailed structural information on the amount of fibrous collagen, the microfibril orientation, and the *d*-spacing.[22] Leather had previously been investigated using SAXS to identify fiber orientation in leather samples under strain,[23,24] although in these studies, the resolution appears to be rather limited (the collagen *d*-period is not easily discerned in the diffraction patterns). SAXS has also been used to investigate the structure of collagenous materials such as those found in tendons,[25,26] bone,[27,28] ligaments,[29] human articular cartliages,[30] breast tissue,[31] mitral valve leaflets[32] and materials for tissue regeneration scaffolds.[33]

Here, we report a study of the structure of ovine and bovine leather in which we attempted to understand the factors that contribute to the strength of leather and which account for the differences between strong and weak leather.

EXPERIMENTAL SECTION

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

Leather was prepared from around 600 pelts. These leathers were generated with a variety of properties by deliberately using a range of processing parameters both during the conventional beamhouse process and then during the application of the conventional tanning processes to those pelts. Briefly, the pelts were depilated using a caustic treatment comprising sodium sulfide ranging from a slow acting paint containing 160 g/L flake sodium sulfide to a quick acting paint containing 200 g/L sulfide and a saturated solution of calcium hydroxide. Depilated slats were then processed to remove the residual wool in a solution of sodium sulfide ranging in concentration from 0.8 to 2.4% for a period ranging from 8 to 16 h at temperatures ranging from 16 to 24 °C. After this treatment the pelts were washed and treated to a proteolytic enzyme process containing either a bacterial enzyme (Tanzyme, Tryptec Biochemicals Ltd.) or pancreatic enzyme (Rohapon ANZ, Shamrock Ltd.) at concentrations ranging from 0.025% to 0.1%, followed, after washing, by pickling in 2% sulfuric acid and 10% sodium chloride solution. The pickled pelts were then pretanned using oxazolidine, degreased with an aqueous surfactant and then tanned using chromium sulfate. The resulting "wet blue" was then retanned using a mimosa vegetable extract and impregnated with lubricating oil prior to drying and mechanical softening.

Tear strengths of the crust leathers were tested using standard methods.[34] In brief, samples were cut from the leather at the official sampling positions (OSP).[35] The samples were then conditioned by holding at a constant temperature and humidity (20 °C and 65% relative humidity) for 24 h, after which time the samples were tested on an Instron strength testing device.

A selection of the 600 leather samples that had been prepared was chosen for the SAXS analysis, with a representative collection of strong and weak leathers. Sample preparation for SAXS analysis began by cutting strips of leather about 1 × 30 mm from the OSP from samples of tanned ovine and bovine leather. The bovine leather had been shaved, resulting in approximately 1.3-mm-thick samples consisting on average of 34% grain and 66% corium. Strips of leather were cut in two perpendicular directions. Each sample was mounted, without tension, in the X-ray beam to obtain spectra for each of three orthogonal directions through the leather (1). For the edge-on analyses (with strips cut in two perpendicular directions) the samples were analyzed from the grain to the corium, with measurements being made every either 0.25 mm or 0.2 mm (ca. 10 measurements in all, per edge). For when the beam was directed flat on (normal to) the surface of the leather, standard samples were cut parallel to the surface, producing a grain sample and a corium sample. These were mounted with the uncut face of the leather directed toward the X-ray beam (ca. four measurements made per sample, in a rectangular grid).

Diffraction patterns were recorded on the Australian Synchrotron SAXS/WAXS beamline, utilizing a high-intensity undulator source. Energy resolution of 10^{-4} is obtained from a cryocooled Si(111) double-crystal monochromator, and the beam size (fwhm focused at the sample) was 250 × 80 μm, with a total photon flux of about 2 × 10^{12} photons·s^{-1}. All diffraction patterns were recorded with an X-ray energy of either 8 or 11 keV using a Pilatus 1 M detector with an active area of 170 × 170 mm and a sample to detector distance of 3371 mm. Exposure time for diffraction patterns was 1 s, and data processing was carried out using the SAXS15ID software.[36]

The analysis of the spectra has been detailed previously,[22] but some key points are described here. The *d*-spacing was determined for each spectrum from Bragg's law by taking the central position of several of the collagen peaks, correcting these for the peak order (usually from n = 5 to n = 10) and averaging the resulting values.

