X-ray diffraction patterns and anatomical properties of claw tissues of beef and dairy cattle

M. P. BROWNE[1], D. W. L. HUKINS[2], J. M. S. SKAKLE[3], C. H. KNIGHT[4],
K. A. K. HENDRY[4], C. J. WILDE[4] AND H. GALBRAITH[1]*

[1] *School of Biological Sciences, University of Aberdeen, 23 St Machar Drive, Aberdeen AB24 3RY, UK*
[2] *School of Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[3] *College of Physical Sciences, University of Aberdeen, Meston Building, Aberdeen AB24 3UE, UK*
[4] *Hannah Research Institute, Ayr KA6 5HL, UK*

(*Revised MS received 6 April 2007; First published online 22 June 2007*)

SUMMARY

Medial claws from the right hind feet were obtained post mortem from four 19–20-month-old beef heifers and from four 28-month-old first-calving dairy heifers 3–4 days postpartum. X-ray diffraction (XRD) studies were undertaken on samples of soft and hard (cornified) integumental tissues of dorsal wall, sole and heel (bulb) for varying sites and planes of exposure. The measurements were interpreted as defining diffraction patterns and intermolecular spacings of cytoskeletal and extracellular fibrous structural proteins. The orientation of these proteins was examined in relation to physical characteristics and function including bearing of body weight by these tissues.

Physical measurements taken included impression hardness which showed typically greater values for wall than sole and variable differences between horn of dairy and beef origin and husbandry systems. Claws from dairy heifers had significantly smaller values for toe (dorsal wall) angle, claw height and heel height and thickness of solear horn and heel soft tissue. Although few were studied, the results reflected typical husbandry origins and indicated the susceptibility to the lesion formation well recognized in postpartum dairy cattle.

Typical XRD patterns for horn samples showed defined arcs of reflectance on the equatorial axis consistent with findings for the presence of α-helices in fibrils reported to occur in other hard-keratin-containing integumental tissues. However, reflectance on the meridional axis also reported for these other tissues was not detected. A similar defined pattern was obtained for less than 0·10 of samples of internal soft pre-cornified epidermal and attached dermal tissue although the values for intermolecular 'd' spacing for these were consistent with those reported for type I collagen. Diffuse reflection patterns were thus evident for the majority of samples of soft tissue epidermis and dermis and also for adipose tissue of the digital cushion.

The formation of defined arcs of reflectance allowed the determination of fibril alignment in wall and solear horn. For the orientated samples of dorsal wall horn tissue, the outer layer showed a longitudinal angle of orientation essentially maintained proximal to distal. This pattern was maintained throughout the depth of horn at the proximal site. In contrast, layers in mid-wall and towards the distal edge showed a greater circumferential (horizontal) orientation in sections collected anterior to posterior towards the inner corial, including laminar, tissues. The orientation of fibrils in inner wall horn appears to relate to the direction of load-bearing forces in connecting horn to the distal phalanx. Horizontal alignment of fibrils was observed in the sole. In presenting the long axis of cells to the ground surface this orientation may facilitate erosive forces and contribute to the thinning of cornified sole horn under adverse underfoot conditions.

INTRODUCTION

Lameness in dairy cattle is a painful and economically important production disease. The incidence of lesions causing lameness varies in different countries

* To whom all correspondence should be addressed.
Email: h.galbraith@abdn.ac.uk

with disorders of claw horn such as solear ulcer and white line disease occurring with incidence rates which can exceed 0·35 of animals in dairy herds (Vermunt 2004). Recent studies on lameness in cattle have given increasing attention to improving knowledge of the biology of claw horn and the underlying epidermis and dermis affected in lesion formation (Tarlton *et al.* 2002; Hendry *et al.* 2003; van Amstel *et al.* 2004*a*, *b*; Galbraith *et al.* 2006*a*). In common with other keratinizing tissues of the integument, claw horn is synthesized from specialized epidermal cells located on the basement membrane adjacent to underlying dermis. Major structural molecules are synthesized during proliferation and differentiation of these cells to form the cornified horn with chemical and physical properties determined by site of production (Budras *et al.* 1998; Tomlinson *et al.* 2004; Galbraith *et al.* 2006*a*). There has been considerable interest in the chemical composition of individual keratin and other proteins expressed by a range of genes in the claw epidermis and in the mechanisms by which they interact to form the cornified horn product (Galbraith *et al.* 2006*a*, *b*). Previous work has described similarities in the expression of gene products in hair and claw horn (Gillespie 1991). More recent studies (Galbraith *et al.* 2006*b*), based on 2D electrophoresis and proteomic analysis, produced protein profiles of extracts of horn and epidermal and dermal soft tissue which demonstrated both trichocyte and cytoskeletal keratins and profiles which were similar to those obtained for hair by Plowman (2003). An additional anatomical feature which links certain hair follicles that produce medullated hair to, for example, wall or sole horn, is the production of medullated tubular horn (Tomlinson *et al.* 2004). As in hair (Galbraith 1998), this develops at the tip of individual dermal papillae. The regulatory mechanisms determining medullation are unknown.

There are also certain clear differences in the function and biomechanical properties of hair and horn. Claw horn has a continuous structure similar to epidermis of skin. The biomechanical properties of horn (Hinterhofer *et al.* 2005) are primarily based on location within the claw, the nature and organization of macromolecules of the cytoskeleton within keratinocytes and in structures of adhesion between these cells (Tomlinson *et al.* 2004).

Of particular interest are the fibrous keratin intermediate filament (IF) proteins which interact with IFI-associated proteins (IFAPs) to form the cytoskeleton in epidermal keratinocytes (Kempson & Logue 1993*a*; Hammond *et al.* 2000; Tomlinson *et al.* 2004). Similarly, vimentin has been described in fibroblasts of the dermis (Galbraith *et al.* 2006*b*).

