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Stiffness as a function of moisture content in natural materials: Characterisation of hoof horn samples

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Hoof horn, which forms the capsule at the lower part of the legs of many grazing animals including equids (horses, donkeys and mules), is a composite natural material based on α-keratin. Its function is influenced by the tubular and intertubular material and is modulated by the moisture content. There is a requirement to adopt a standard approach to drying regimes and sampling protocols in order to make progress in understanding how the biomechanical properties of hoof horn are related to its structure. In this work the stiffness of donkey hoof has been examined using a three point bending technique and the effect of hydration has been investigated. Also the tubule density properties of this hoof horn material are reported. © *1998 Kluwer Academic Publishers*

1. Introduction

The study of hoof horn provides materials scientists with a challenging insight into natural materials, but why approach the study of hoof horn from the materials science base? Hoof horn may be described as a naturally occurring composite material which includes keratin arranged in a tubular and intertubular form [1]. Its biofunctional role is not yet completely understood. Each tubule consists of a medulla (possibly hollow) which is surrounded by a cortex of keratinised cells [2]. Thus materials scientists may think of hoof horn as a mixed morphology material whose mechanical properties are very dependent on the structure [3]. The properties of hoof horn are of potential interest to conservators and restorers, but much more importantly to those concerned with animal husbandry and welfare. Hoof management has tended historically to be a skill rather than a science and consequently there is a paucity of information concerning structure-property relationships in the scientific and veterinary literature. The need to assess critically the scientific information that has been reported is highlighted in a recent review by Reilly [3] who comments also on such issues as the inadequate numbers in sample populations together with a lack of funding for focussed research.

Materials science provides a platform for investigating the effects of environment, nutrition and management on hoof horn structure and aspects of genetic influences can be included by careful experimental design. Is equine hoof different from bovine hoof in its structural aspects; do other equids such as donkey have the same hoof horn structure as horse? In order to answer these and other related questions it is important to generate a sound knowledge base of hoof horn characteristics for different species based on controlled trial situations [3], and in this respect materials scientists have a role to play alongside veterinary practitioners and biochemists [4]. Baillie and Fiford [5], for example, have analysed hoof structure-property relationships in order to understand the nature of cattle hoof horn as a material. Animals are reliant on the hoof horn capsule for effective locomotion and the mechanical properties of hoof horn are therefore important [1]. The mechanical properties of hoof horn and other biomaterials can be routinely investigated by using tensile, compression and hardness testing methods and this is recognised in the materials science approach to biomechanics outlined by Vincent [6], and Fraser and Macrae [7]. Hoof horn is designed to cope with different types of natural impact and an understanding of its structure-property relationships will facilitate in biomimetic design as envisaged by Vincent [6]. For example, measurement of stiffness of horn sheath keratin by three point bending experiments have been reported by Kitchener and Vincent [8], and Kitchener [9]. Stiffness, which is the resistance to deformation, can be measured by the deformation of a material in uniaxial tension or compression using strain gauges or extensometers, and also by bending. Three point bending measurements are of particular relevance to hoof horn material because the technique mimics the *in vivo* situation in the hoof capsule as compression occurs in the

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hoof wall resulting from its reaction with the ground [10, 11].

Examination of samples by various forms of microscopy is also important. For example, the nature of tubules and the intertubular matrix can be examined by scanning electron microscopy (SEM) and elemental mapping by energy dispersive X-ray analysis. The tubular density within the hoof horn matrix is important and it has been shown that there is a zonal arrangement [12] for horse hoof horn and that the type of tubule varies according to position in the hoof wall [11, 13]. Wilkens [14] has described the tubular nature of bovine hoof horn but did not quantify the distribution of tubules. An alternative to the tried and tested staining of microtomed sections followed by manual counting methods is to develop computer imaging techniques.