The orientation index (OI) is defined as (90° − OA)/90° where OA is the minimum azimuthal angle range, centered at 180°, that contains 50% of the microfibrils. OI is used to give a measure of the spread of microfibril orientation (an OI of 1 indicates the microfibrils are completely parallel to each other and the leather surface; an OI of 0 indicates the microfibrils are completely randomly oriented). The OI is calculated from the spread in azimuthal angle of the most intense *d*-spacing peak (at around 0.059−0.060 $Å^{-1}$). Note that this differs from our previous use of the term orientation index in this context[22,33] where we had used the azimuthal angle range rather than this true index.

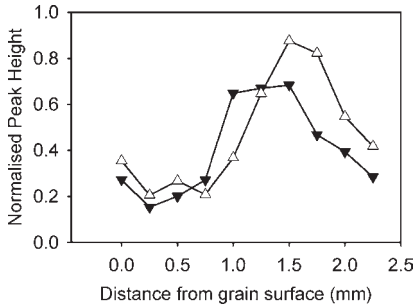

Figure 2. Amount of collagen through the thickness of ovine leather parallel to the backbone (averages of 12 samples each): (▼) strong leather; (△) weak leather.

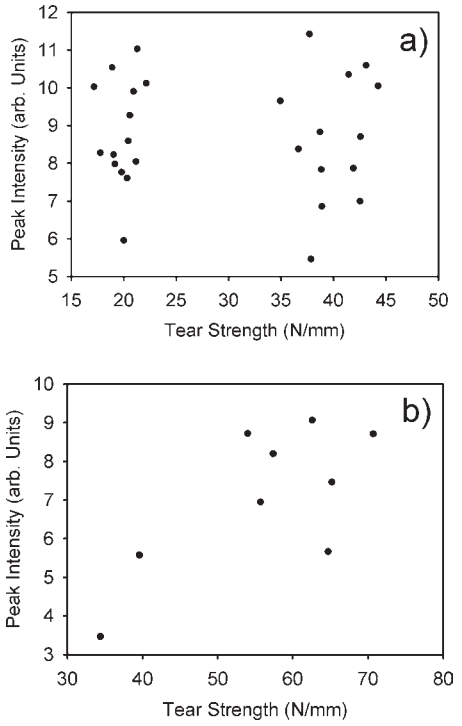

Figure 3. Amount of collagen determined from the intensity of the primary collagen *d*-spacing peak (at around 0.059−0.060 $Å^{-1}$) versus tear strength for (a) ovine leather; (b) bovine leather.

The amount of collagen was estimated by taking the area above the baseline of the fifth-order collagen *d*-spacing peak divided by the intensity of baseline under the peak.

For the plots of OI and *d*-spacing, each point on a plot represents the average of the four (normal) or about 10 (edge) measurements from different points on the sample (see above). For the plots of the amount of collagen (i.e., peak intensity), each point on the plot represents the average of measurements taken in the three orthogonal directions (which are in turn averages of four or 10 measurements as for the OI and *d*-spacing).

RESULTS AND DISCUSSION

Variation in Tear Strength of Leathers. A selection of leathers was chosen from those tested to have a representative sample of ovine leather of lower strength (17−22 N/mm), ovine leather of higher strength (35−44 N/mm) and bovine leather in the 32−71 N/mm strength range (although most of the last was predominantly in the upper end of this range). A list of tested samples is tabulated in the Supporting Information, and includes a sample's absolute strength, its thickness, and its relative strength (tear strength divided by thickness, which is the strength measurement used throughout this work).

Concentration of Crystalline Fibrillar Collagen. The amount of crystalline collagen varied through the thickness of ovine leather, with a greater concentration being measured toward the corium side (2). The corium is known to impart more strength to the leather than the grain,[16] and the above finding—of three or four times more fibrillar collagen in the corium region than in the grain region—may be part of the reason for this extra strength. However, the total amount of crystalline collagen in leather samples was not correlated with strength (Figure 3) (instead, strength was correlated with microfibril orientation; see below). An average value of about 8.8 was found for collagen, with no variation with strength. This finding was rather surprising as it is generally believed collagen is the structural material that contributes most to the strength of skin.