Such interactions between IFs and IFAPs are poorly understood for claw tissue but have been the subject of considerable historical research on biological materials such as sheep wool and porcupine

quill, using techniques which include electron microscopy and X-ray diffraction (XRD). The XRD approach has been used, for example, to investigate the role of 'hard' trichocyte α-keratin IFs in forming α-helical structures of varying complexity, with stability provided by di-sulphide bonding within and between protofilamentous networks (Parry 1996). Studies on side chains have been reported by Busson *et al.* (1999), Briki *et al.* (2002) and, in a recent study which described α-helix to β-sheet transformation in stretched horse hair fibres, Kreplak *et al.* (2004). Further reports on properties of different IFs and characteristics of human hair keratin-associated matrix proteins which contribute to cytoskeletal integrity include those of Fraser & Parry (2006) and Parry *et al.* (2006), respectively. Similarly, in considering the role of the dermis of the claw in the suspension of body weight (Tarlton *et al.* 2002), fibrous macromolecules such as collagens (e.g. I, IV and VII (Crespo *et al.* 1999)) are recognized as essential components of this system, in connecting internal bone of the limb to the external horn of the claw (Westerfield *et al.* 2000).

The properties of such collagenous tissues have also been investigated by XRD in a range of tissues (Lees & Hukins 1992; Purlslow *et al.* 1998; Wilkinson & Hukins 1999; Wess & Cairns 2005; Orgel *et al.* 2006).

There has thus been particular interest in the use of XRD to determine orientational or spatial properties of structurally important molecules of intracellular cytoskeleton (e.g. keratins) and extracellular connective tissue (e.g. collagens). The technique involves the passage of a beam of monochromatic X-rays through uniformly prepared biological material. The emergent diffraction pattern characteristic of the molecular structure of the specimen may then be used to determine the predominant orientation and spacing of molecules within a fibril according to Bragg's law. Such measurements include those for the intense arcs on the equator of the pattern which describe the relative side-to-side packing of the molecules. As the molecules are aligned almost parallel to the fibril axis, these equatorial arcs can be used to show the direction of the fibril axis which is perpendicular to the bisector of these arcs.

Such an approach has been used to determine the predominant direction of orientation of fibrous proteins such as α-keratins in avian feathers (Cameron *et al.* 2003) and horse hair and porcupine quill (Busson *et al.* 1999; Briki *et al.* 2002). These authors have described the physical structure of α-keratin fibres as arising from the wrapping of two or more keratinous α-helices into α-helical coiled coils. They describe XRD patterns for α-keratins as displaying (a) equatorial (horizontal) reflectance relating to the distance between the α-helical axes and (b) reflections on the meridional (vertical) axis and

arising from (i) the axial rise per residue and (ii) the projection of the α-helical pitch, along the coiled coil axis. Similarly, equatorial and meridional reflection have been associated with structural properties of collagens (Wilkinson & Hukins 1999; Wess & Cairns 2005).

The anatomical and biomechanical properties of claw horn and underlying connective tissue are important determinants of claw health. These are frequently compromised in dairy cows (Vermunt 2004), particularly in the immediately postpartum time period (Tarlton *et al.* 2002), but are rarely described in female beef cattle of similar age. Major aims of the current work were therefore to test the hypotheses that (i) the structural anatomy of claws of dairy heifers immediately postpartum differs from non-parous beef heifers and is likely to predispose to lesion formation and lameness later in lactation, (ii) that keratin-containing cornified horn exhibits XRD behaviour similar to that of other tissues of the integument which contain fibrous α-helical molecular structures and (iii) that the orientation of structural macromolecules in cornified horn and underlying epidermal and dermal soft tissue is determined by location and function in the bovine claw. Measurements, using XRD analysis and not previously undertaken for these tissues, were made on samples from a range of sites within the claw.

These measurements were made in the context of other anatomical properties such as hardness and dimensions of horn and underlying tissues which affect functional geometry and are important for claw health. Claws of female beef and dairy cattle were used in the expectation of providing differences in study material based on intrinsic genotype and extrinsic production environment and likely susceptibility to development of claw horn lesions.

MATERIALS AND METHODS

Collection, impression hardness and anatomical dimensions of claws

Medial claws from the right hind feet of eight heifers were used. Four animals were from a uniform group of dairy heifers aged approximately 28 months and slaughtered 2 or 3 days after calving. These control animals had been offered a conventional dairy heifer diet based on grass silage and 1 kg/day concentrate mix and were part of a study investigating the effect of nutritional supplementation of methionine or melatonin throughout pregnancy on biology of claw horn. The claws of the animals had been functionally trimmed 4–6 weeks before slaughter. The underfoot conditions were a straw-bedded standing/lying area with concrete flooring providing access to the feeding barrier and trough. Data from studies on characteristics of hind right lateral hind claws and including
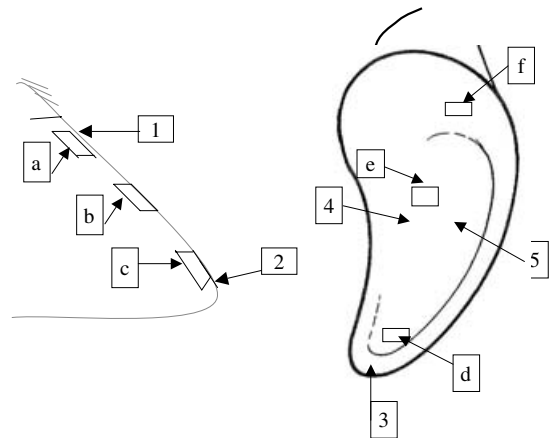

Fig. 1. Location of positions of (i) measurement of impression hardness (dorsal wall: 1, proximal and 2, distal; sole apex: 3; pre-bulbar: axial, 4 and abaxial aspect, 5) and (ii) collection of samples for measurement of anatomical dimensions or XRD studies (a, b, c proximal, mid- and distal wall respectively and d, apex; e, sole–bulb junction and f, bulbar regions). See Materials and Methods section for details.

sulphur amino acid composition have been reported previously (Galbraith *et al.* 2006*a*). The other four animals (two Charolais X and two Simmental X) were beef heifers aged 19–20 months at slaughter and were conventionally reared on a commercial farm. Following slaughter of all animals at a commercial abattoir, claws were quickly separated from the leg, chilled in plastic bags on ice and frozen individually at $-20\,^{\circ}\mathrm{C}$ within 1 h.