The properties of keratinous materials are strongly influenced by their state of hydration [8] and it is well established that water will modulate many of the properties of hoof horn. For example, the effect of water on the stiffness and viscoelasticity of horn sheath material has been reported by Kitchener [9]. Similarly, the mechanical properties of hoof horn have been shown to vary with hydration by Bertram and Gosline [15] and it has been reported that the variation in tensile stiffness of hoof keratin with hydration level may be more important than its anisotropy [16]. Definition of moisture content before assessment of material properties is therefore important as the subjective "quality" of the hoof horn and the mechanical function of the material are likely to be influenced by this property. Moisture contents in the range 25–35.5% have been reported but the results depend strongly on the drying method used and the sampling protocol adopted. Moisture content is dependent on relative humidity and the reports in the literature [8, 15] for the results of mechanical testing of biological samples at particular relative humidities suggest an exponential uptake of water with time.

Whilst it is possible to examine morbid hoof samples, the most readily available samples come from clippings associated with routine hoof management which are noninvasive to the animal. The materials science knowledge base needs to be built up from a range of clipped samples which have been collected and stored according to a clearly specified set of protocols, and samples for scientific study must come from defined points on the macrosample. Throughout the scientific and veterinary literature there is a lack of detailed information regarding experimental methodologies and comparative studies are therefore difficult. Weaver [17] reported that the physical properties of hoof horn from cattle are not easy to measure and that difficulties multiply when attempts to make comparisons are hampered by both the lack of standard approaches and by variables such as the selection of test sample from the hoof and the environment of the stock.

There are approximately 41 million donkeys[1] in the world compared with about 65 million horses [18] and despite the fact that there are obvious differences in hoof size and angle of hoof wall [19], and sole thickness [20] there is little reported work on donkey hoof. The Donkey Sanctuary, Sidmouth, UK houses a large donkey population which is generally unshod, from which a representative sample has been identified. This paper reports the initial results for selected techniques from experiments on donkey hoof horn samples.

2. Experimental

2.1. Samples

Suitable clippings of hoof horn from the right foreleg were obtained by farriers during regular hoof maintenance and sharp hoof cutters were used in order to prevent tearing of the sample. The samples were wrapped immediately in three overlapping layers of Parafilm (Parafilm "M" Laboratory Film, American National Can^{TM}, CT 06836, USA) to make an airtight seal which moulded to the shape of the sample. The labelled, wrapped samples were then stored at 4 °C prior to examination. The portions removed for testing were from the midline dead centre site (MDC) [12] as shown in Fig. 1.

2.2. Determination of moisture content and effect of relative humidity

Clippings were obtained from an identified population of 31 donkeys from Sidmouth. The following drying regimes were employed:

- Room temperature
- Drying under vacuum at room temperature
- Placing over phosphorus pentoxide
- Freeze drying
- Oven drying at 90, 100, 105, 110 and 120 °C.

Masses were recorded daily until a constant level was observed and moisture contents were determined as a percentage of the dry mass.

Similar samples were prepared and subjected to different environments having relative humidities in the range 6.5–93% by suspending them over saturated solutions of specified salts. Again, masses were monitored daily until constant and then the samples were dried over phosphorus pentoxide at room temperature.

In an alternative approach to investigating the effect of relative humidity, samples were initially dried over phosphorus pentoxide and then subjected to environments having relative humidities in the range 3.2–99.2% provided by aqueous sulphuric acid solutions.

2.3. Tubule density

Transverse sections (thickness 10–12 μm) from the MDC samples were obtained using a microtome/cryostat at -20 °C. Typically, the sections were stained using haematoxylin, eosin and alcian blue-PAS. A tubule count per unit area to give a tubule density was then carried out on photographs taken of the slides by macrophotography using the principle outlined by Reilly *et al.* [12].

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[1] Horses and donkeys both belong to the generic category of equids but donkeys are physiologically different from horses. Only the latter are equines.