Orientation of Collagen Microfibrils Correlated with Tear Strength. The orientation and OI of the fibrils in leather are different when observed from different directions (Figure 4), making up a complex three-dimensional structure. Statistically significant differences in OI were observed between weak and strong ovine leather. Specifically, for ovine leather measured edge-on, the OI values were higher for the higher strength ovine leather. For ovine edge-parallel measurements, the average OI was 0.422 (variance = 0.033) for low strength (19.9 N/mm) ovine leather, which was significantly less than for high strength (39.5 N/mm) ovine leather (0.452 (0.033)) (*t*-stat = −2.06, *t*-crit =2.00, $P = 0.044$). Likewise, the OI of edge-perpendicular measurements of low-strength ovine leather was significantly less than of the corresponding high-strength leather (i.e., 0.578 (0.025) and 0.630 (0.016) respectively with *t*-stat = −3.21, *t*-crit = 2.01, $P = 0.0023$). These findings reveal that stronger ovine leather was characterized by having more collagen microfibrils that were in one plane (i.e., parallel to the surface), with fewer crossing between layers of the leather (referred to in the industry as a lower "angle of weave"). With the X-ray beam normal to the surface of the leather, the OI of ovine leather was around 0.248 (0.016) for grain and 0.329 (0.010) for corium, and it did not vary with strength (Figure 4c). In contrast, similar analysis of bovine leather yielded an average OI of 0.184 (0.026) in the grain and 0.364 (0.017) in the corium (Figure 4d). The microfibrils were aligned in the direction approximately parallel to the backbone of the animal (not shown). These OIs represent a moderate degree of alignment of the fibers in the direction of the backbone, with greater alignment in the corium in both ovine and bovine leather.

As for bovine leather, the OI measurements taken normal to the bovine leather surface were not strongly correlated with tear strength (Figure 4d), however, the greater alignment in the bovine corium is consistent with other observations described below. According to pooled edge-on measurements of bovine leather, strong bovine leather (61.5 N/mm) had a higher OI than strong ovine leather, of 0.493 (0.016) (*t*-stat = −2.20, *t*-crit = 2.01, $P = 0.03$) (Figure 5). There was not a strong trend visible within the limited bovine data (Figure 4b,d,f), however it is clear that the bovine leather was more aligned in one plane than was the ovine leather. This result, combined with the trend seen within the ovine leather, leads us to propose that fibril orientation is a determiner of strength in leather (and possibly, by analogy,