After thawing, impression hardness measurements were taken (Fig. 1) in triplicate by a single operator under uniform atmospheric conditions, at two sites on the dorsal wall which were approximately 5 mm below the lower periopic line and 5 mm above the distal edge, respectively and three sites on the sole (sole apex and axial and abaxial aspects of the pre-bulbar region, at 5 mm from the white line). Measurements were made using a Shore D meter (Durotech Shore D durometer, model B101D), which records the relative resistance force against penetration of a test material by a sharply pointed pin attached to the measuring device. Units are given on an arbitrary scale from 1 increasing to 100 for the hardest material. Digital images of each claw were captured in four different planes using an Olympus Camedia C2500l digital camera. Photographic images were processed (Image Pro Plus 3.0 software) and conventional dimensions measured (Fig. 2) including claw length (at dorsal border), claw height, heel height, and toe (dorsal) angle, claw width (Greenough 1997*a*), solear surface area and its percentage of erosion (as depressions on solear surface).
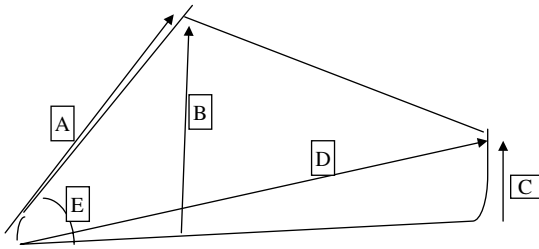
Fig. 2. Conventional claw measurements: length (at dorsal border) (A), height (B), heel (bulb) height (C), diagonal (D) and toe (dorsal) angle (E) (Greenough 1997).

Fig. 3. Schematic representation of the claw section showing the planes of orientation through which the X-ray beams passed (R = radial: L = longitudinal).

Preparation of sections

A 10 mm wide section of the claw was cut by autopsy saw extending from the periopic segment of the wall down its length and across the centre of the solear surface to the periopic border at the heel. Digital images were taken as described above for measurement of anatomical dimensions and the outer horn and underlying soft tissue was separated from the distal phalanx.

On average, 100 sub-sections from each claw were prepared to fit into a 10 mm² well in Perspex cells. The sub-sections measured 1–2 mm in depth and width and up to 10 mm in length. They comprised regions of cornified hard tissue, soft tissue dermis with attached pre-cornified epidermis, in wall and sole or underlying adipose tissue (digital cushion) of the bulb (Fig. 1). Sub-sections were prepared in different planes to enable the X-ray beam to pass through 1–2 mm thickness of tissue and along either the longitudinal (L) or radial (R) axes (Fig. 3). All material was prepared
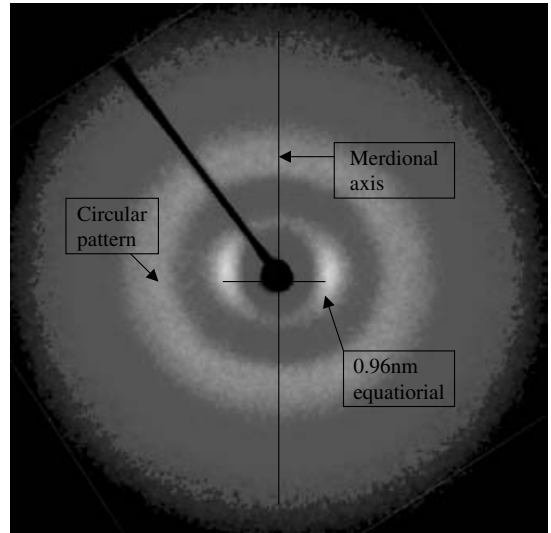

Fig. 4. Typical XRD pattern of a section of cornified horn showing the presence of arcs on the equatorial (horizontal) axis at maxima of approximately 0·96 nm (arrowed) typical of hard α-keratin. Note the diffuse disordered circular pattern (arrowed) and the absence of specific areas of intensity on the meridional (vertical) axis perpendicular to the equatorial axis.
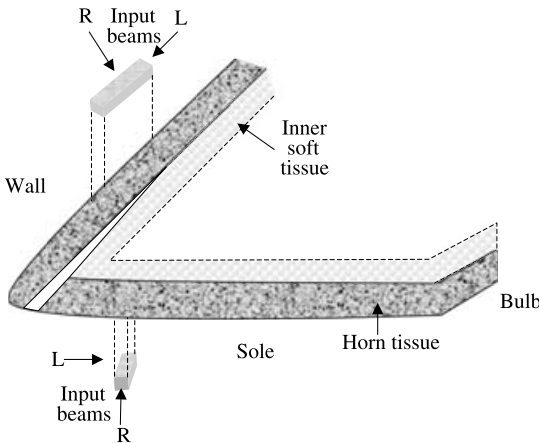
under similar environmental conditions and stored in plastic bags at $-20\ °C$ until use.

XRD

The XRD patterns were obtained using a single crystal diffractometer (SMART 1000 CCD Area Detector, Bruker AXS) which used molybdenum Kα radiation of 0·071073 nm wavelength, λ. Diffracted rays were detected by a phosphor scintillation screen coupled to an array of solid state photo-detectors that recorded a digitized diffraction image. A specimen-to-detector setting (R) of ~ 50 mm was used with precise calibration undertaken daily using finely ground silicon powder. The angle of diffraction, θ, was calculated by geometry from the diffraction pattern using image analysis software (Image Pro Plus 3·0-Media Cybernetics, USA). Intermolecular spacings, d, were then calculated from θ using Bragg's law ($\lambda = 2d\sin\theta$). Collagen tape with parallel fibres (Hukins 1977; Wilkinson & Hukins 1999) was used as a reference to validate the technique being used.