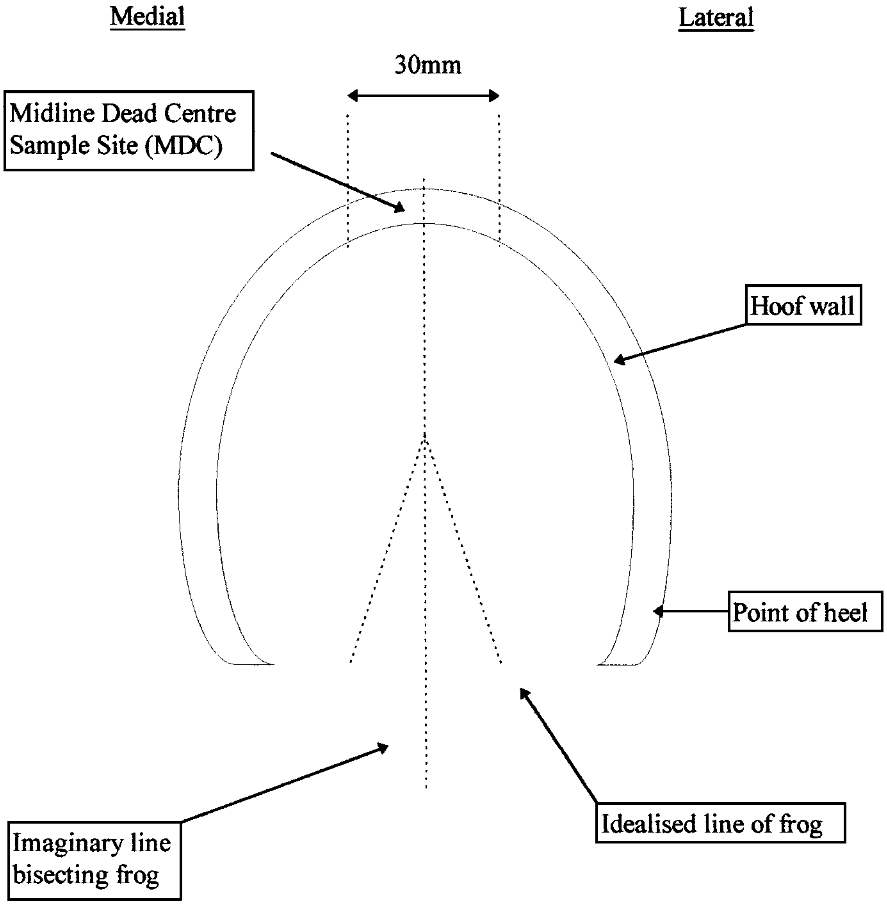

Figure 1 Position of sample sites on clipping (As seen from the underside of the donkey foot. Note to scale).

2.4. Three point bending

In order to avoid possible differences in stiffness due to tubular orientation, samples were taken from the MDC of clippings and milled into beams (hoof wall width × 30 mm × 2 mm) cut perpendicular to the line of the tubules. Samples were subjected to three point bending at 20 °C and 60% relative humidity using an Instron 4302 with a 100 N loadcell and a crosshead speed of 2 mm min^{-1} [6]. Samples with a span of 24 mm were tested to a deflection of 0.5 mm following preloading to ~ 0.04 N in order to minimise the effects of backlash and specimen curvature. Samples were tested as follows: fresh (stored as described previously to prevent loss of water); dried over P_2O_5 to constant mass; fully hydrated (placed in distilled water until constant mass); subjected to an environment of 75% relative humidity (until constant mass).

Compliance for the testing system was determined by carrying out an experiment with a rigid aluminium specimen which had a stiffness greater than that of the load frame. A correction factor was then applied to the results for the stiffness measurements on the hoof horn samples.