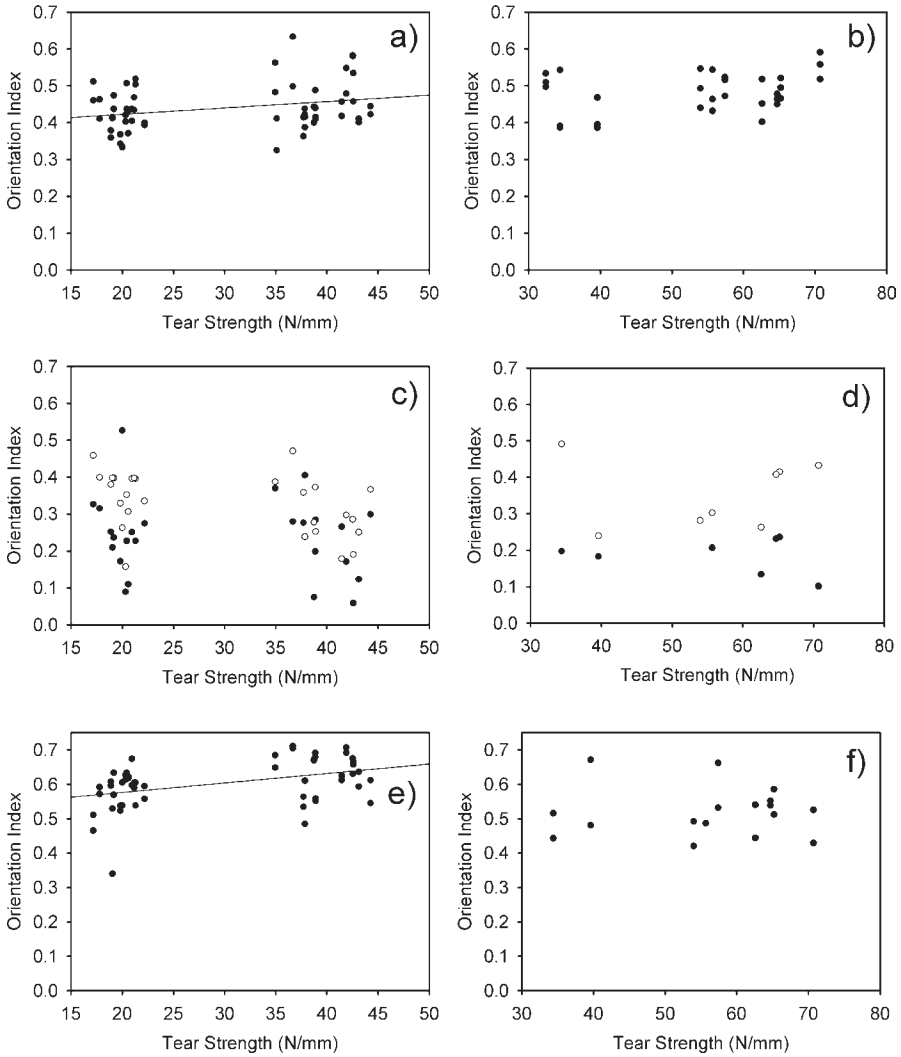

Figure 4. Orientation index versus tear strength for OSP samples of leather: (a) ovine, edge, parallel to backbone; (b) bovine, edge, parallel to backbone; (c) ovine, flat, (●) grain, (○) corium; (d) bovine, flat, (●) grain, (○) corium; (e) ovine, edge, perpendicular to backbone; (f) bovine, edge, perpendicular to backbone. A higher OI indicates a greater degree of fiber alignment.

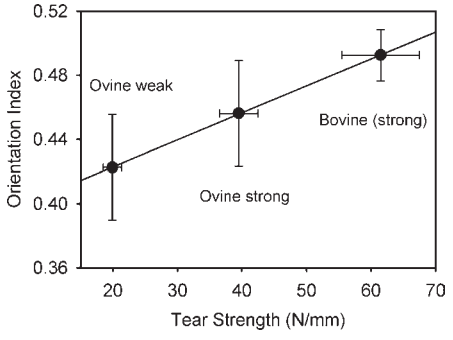

Figure 5. Orientation versus tear strength for the averages of each of the leather types measured through the edge parallel to the backbone. Error bars for one standard deviation.

in other tissues), and is not merely correlated with it. This is a key finding of this work.

A statistically significant difference was observed between ovine leather of low and high tear strength and collagen microfibril OI for edge-on measurements, with higher strength leather having a higher OI (Figure 4a, Figure 5). A similar correlation was found when the measurements for bovine leather were included in the data set. A regression line fitted to the averages of the three groups of data for OI and tear strength (low strength ovine, 15 samples, 228 analysis points; higher strength ovine, 14 samples, 249 analysis points; and bovine, 10 samples, 167 analysis points) has a slope of 1.708×10^{-3} mm/N, $r^2 = 0.20$, $p = 4.45 \times 10^{-5}$ (Figure 5). The relationship between OI and tear strength documented within species (i.e., sheep) is maintained between species (i.e., sheep and cattle). This finding points to a universal property of leather: that strength is determined by fibril orientation, such that stronger leather has the fibrils arranged mostly parallel to the plane of the leather surface (low angle of weave), while weaker leather has more out-of-plane fibrils (higher angle of weave) (Figure 6). It is possible that this relationship may extend to a large number of animal leathers. Work is currently underway to investigate this further.

There is likely to be a upper limit for OI because, with all fibers parallel, there might be little mechanical connection between the

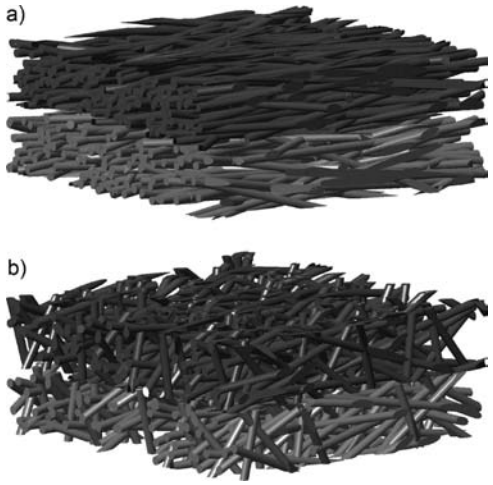

Figure 6. Sketch of fiber orientation in (a) stronger and (b) weaker leather. In the stronger leather the angle of weave is smaller (the fibers are contained more in planes parallel to the leather surface). The orientation change is exaggerated to better illustrate the difference.