Data preparation, interpretation and statistical analysis

Diffraction patterns were saved as bitmap files and processed by image analysis software (UTHSCA

Table 1. *Mean values (with pooled S.E.D.) for impression hardness measurements on claws of 8 animals at 5 sites*

	Position of hardness measurements					
Breed	Sole (apical)	Sole (axial)	Sole (abaxial)	Wall (proximal)	Wall (distal)	S.E.D.
Dairy	41[bc]	43[c]	43[c]	64[d]	71[e]	2·3
Beef	37[b]	32[a]	40[bc]	71[e]	74[e]	

Means with dissimilar superscripts differ significantly ($P < 0.05$).

Table 2. *Mean values (with S.E.M.) for external claw measurements of dairy and beef heifers*

Variable	Dairy	Beef	Statistical significance
Dorsal angle (°)	39 (2·0)	46 (0·8)	$P < 0.001$
Wall length (mm)	68 (2·5)	71 (4·8)	N.S.
Sole width (mm)	49 (2·1)	54 (2·9)	$P < 0.05$
Sole length (mm)	109 (7·9)	90 (9·8)	N.S.
Heel height (mm)	23 (4·7)	39 (7·1)	$P < 0.01$
Claw height (mm)	89 (1·5)	99 (3·2)	$P < 0.05$
Sole area (mm²)	3860 (849)	4411 (621)	N.S.
Eroded sole area (%)	30 (8·8)	33 (12)	N.S.

Table 3. *Mean values (mm with S.E.M.) for internal claw measurements of dairy and beef heifers*

Variable	Dairy	Beef	Statistical significance
Proximal wall			
Horn tissue	5·5 (2·08)	5·00 (1·41)	N.S.
Soft tissue	5·3 (0·96)	5·8 (2·06)	N.S.
Total	10·8 (1·71)	10·8 (1·26)	N.S.
Distal wall			
Horn tissue	7·5 (1·29)	7·3 (1·71)	N.S.
Soft tissue	3·5 (0·58)	3·5 (0·58)	N.S.
Total	11·0 (1·15)	10·8 (1·89)	N.S.
Sole (apical)			
Horn tissue	5·3 (3·40)	13·5 (3·0)	$P < 0.01$
Soft tissue	2·3 (1·26)	3·0 (1·15)	N.S.
Total tissue	8·0 (3·56)	16·5 (2·52)	$P < 0.01$
Sole-bulb			
Horn tissue	6·25 (2·06)	13·8 (3·3)	$P < 0.01$
Soft tissue	6·5 (1·29)	8·0 (1·41)	N.S.
Total tissue	12·8 (2·06)	21·8 (3·3)	$P < 0.01$
Bulb			
Horn tissue	7·5 (1·29)	9·5 (1·91)	N.S.
Soft tissue	6·5 (1·73)	13·0 (2·58)	$P < 0.01$
Total tissue	14 (1·83)	22·5 (1·91)	$P < 0.01$

with least significant differences calculated between mean values where main effect statistical significance (at least $P < 0.05$) was obtained.

Image tool 2.00, Texas Health Science Centre, USA). The relative intensity of images was altered by thresholding and the co-ordinates and intensities of the peaks in the diffraction patterns determined. These were then inputted into Microsoft Excel and the orientation of the sample determined with respect to the collagen tape and to the position within the claw. For oriented samples, arcs in the XRD patterns on the equatorial axis were detected (e.g. Fig. 4). The predominant orientation was measured from the centre position of the arcs and the average between the extremes of the arc. Such equatorial XRD maxima correspond to the spacings between molecules perpendicular to the fibre axes and thus are used to determine the predominant direction of the fibres. The angular extent of the arcs give an indication of the relative alignment of the fibrils: total alignment would result in an extremely narrow arc, whereas random alignment results in a circular diffraction pattern. The data for internal and external claw dimensions were examined statistically using Students *t*-test for comparisons of differences due to breed. For other measurements, two-factor analysis of variance (Minitab version 13.1) was applied to examine main effects due to breed and site of collection of sample

RESULTS

Hardness measurements

Statistical analysis indicated significance ($P < 0.05$) for the main effect for position of impression hardness measurement and interaction between this factor and breed (Table 1). Mean values were significantly greater on wall sites than sole. The significantly smaller value at the axial solear site for beef claws was associated with an appearance of external horn described as 'powdery' in nature. Hardness values at the proximal wall site were significantly greater on average for beef cattle claws than for those of the dairy cattle.

Claw dimensions

Examination of individual means showed that values for external measurements of dorsal angle, sole width, heel height and claw height were significantly greater for claws from beef than from the dairy heifers with no differences recorded for solear surface area and percentage of erosion (Table 2). Comparisons (Table 3; Fig. 5) of internal measurements showed no differences in thickness of soft and cornified tissue
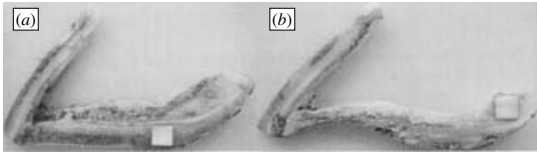
Fig. 5. Digital images showing typical sections of claw from (*a*) beef and (*b*) dairy production types.

between breeds for the wall region. In contrast, mean values were greater (at least $P < 0.05$) for beef cattle claws, consistently for total thickness and for thickness of horn although not underlying soft tissue, at solear sites. There was a difference (significant, $P < 0.01$, beef > dairy) in soft tissue dimension at the heel site. It was also notable that symptoms of claw horn disorder, such as bruising of the sole and white line or more severe lesions (Anon 2006) were not visually evident at any of the sites in the claws studied.

XRD and d spacing

XRD results indicate both the intermolecular separation and also the degree of alignment of long organic molecules such as those containing keratins within cells and collagens in extracellular connective tissue. In examining diffraction patterns from cornified horn tissue in wall and sole, there was consistent evidence of predominant orientation of these molecules as indicated by the narrow arcs on the equatorial axis (Fig. 4). The patterns for the tissue produced arcs from which the intermolecular (*d*) spacing was calculated. Values for cornified horn tissue derived from such arcs were typically in the range of 0·96–1·0 nm. In addition, a diffuse circular diffraction pattern, typical of a disordered arrangement of molecules, with *d* value of approximately 0·5 nm was also observed in these samples (Fig. 4). There was no evidence of arcs indicating defined areas of intensity on the meridional axis of the samples studied.