3. Results

3.1. Determination of moisture content and effect of relative humidity

Constant sample masses for 31 samples were obtained after about 5 days using freeze drying and 24 hours using oven drying regimes, whereas similar observations were made after about 10 days for room temperature drying and drying over phosphorus pentoxide. Moisture contents were in the range 25–35% and are summarised in Fig. 2. In both approaches the moisture content at room temperature rapidly increased with increasing relative humidity and this is shown in Fig. 3.

3.2. Tubule density

A photograph of a typical stained microtomed section of donkey hoof wall is shown in Fig. 4. For the purposes of comparison with the results for pony hoof previously reported by Reilly [12] the hoof wall was divided into four zones. A mean tubular density of 19 tubules mm^{-2} was obtained for zone 1 of the outer hoof wall with the remaining three zones having 8–9 tubules mm^{-2}. The results are summarised in Table I which includes a comparison with pony hoof.

3.3. Three point bending

The plots of stress vs deflection show a Hookean relationship. Typical stress-deflection plots for samples stored under different environments are shown in Fig. 5. The results for the modulus of elasticity calculated from these plots are summarised for the different storage regimes in Fig. 6. In some cases after drying over P_2O_5 the samples were too curved for the experiment to be performed effectively. The data set for samples tested

TABLE I Tubule density within the donkey hoof wall of MDC samples

Samples	Outer wall			Inner wall	
Donkey $n = 7$	Zone 1 Tubules mm^{-2}	Zone 2 Tubules mm^{-2}	Zone 3 Tubules mm^{-2}	Zone 4 Tubules mm^{-2}	Tubules mm^{-2} (full wall width)
Mean	19	8	8	9	11
Range	7–41	3–14	3–12	4–14	3–41
Ponies*	>27	16–27	8–16	<8	16

*The zones used by Reilly [12] do not relate exactly to the zones used above but provide a general guideline. Ponies are usually defined as horses under 14.2 hands at the withers.

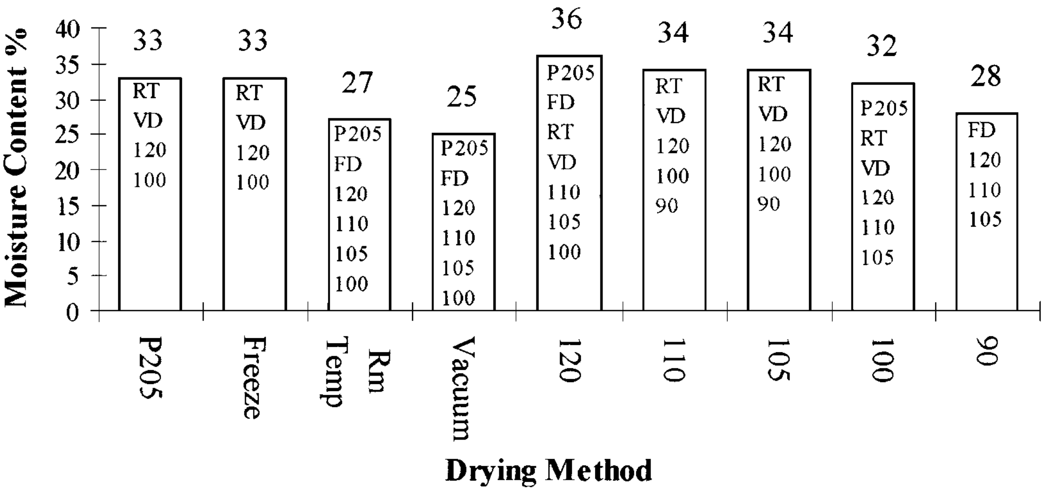

Figure 2 Comparison of median ($n = 31$) moisture content for donkey hoof horn for different drying methods. The list of techniques within each individual bar indicates significant differences ($p < 0.05$), e.g. P_2O_5 drying is significantly different from drying at room temperature, 100 and 120 °C, and by drying under vacuum.