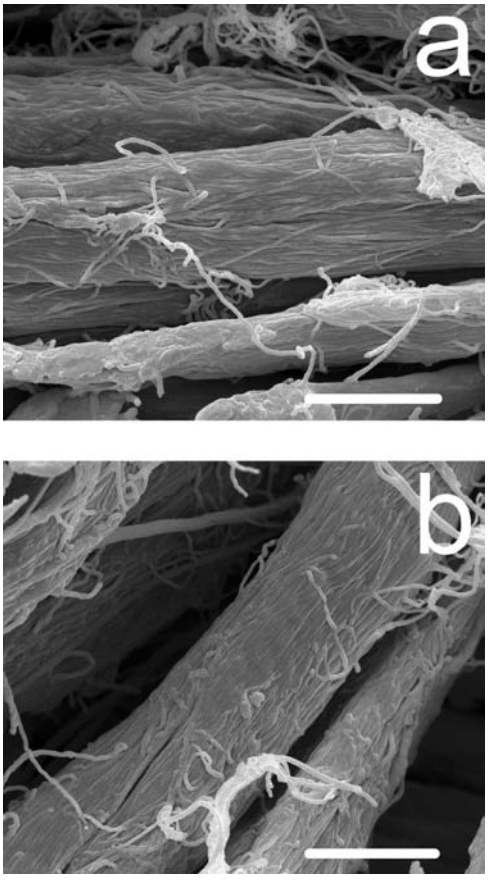

Figure 7. Representative SEM images of sections of leather observed parallel to the backbone: (a) weak ovine; (b) strong ovine. Scale bars 10 μm.

layers and delamination might be more likely. Other factors clearly are also important as the OI versus tear strength data have scatter.

SEM Images of Fibers and Crimp. We considered the possibility that spread of orientation we measure with SAXS may have a contribution from what is known as crimp, which consists

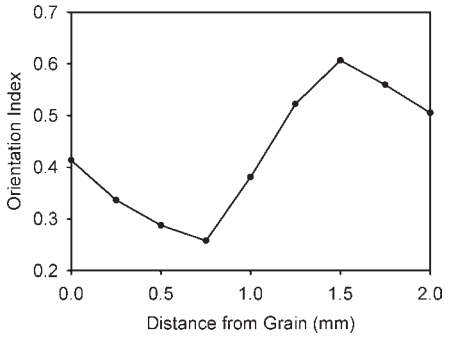

Figure 8. Average orientation index across the thickness of ovine leather measured parallel to the backbone. An average of 24 leather samples (1 measurement per sample).

of a wavy texture to the fibers with a period of several micrometers. One might imagine that increased crimp could result in a larger OI since the fibers and therefore the fibrils are tilted through a range of directions as they bend. We therefore used SEM to investigate ovine and bovine leathers with a range of OI determined by SAXS (Figure 7). The SEM images show no crimp for the ovine samples but some crimp in the bovine sample. Therefore if crimp were a significant contributor to the OI, then the trend on OI with strength would likely be in the opposite direction to that which we observe. We therefore believe that crimp is not a significant contributor to the measured OI and our observations on the correlation of OI to strength relate to overall fiber orientation rather than any effect of crimp.

Orientation of Collagen Microfibrils across the Thickness of Leather. Collagen microfibril alignment was found to vary considerably through the thickness of both ovine and bovine leather. Measured flat (normal to the surface) ovine leather is more aligned in the corium OI = 0.329 than the grain OI = 0.248 (t-stat = −3.7, t-crit = 2.1, P = 0.001) (Figure 4c); bovine leather is also more aligned in the corium OI = 0.364 than the grain OI = 0.184 (t-stat = −4.7, t-crit = 2.45, P = 0.003) (Figure 4d). The orientation was also measured edge-on parallel to the backbone with a series of points taken across the thickness of the leather (Figure 8). For ovine leather, the microfibrils were poorly aligned with each other edge-on in the grain (OI = 0.29−0.41), compared to the corium (OI = 0.50−0.61) (for ovine leather: t-stat = −12.9, t-crit = 2.05, $P = 2 \times 10^{-13}$). If, as suggested by our findings, leather strength depends on a high level of alignment of fibers within the plane of the leather, then these OIs suggest that the corium should be stronger than the grain in the ovine leather, as has been recognized by others.[16]