In contrast to results for cornified horn, epidermal–dermal soft tissue and adipose tissue of the digital cushion of the heel bulb gave most frequently diffuse circular patterns which indicated the absence of any consistent predominant orientation of fibrous components (Fig. 6*a*, *b* respectively). The majority of patterns from the soft non-cornified tissue consisted of a diffuse ring centred around 0·36–0·37 nm. Exceptions were a small number (4/48, not shown) with evidence of equatorial arcs and *d* values in the range of 1·45–1·56 nm.

Angles of orientation of molecules within horn tissue

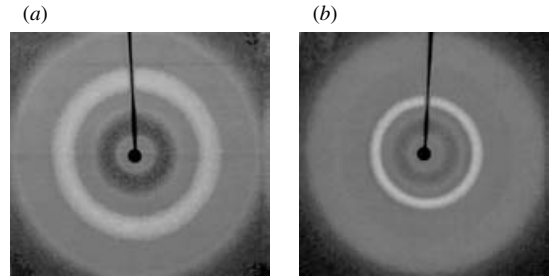The orientation of the aligned molecules was calculated from X-ray patterns such as that shown in

Fig. 6. Typical diffraction patterns showing the absence of orientation of fibrils produced by (*a*) corial soft tissues and (*b*) fat pad at heel.

Fig. 4. The alignment is perpendicular to the line joining the mid points of the arcs, and samples were carefully aligned within the beam so that the alignment was measured relative to the longitudinal and radial axes.

Mean values indicating a predominantly longitudinal alignment of keratin molecules were essentially similar in cornified horn sections taken along the whole length of the outer horn along the dorsal border of the wall (Tables 4 and 5). However, the data indicate a significant main effect ($P < 0.05$) according to site of measurement for changes in orientation of horn with progression from outer surface to inner region (see Fig. 7 for diagrammatic representation). Values for the proximal wall sites measured in the radial plane averaged angles in excess of 75° indicating a predominantly longitudinal alignment with little change in more internal sections. In contrast, the orientation of molecular alignment in mid-wall and distal-wall sites changed markedly towards a more circumferential (horizontal) direction in sub-sections positioned closer to internal tissue. Results indicating changes at the distal wall site in response to anterior to posterior progression were confirmed by measurement of alterations in diffraction in response to the X-ray beam travelling in the longitudinal plane (Table 5). The angle of orientation was shown to change towards a horizontal direction in progressing from outer to inner segments and effectively from coronary horn through to horn tissue of the white line.

Comparisons were also made for horn sections from the upper wall site and sole (apical site) (Table 6). The main effect due to site of collection was not statistically significant. The small angle of orientation in response to longitudinal application of X-rays is shown to confirm longitudinal alignment for proximal wall and horizontal alignment on longitudinal exposure of sole, to the X-ray beam.

There were no statistically significant effects for breed, or interactions between breed and site of horn collection for any of the data obtained by XRD.

Table 4. *Mean values (with S.E.M.) for angle of orientation of the proximal- and mid-wall sections progressing from outer to inner horn with radial X-ray beam passing from the outer to the inner surface of the wall. (S.E.D. value between individual means for site of collection = 31·5 (18 DF))*

Breed	Proximal wall (outer)	Proximal wall (inner)	Mid-wall (outer)	Mid-wall (mid-internal)	Mid-wall (inner)
Dairy	91 (1·5)	78 (11·5)	87 (2·9)	44 (16·0)	29 (13·1)
Beef	66 (11·2)	85 (4·7)	52 (19·0)	76 (3·1)	13 (3·4)
Mean	79 (7·0)	81 (5·9)	72 (10·3)	60 (9·7)	12 (6·9)

Table 5. *Angle of orientation (mean with S.E.M.) from outer to inner wall sections of the distal wall site determined by X-rays applied in radial and longitudinal planes. (S.E.D. values between individual means for site of collection for (a) = 26·9 (18 DF) and (b) = 48·7 (18 DF))*

Breed	Radial plane (a)			Longitudinal plane (b)		
	Outer	Mid – internal	Inner	Outer	Mid-internal	Inner
Dairy	80 (1·6)	47 (18·6)	43 (14·2)	11·4 (1·87)	3·0 (1·63)	65 (22·8)
Beef	76 (3·0)	24 (12·6)	29 (9·5)	6·0 (1·63)	4·8 (1·87)	47 (30·9)
Mean	78 (1·7)	35 (11·2)	36 (8·4)	9·1 (1·73)	3·9 (1·16)	56 (17·5)

DISCUSSION

Anatomical measurements according to origins of claw and known susceptibility to formation of lesions

The anatomical structure of the bovine claw is well recognized as an important intrinsic factor in determining resistance to formation of lesions. Important factors include the properties of the horn capsule as a protective barrier to external environment, its role in supporting the body weight of the animal by the laminar region, weight bearing on the palmar and plantar surface and shock absorption at the bulb of underlying soft tissue. The small numbers in the present study preclude general conclusions about generic differences between claws of beef and dairy origin. However, the results may be interpreted as indicating some important characteristics with potential significance for susceptibility to lameness. This is particularly the case for dairy heifers in the present study. These animals were euthanased 2 or 3 days after calving and in advance of the time period after parturition in which the incidence of formation of claw lesions typically increases (Tarlton *et al.* 2002). For example, the dairy animal claws had significantly lower values for dorsal angle, claw (apical), height and heel (bulb) height which are all associated with a greater predisposition to formation of claw disorders (Vermunt & Greenough 1995). It may also be speculated that such anatomical characteristics for the medial claw may result in a more limited contribution to load bearing of body weight and so may increase susceptibility of the more frequently affected lateral claw to formation of lesions. This approximates to the converse of relieving weight bearing by the lateral claw following the placing of blocks to raise the height of medial claws during, for example, treatment for solear ulcers.