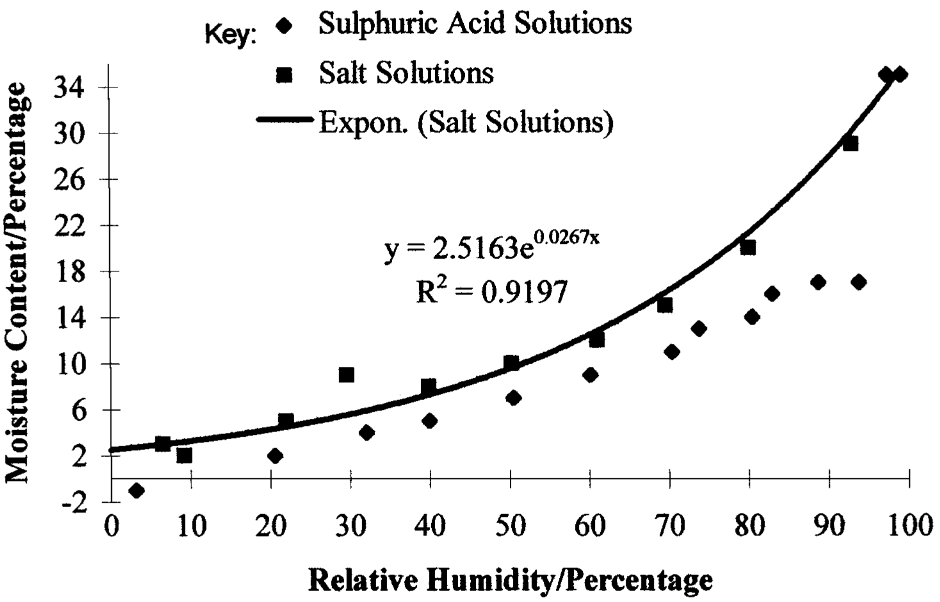

Figure 3 Plots of moisture content vs relative humidity for donkey hoof horn samples.

with a fresh moisture content showed a non-normal distribution ($p < 0.05$) but the data set after correction for moisture showed a normal distribution. Mann Whitney U tests showed differences between all data sets ($p < 0.0001$) except for the comparison of data from the measurements of stiffness on fresh and fully hydrated samples ($p > 0.05$).

4. Discussion

To understand the biomechanical function of hoof horn material it is important to establish the relationships between moisture content, relative humidity, tubular density, structure and mechanical properties. In order to make progress the fundamental issues associated with each of these areas of interest must be fully examined.