Internal Structure (d-Spacing) of Collagen Microfibrils Correlated with Tear Strength. Because of the nature of the molecular overlap in the collagen structure, the d-spacing gives a measure of the sum of the length of the collagen molecule plus the gap between two end-aligned molecules. Therefore, a difference in d-spacing indicates either a difference in the length of the molecules that make up the microfibrils or a difference in the amount of overlap of those molecules (less overlap means a longer d-spacing). d-Spacing varies with leather age.[37]

The d-spacing of ovine leather (64.3 (0.5) nm) differed significantly different from that of bovine leather (63.5 (0.9) nm) (t-stat = −11.58, t-crit = 2.04, $P = 1.34 \times 10^{-12}$).

For ovine edge-on measurements, there was no significant difference in the d-spacing between the weak and the stronger leathers (Figure 9). However, for normal ovine measurements of

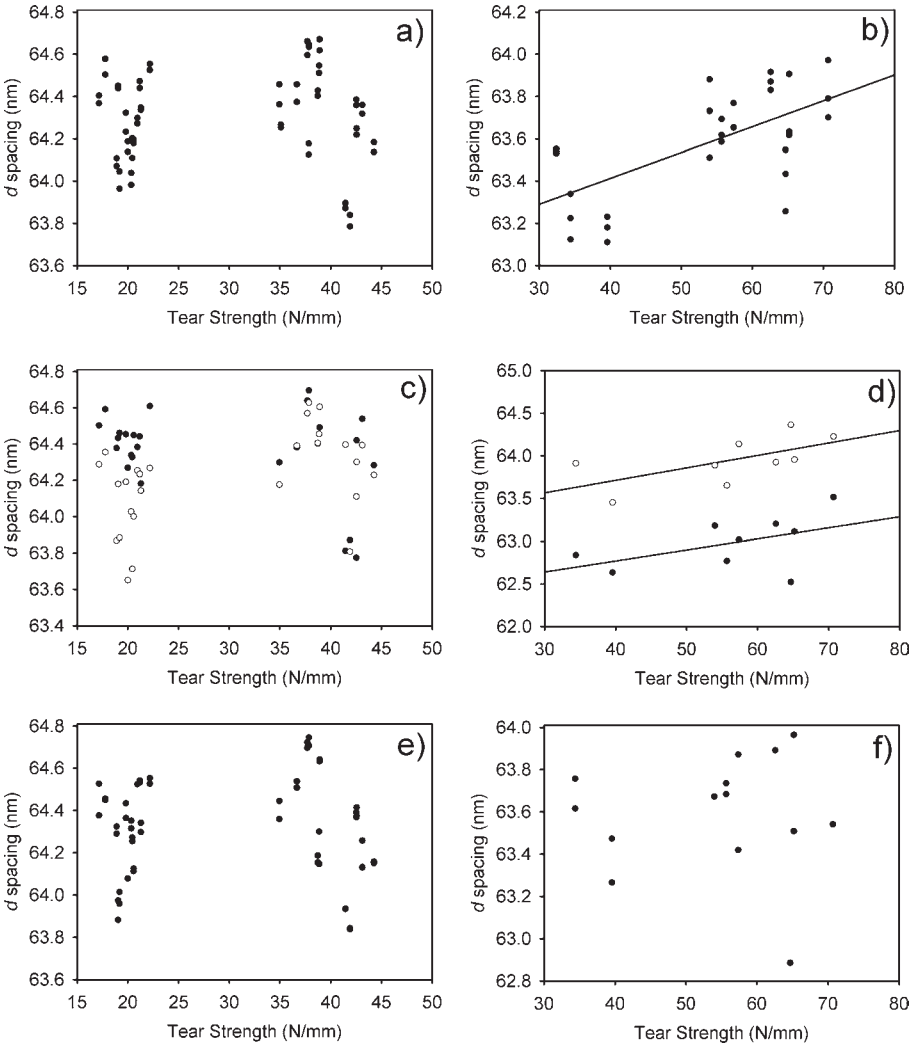

Figure 9. *d*-Spacing verses tear strength for (a) ovine, edge, parallel to backbone; (b) bovine, edge, parallel to backbone; (c) ovine, flat, (●) grain, (○) corium; (d) bovine, flat, (●) grain, (○) corium; (e) ovine, edge, perpendicular to backbone; (f) bovine, edge, perpendicular to backbone.

the weak leather, the *d*-spacing of the corium (64.1 nm) was significantly lower than that of the grain (64.4 nm) (*t*-stat = −5.08, *t*-crit = 2.06, $P = 2.75 \times 10^{-5}$). For the stronger ovine leather, the observed difference was not significant.