Examination of the thickness of the cornified horn capsule and underlying epidermal and dermal soft tissue showed no differences for the wall region but smaller thickness of sole ($P < 0.05$) in dairy cattle claws. Part of the difference in solear cornified horn may be attributed to claw trimming, which is not generally practised in commercial beef cattle. Additional influences on the horn characteristics of the dairy cattle studied include the effect of pregnancy, which has been associated with effects on properties of the white line postpartum (Kempson & Logue 1993*b*) and parturition which, in reducing the supportive capacities of laminar connective tissue (Tarlton *et al.* 2002), may impose additional stresses on horn and horn-forming epidermis.

The average value for thickness of soft tissue at the sole apical region (2·3 mm for dairy) is less than that reported by van Amstel *et al.* (2004*a*) (3·92 mm by ultrasound) for Holstein cattle, although the mean value of 5·3 mm for horn thickness at this region is consistent with the value of 5·15 mm reported for 'thin soled' dairy cattle by these workers (van Amstel *et al.* 2004*b*). These authors have also made the association between such thin soles which had greater
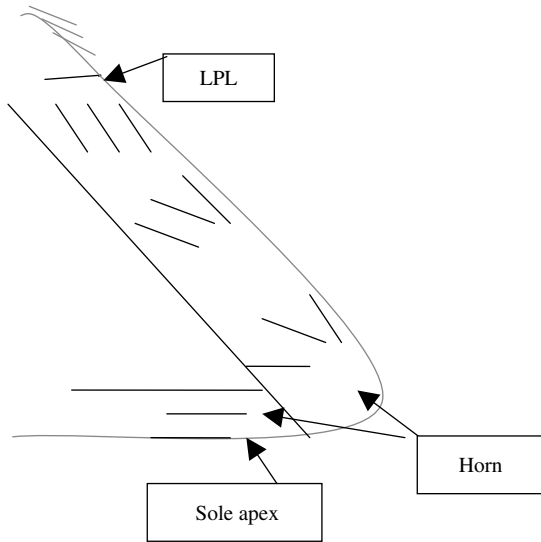
Fig. 7. Saggital representation of alignment of fibrils in horn of (i) dorsal wall from the lower perioplic line (LPL) to the dorsal border and (ii) apex of the sole.

Table 6. *Angle of orientation (mean with* s.e.m.*) of the proximal wall horn and the apex of the sole with the X-ray beam passing in the longitudinal plane proximal-distal for the wall and anterior–posterior for the sole*

Breed	Proximal wall	Sole (apex)
Dairy	8·4 (3·14)	6·0 (2·97)
Beef	9·3 (0·25)	7·8 (2·14)
Mean	8·8 (1·47)	6·9 (1·73)

values for moisture content and which were thought in turn to produce softer horn and to lead to the observed greater disposition towards lameness. Although dry matter was not measured in the present study, there was no evidence of reduced (directly measured) impression hardness for horn in soles of the dairy cattle at the three sites tested compared with beef cattle with similar or greater thickness of horn and which gave lower hardness values. There was also visual evidence of the retention of powdery horn on the axial sole surface of beef cattle claws, which may have been associated with limited abrasion on the floor surface provided by straw bedded courts typically used to house cattle in the local geographical area. It is apparent that the interactions between claw horn growth and wear, absence of trimming and underfoot conditions have contributed to the results for beef cattle to produce the differences observed on comparison with dairy animals with different background of production. Similarly, differences in the most recently produced upper (proximal) wall coronary horn between the breed types may reflect variation in hydration and/or capacity for disulphide bond formation related to synthesis of cytoskelal keratins and associated proteins (Hepburn *et al.* 2007). For the dairy cattle, such effects may be related to nutritional or endocrine influences of pregnancy affecting proliferation and differentiation of keratinocytes. Greenough (1997*b*) has described more extreme forms of stress responses in the proximal wall region as being involved in the production of 'horizontal grooves' in horn.

The significantly smaller thickness of total tissue in the heel region of dairy cattle is largely due to a thinner underlying soft tissue including the digital cushion. The digital cushion has an important role in absorbing the impact of transmission of body weight by the distal phalanx during locomotion and is considered to increase in deposition of adipose tissue with increasing age (Lischer & Ossent 2000). However, this factor alone is inadequate to explain the difference between the younger beef and older dairy claws tested. The dairy heifers, which were euthanased 3–4 days after calving, thus appeared to be disadvantaged very early in lactation by having (a) thinner solear horn, (b) reduced shock absorbing capacity of the heel region and (c) increased concentration (due to lesser toe angle) of weight-bearing forces posteriorly towards the sole–bulb junction and recognized sites of greater tissue compression by the distal phalanx. Such changes in positioning of the distal phalanx with respect to vascularized dermis and avascular epidermis are known to predispose to bruising and other lesions (Lischer & Ossent 2000). Such disadvantages appear consistent with reported sensitivity of dairy heifers to lesion development postpartum and which has been well documented (Kempson & Logue 1993*b*; Webster 2002).

Results for impression hardness also showed a typical order for horn of cattle claws according to functional site with harder horn in the wall than sole (Galbraith *et al.* 2006*a*).

XRD

Cornified horn is composed of de-nucleated squamous epithelial cells which are largely composed of a proteinaceous cytoskeleton containing keratins and associated molecules arranged into IFs 'tonofilaments' of diameter *c.* 8–10 nm. Tonofilaments are generally arranged in parallel to the long axis of the cell and have properties which along with those of cell–cell adhesion are important in determining structural strength of the horn capsule (Hendry *et al.* 1997; Budras *et al.* 1998). Such mechanical strength is important in the claw wall region to maintain effective

suspension, via the bones of the limb, of the body weight of the animal. The success of the suspensory system in maintaining functional geometry in the hoof is affected by the direction of mechanical forces and position of load-bearing structures within the claw.