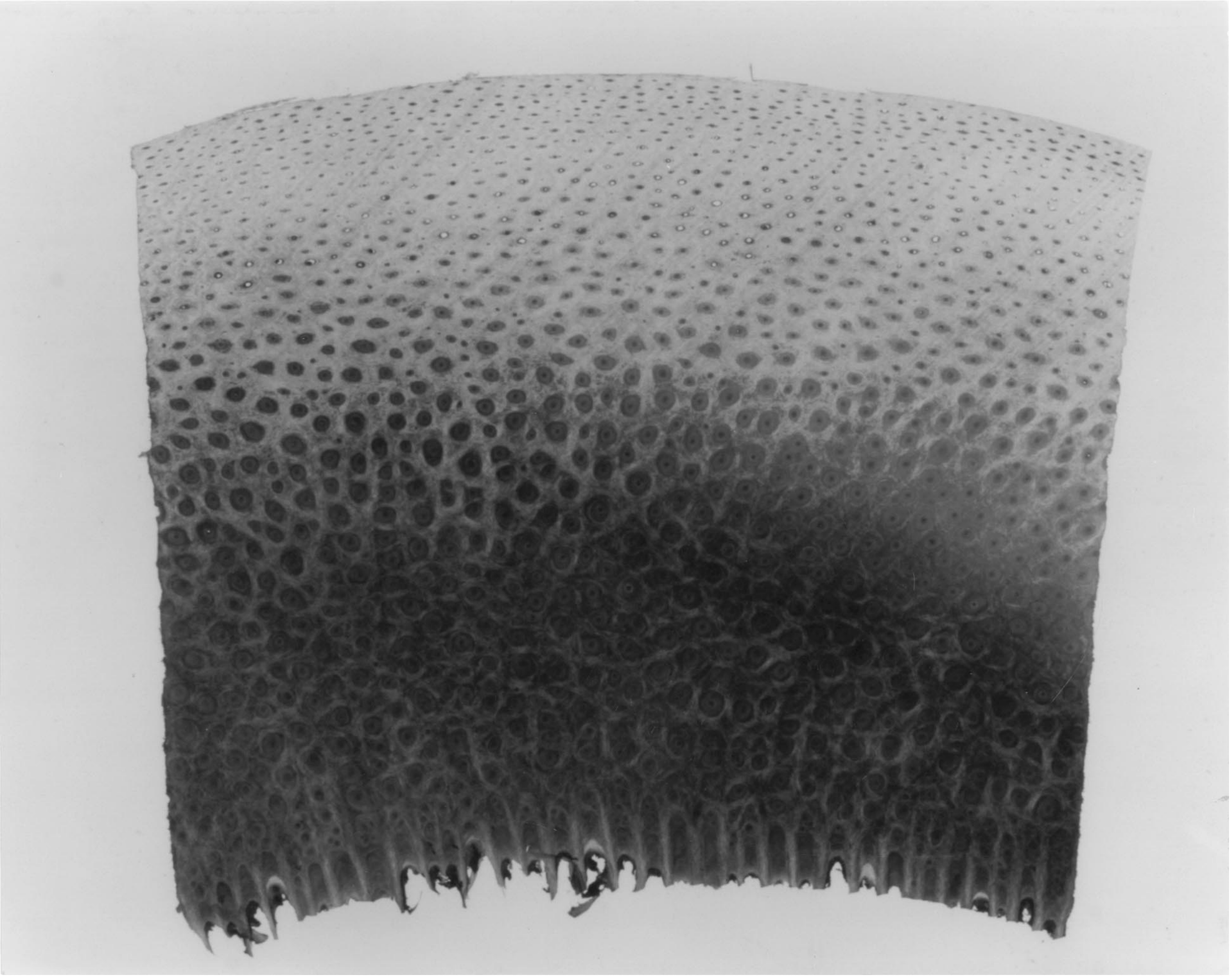

Figure 4 Photograph of stained transverse section of donkey hoof horn taken from a mid wall midline dead centre (MDC) section. Scale: |———| 1 mm.

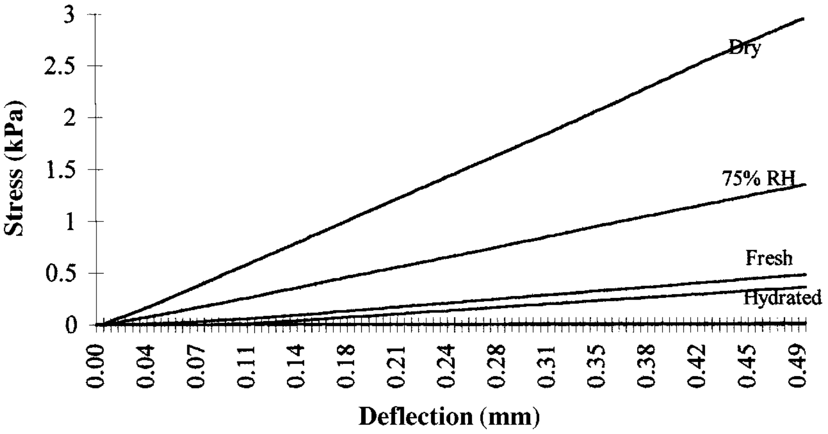

Figure 5 The effect of moisture on plots of stress vs deflection for donkey hoof horn.