For bovine leather measured edge-on parallel, there was a consistent increase in *d*-spacing with increasing strength (least-squares fit slope = 0.014 nm·mm/N, $r^2 = 0.53$, $P = 2.98 \times 10^{-4}$), although the number of samples measured was fewer than for the ovine leather. For the normal bovine measurements, the corium had a larger *d*-spacing (63.9 (0.4) nm) than the grain (63.0 (0.3) nm) (*t*-stat = −6.91, *t*-crit = 2.12, $P = 3.49 \times 10^{-6}$), but bovine values were less than most ovine values. Least squares fits to the bovine data gave the following: for corium, slope = 0.015 nm·mm/N, $r^2 = 0.40$ and $P = 0.07$, and, for the grain, slope = 0.013 nm·mm/N, $r^2 = 0.25$ and $P = 0.17$ (this last suggesting that this correlation in the grain is not particularly significant). However, the changes in *d*-spacing across the bovine samples were not large compared to the differences between bovine and ovine leather.

There are advantages to calculating average *d*-spacing for each piece of leather, despite the fact that it obscures variation in leather across its thickness. Although each skin gave a different profile of

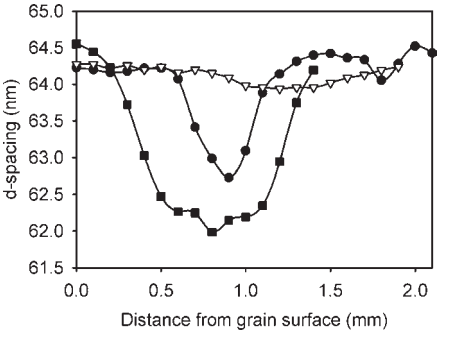

Figure 10. Variation in *d*-spacing through representative samples of (▽) weak ovine, (●) strong ovine and (■) bovine leathers.

d-spacing through the leather, a fairly consistent difference was observed between weak ovine, strong ovine and bovine leathers (Figure 10). Weaker leather had less variation in *d*-spacing compared to stronger leather, which had a dip of up to 3 nm in *d*-spacing in the central region of the leather. These differences in *d*-spacing may indicate differences in protein composition or fibril structure.

Our current research indicates that *d*-spacing responds to tensile stress applied to leather and gives a measure of the force applied to individual fibrils. We will present results from stress measurements on OI and *d*-spacing in leather in a subsequent paper.

The observed differences in *d*-spacing between ovine and bovine leathers cannot yet be ascribed to structures controlling leather strength. However, we expect that current tensile studies on these materials will provide further insight into the mechanisms of strength in collagen-based materials.

In summary, a correlation has been found between the tear strength in ovine leather and the orientation of collagen microfibrils in that leather. Stronger leather was found to have more fibrils aligned with the surface of the leather, with less crossover between layers, than weak leather (i.e., strong leather had a lower angle of weave). This trend was also apparent in bovine leather, which is stronger than ovine leather, with bovine leather having even more aligned fibrils. The total quantity of crystalline collagen was found, surprisingly, to not be correlated with strength. The *d*-spacing of bovine and ovine leather was found to be variable, reflecting differences in the molecular structure of collagen. A difference in the profile of the *d*-spacing was found between weak and strong ovine leather and between these leathers and bovine leather. We have not yet been able to ascribe these differences in *d*-spacing to structures that control leather strength. The discovery of the relationship between collagen microfibril alignment and leather tear strength has provided a clear understanding of a basis of the differences in strength between different leather types. We speculate that this relationship is a general one, which applies not only to skin of sheep and cattle but to skin of other animals also, including humans, and that this relationship may extend to other tissues.

■ ASSOCIATED CONTENT

Supporting Information. Table of tested leather samples and their absolute strength, thickness, and relative strength. This material is available free of charge via the Internet at http://pubs.acs.org.

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