Outputs from measurement of XRD patterns in bovine claw horn have not been described previously. Results from the present study describe XRD patterns with features which are similar to those described for porcupine quill (Busson *et al.* 1999) and horse hair (Briki *et al.* 2002). In particular, there was clear evidence in cattle horn tissue of reflections in the equatorial plane in the range of 0·96–1·06 nm (Fig. 4) and so encompassing the value of 0·96 nm published for α-keratin in horse hair (Kreplak *et al.* 2004): this value relating to the mean distance between α-helical axes. The results also identified a diffuse circular 'halo' with '*d*' value approximating to 0·5 nm which was similar to that suggested by Kreplak *et al.* (2004) as being due to the presence of less ordered polypeptide structures in coiled coils. However, there was no evidence of the meridional (vertical) axis 0·52 nm spacing which corresponds to the α-helical pitch projection along the coiled coil axis.

The orientation of the aligned molecules in the samples studied was calculated from XRD patterns such as that shown in Fig. 4. The outermost horn layer was shown to have a predominantly longitudinal angle of orientation which was essentially maintained proximal to the distal edge of wall horn. This arrangement, although longitudinal in inner layers of the upper wall, contrasted with the inner layers in mid-wall and lower-wall regions which changed to a more radial orientation. This was confirmed for the lower wall by XRD of radial as well as longitudinal planes. These results have consistency with the observation of Kempson & Logue (1993*a*), who described keratin bundles in keratinocytes in laminar horn leaflets, although not those of interdigitating horn, as being parallel to the orientation of the leaflet structures. In their studies, the organization of epidermal fibrils in inner cornified horn is thus seen to orientate, at least partially, towards the direction of force exerted by the distal phalanx via laminar attachments. Similar observations focusing on relationships between the plane of the axis of tubular and intertubular horn and alignment of IFs in equine lamellar wall horn have been made by Kasapi & Gosline (1997).

Results were also available for solear horn at the apical region which suggested a horizontal orientation of fibrils. This arrangement is consistent with the presence of fibrils positioned along the long axis of solear squamous epithelial cells. It may be speculated that this arrangement confers reduced resistance to abrasion and may contribute to the extent of erosion recorded for all claws. It may also be a factor contributing to solear thinning (van Amstel *et al.* 2004*b*) under conditions of exposure to concrete flooring and seasonally-affected reductions in claw horn synthesis (MacCallum *et al.* 2002).

The soft tissue examined in the present study included pre-cornified epidermis attached to dermal connective tissue. The dermis has a particular role in bridging the tissues of the wall horn and distal phalanx (Lischer & Ossent 2000; Ossent *et al.* 2000; Tarlton *et al.* 2002). Its effectiveness is determined by macromolecular composition of the extracellular matrix and properties such as angular orientation and spaces (*d*-spacing) between individual collagen molecules (Westerfield *et al.* 2000). Although these properties have been studied by XRD and described in tissues such as the cornea of the eye (Quantock *et al.* 1998), skin and muscle perimysium (Purslow *et al.* 1998) and rat tail tendons (Orgel *et al.* 2006), they have not been studied previously in bovine claw tissue. In contrast to results for cornified horn, typical diffraction patterns for epidermal–dermal soft tissue and adipose tissue of the digital cushion of the heel bulb did not indicate consistent predominant orientation of fibrous components (Fig. 6*a*, *b* respectively). The majority of patterns from the soft non-cornified epidermis and dermis tissue consisted of a diffuse ring centred around 0·36–0·37 nm. This value approximates to the pattern for reflectance (*d* spacings of 0·29–0·40) described by Meek *et al.* (1991) for steer-skin and rat-tail tendon, but which was orderly in the meridional axis and was associated with axial molecular organization. These authors also describe a diffuse ring at 0·33 nm in heavily hydrated bovine cornea; they relate this to scattering properties of water, which is a major component of the tissue under these conditions and which would be expected to be present in bovine soft tissue under the conditions of the present study.

Exceptions in the present study were a small number (4/48) which demonstrated defined arcs and for which *d* values in the range of 1·45–1·56 nm were obtained. These are more consistent with the values for equatorial reflectance for intermolecular Bragg spacing ranging from 1·15 nm in collagen of dry corneal tissue to 1·60 nm at approximately normal physiological levels of hydration and described by Meek *et al.* (1991). The complexities of elucidating the molecular structure of collagen type I *in situ* has been the subject of recent consideration by Orgel *et al.* (2006). As regards keratins, it is also possible that increases in intermolecular spacings of these molecules by hydration (Kreplak *et al.* 2004) may also have produced a similar pattern in the soft tissue epidermis component of the samples tested.

The general absence of a consistent orientation of diffraction patterns in the soft tissue sites tested suggested a random network of fibres such as found

in skin and artery wall (Purslow *et al.* 1998). However, the question of whether orientation is a factor in load-bearing function applicable *in vivo* was not tested in the unloaded post mortem claws of the present study.

CONCLUSIONS

In conclusion, the present results describe differences in the structural anatomy of claws derived from beef and dairy cattle. In particular, dairy claws exhibited lower values for dorsal angle, claw height and bulb (heel) height with reduced thickness of solear claw horn and digital cushion at the bulb. These anatomical properties appear typical of those expected to determine susceptibility to development of lesions which cause lameness under conditions of dairy husbandry. While, from XRD measurements, there was limited evidence of orientation of macromolecules in soft tissue epidermis and dermal connective tissue, there was clear evidence of site-specific alignment of fibrils in cornified horn in sole and wall. The latter appeared to relate to load-bearing function. Future studies could usefully examine the effects of load bearing on macromolecular fibril orientation under conditions of tensile stress using, for example, the techniques of Purslow *et al.* (1998).

REFERENCES

ANON. (2006). *The Lamecow Project*. Available online at http://template.bio.warwick.ac.uk/E+E/lamecow/public_html/lesions.html (accessed April 2007, verified 15/05/2007).

BUSSON, B., BRIKI, F. & DOUCET, J. (1999). Side-chains configurations in coiled coils revealed by the 5·15-angstrom meridional reflection on hard alpha-keratin X-ray diffraction patterns. *Journal of Structural Biology* **125**, 1–10.

BRIKI, F., DOUCET, J. & ETCHEBEST, C. (2002). A procedure for refining a coiled coil protein structure using X-ray fiber diffraction and modeling. *Biophysical Journal* **83**, 1774–1783.