Comparison with previously reported work on moisture content is difficult as a variety of different drying techniques have been employed. Reported moisture contents for horse hoof include 27.1% by Miyaki *et al.* [21], 27.9% (outer wall), 35.5% (inner wall) by Douglas *et al.* [22] and 17–24% by Leach [11] but there appear to be no reported comparisons with moisture content in donkey hoof horn. The results for the donkey hoof samples examined in this work broadly agree with those reported for horse hoof, although the experimental approaches and sampling protocols used by different workers vary.

The level of hoof wall hydration can be adjusted internally by fluids from within the dermis, from direct contact with water [15] or possibly by the relative humidity of the environment. Bertram and Gosline [15] deduced that the *in vivo* moisture content of the horse hoof was in the range 65–83% relative humidity and that maximum fracture toughness existed at this level. Using the principle outlined by Bertram and Gosline

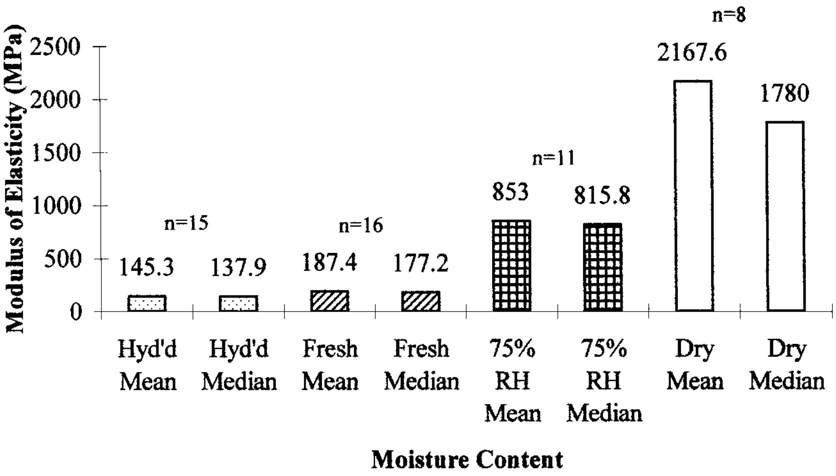

Figure 6 Comparison of mean and median modulus of elasticity for samples of donkey hoof horn having different moisture contents.

[15] for a hoof moisture sorption isotherm, the results shown in Fig. 3 indicate that a moisture content of 33% in donkey hoof corresponds to a relative humidity environment of about 96%. It is possible, however, that differences are due to different drying techniques and/or sample sites from the hoof clippings. The apparent negative result for the donkey hoof sample equilibrated in the 3.2% relative humidity environment provided by the vapour above sulphuric acid is because after drying of the sample over P_2O_5 there was a mass loss rather than a mass gain. Thus there must have been a transpiration of water vapour from the sample to the atmosphere in the humidity chamber in order to preserve the equilibrium state. Hysteresis effects are well known in absorption/desorption experiments carried out on the same sample and the differences between the two sets of data on Fig. 3 may well be a consequence of this phenomenon: samples equilibrated in relative humidity environments provided above the saturated salt solutions were subsequently dried, whereas those subjected to the relative humidity environments provided above sulphuric acid solutions were dried before exposure.

It is clear that in order to compare samples a standard drying approach must be used. This study shows that different amounts of water can be extracted from the hoof material by different drying regimes. There is a longer equilibrium time, typically 10 days, associated with drying over P_2O_5. Air drying leads to a larger variation and the results are dependent on the relative humidity. Fig. 2 shows the difference between air drying and drying over a P_2O_5 desiccant. For laboratory approaches to the drying of samples, processing times may be of importance. Techniques such as oven drying at high temperatures to ensure a fast drying time may lead to volatilization or decomposition of the sample. The loss of mass in these instances may, however, represent a loss of very tightly bound water or possibly some other volatile component of the tissue [15, 23]. This may not therefore be the ideal method to attain dry samples if mechanical testing is to follow as fibres in the drier outer layers may be torn apart and splitting may occur internally or externally if the moisture gradient becomes too steep. This phenomenon has previously been demonstrated for wood [24]. Mechanical testing following this drying may then yield results from an already stressed material.

Tubular density within hoof horn is alleged to be important in hoof "quality" and to influence hoof hardness [25]. Tubule densities have been reported for several species but details of the sampling methodologies are not always clear. These initial results suggest that there may be differences between the zonal tubular densities of pony and donkey hoof horn although both have higher tubular densities in the outer hoof wall. Tubular density may well have an influence on the mechanical properties of the hoof horn material and thus the difference between hydration effects between equine and donkey hoof horn may be a consequence of the differences in tubular characteristics. The tubular density pattern for pony hoof previously reported by Reilly *et al.* [12] showed a distinct arrangement of four zones and this zonal arrangement was not seen in these donkey hoof samples although there are similarities in the outer zone. These differences in tubule density across the hoof wall may be linked to the differences in moisture content.

The stress vs deflection plots show a Hookean relationship for donkey hoof horn, which is in agreement with results of work previously reported by Leach and Zoerb [26] and Landeau *et al.* [27]. From their work on stress-relaxation compression tests, Landeau *et al.* [27] concluded that equine hoof horn material behaves as a linear viscoelastic material. The results of the bending experiments reported here confirm that samples should be controlled for moisture content as there are clear differences in the force vs extension plots, shown in Fig. 5, for samples which have been subjected to different environments. Bertram and Gosline [15] have reported that horse hoof exists with a moisture content corresponding to $\sim 75\%$ relative humidity and yet for this study on donkey hoof it can be seen from Fig. 6 that there are considerable differences in stiffness between samples having a fresh moisture content and those at 75% relative humidity ($p < 0.0001$). In comparison, there is no significant difference between the results for samples with a fresh moisture content and those which have been hydrated ($p > 0.05$). This observation reinforces the fact that the results from moisture content studies indicate that donkey hoof has an *in vivo* moisture content which corresponds to a relative humidity environment of $\sim 96\%$. As expected, moisture content clearly has an influence on the stiffness of hoof horn and this is seen in Fig. 6 where stiffness of dry samples is about 10–15 times greater than those with fresh moisture content. Similar trends have been reported by other workers [8, 15, 22]. For example, Kitchener and Vincent [8] reported an increase in stiffness by a factor of about 40 for gemsbok horn when fresh and dried samples were compared. The level of difference between fresh and dried samples may be due to the use of different drying regimes (gemsbok horn was dried at 110 °C for 24 hours) but it may also be associated with the different anatomy and functionality of the horn material. As with moisture content studies, comparison with other studies reported in the literature is difficult as the descriptions of sampling, sample preparation and testing are unclear. Variables include: the type of mechanical test used to evaluate the modulus of elasticity, E, as it is not uncommon for the tensile strength in bending to be considerably higher than that in direct tension; the level of hydration of the sample; the area of hoof tested – different areas of the hoof may have different levels of hydration; the cross head speed used in bending measurements.

The experimental work reported in this paper has been focussed on the specific property of stiffness as a function of moisture content and the tubular structure of the hoof wall has been discussed in relation to mechanical properties and moisture content. Clearly the way forward involves a more detailed structure-property analysis which takes account of the composite nature of this natural material. Thus, not only the tubules, but other structural features should be considered in further work.

5. Conclusions

Hoof horn material is a composite natural material whose normal characteristics have not yet been properly defined. The interdependence of moisture content and role of water, morphology and biomechanical properties can only be explored by investing in links between veterinary science and materials science to provide a better understanding of the structure-function relationships.

- It is important to establish the sampling protocols in order to investigate moisture content and the effects of relative humidity.
- The results from tubular density measurements indicate that there may be differences between the morphology of hoof horn from different equids.
- The results from three point bending experiments show that stiffness is a function of moisture content.

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