BUDRAS, K. D., GEYER, H., MAIERI, J. & MÜLLING, C. K. W. (1998). Anatomy and structure of hoof horn (Workshop Report). In *10th International Symposium on Lameness in Ruminants* (Eds Ch. J. Lischer and P. Ossent), pp. 176–199. Lucerne, Switzerland.

CAMERON, G. J., WESS, T. J. & BONSER, R. H. C. (2003). Young's modulus varies with differential orientation of keratin in feathers. *Journal of Structural Biology* **143**, 118–123.

CRESPO, R., GALBRAITH, H. & FINN, D. (1999). Expression of extracellular matrix proteins in solear tissues of Holstein–Friesian dairy cattle. In *Proceedings of the Symposium on Metabolic Stress in Dairy Cows*. BSAS Occasional Publication No. 24, pp. 209–214. Penicuik, Midlothian: British Society of Animal Science.

FRASER, R. D. B. & PARRY, D. A. D. (2006). The three-dimensional structure of trichocyte (hard alpha-) keratin intermediate filaments: The nature of the repeating unit. *Journal of Structural Biology* **155**, 375–378.

GALBRAITH, H. (1998). Nutritional and hormonal regulation of hair follicle growth and development. *Proceedings of the Nutrition Society* **57**, 195–205.

GALBRAITH, H., RAE, M., OMAND, T., HENDRY, K. A. K., KNIGHT, C. H. & WILDE, C. J. (2006a). Effects of supplementing pregnant heifers with methionine or melatonin on the anatomy and other characteristics of their lateral hind claws. *The Veterinary Record* **158**, 21–25.

GALBRAITH, H., FLANNIGAN, S., SWAN, L. & CASH, P. (2006b). Proteomic evaluation of tissues at functionally important sites in the bovine claw. *Cattle Practice* **14**, 127–137.

GILLESPIE, J. M. (1991). The structural proteins of hair. Isolation, characterisation and regulation of biosynthesis. In *Physiology, Biochemistry and Molecular Biology of Skin* (Ed. L. A. Goldsmith), pp. 625–659. New York: Oxford University Press.

GREENOUGH, P. R. (1997a). Conformation, growth and heritable factors. In *Lameness in Cattle*, 3 edn (Eds P. R. Greenough & A. D. Weaver), pp. 71–86. Philadelphia, USA: W. B. Saunders.

GREENOUGH, P. R. (1997b). Horizontal grooves and fissures. In *Lameness in Cattle*, 3 edn (Eds P. R. Greenough & A. D. Weaver), pp. 111–113. Philadelphia, USA: W. B. Saunders.

HAMMOND, A., BIRNIE, M., KING, T. P. & GALBRAITH, H. (2000). Expression of actin, vimentin and keratins in solear dermis and epidermis of Holstein × Friesian dairy cattle. In *XI International Symposium on Disorders of the Ruminant Digit* (Eds C. M. Mortellaro, L. de Vecchis & A. Brizzi), pp. 119–121. Parma, Italy.

HENDRY, K. A. K., MACCALLUM, A. J., KNIGHT, C. H. & WILDE, C. J. (1997). Laminitis in the dairy cow: a cell biological approach. *Journal of Dairy Research* **64**, 475–486.

HENDRY, K. A. K., KNIGHT, C. H., GALBRAITH, H. & WILDE, C. J. (2003). Basement membrane integrity and keratinization in healthy and ulcerated bovine hoof tissue. *Journal of Dairy Research* **70**, 19–27.

HEPBURN, N. L., GALBRAITH, H. & KINNINMONTH, L. (2007). Pigmentation, impression hardness and the presence of melanosomes in bovine claw tissue. *Journal of Agricultural Science, Cambridge* **145**, 283–290.

HINTERHOFER, C., FERGUSON, J. C., APPRICH, V., HAIDER, H. & STANEK, C. (2005). A finite element model of the bovine claw under static load for evaluation of different flooring conditions. *New Zealand Veterinary Journal* **53**, 165–170.

HUKINS, D. W. L. (1977). X-Ray diffraction by collagen tape shows that type I collagen fibrils need not have a three-dimensional lattice. *Biochemical and Biophysical Research Communications* **77**, 335–339.

KASAPI, M. A. & GOSLINE, J. M. (1997). Design complexity and fracture control in the equine hoof wall. *The Journal of Experimental Biology* **200**, 1639–1659.

KREPLAK, L., DOUCET, J., DUMAS, P. & BRIKI, F. (2004). New aspects of the α-helix to β-sheet transition in stretched hard α-keratin fibres. *Biophysical Journal* **87**, 640–647.

KEMPSON, S. A. & LOGUE, D. N. (1993*a*). Ultrastructural observations of hoof horn from dairy cows – the structure of the white line. *The Veterinary Record* **132**, 499–502.

KEMPSON, S. A. & LOGUE, D. A. (1993*a*). Ultrastructural observations of hoof horn from dairy cows: changes in the white line during the first lactation. *The Veterinary Record* **132**, 524–527.

LEES, S. & HUKINS, D. W. L. (1992). X-Ray diffraction by collagen in the fully mineralised cortical bone of cow tibia. *Bone and Mineral* **17**, 59–63.

LISCHER, C. J. & OSSENT, P. (2000). Sole ulcers in dairy cattle – what's new about an old disease. In *III International Conference on Bovine Lameness* (Eds C. M. Mortellaro, L. de Vecchis & A. Brizzi), pp. 46–55. Parma, Italy.

MACCALLUM, A. J., KNIGHT, C. H., HENDRY, K. A. K., WILDE, C. J., LOGUE, D. N. & OFFER, J. E. (2002). Effects of time of year and reproductive state on the proliferation and keratinisation of bovine hoof cells. *The Veterinary Record* **151**, 285–289.

MEEK, K. M., FULLWOOD, N. J., COOKE, P. H., ELLIOTT, G. F., MAURICE, D. M., QUANTOCK, A. J. & WALL, R. S. (1991). Synchrotron x-ray-diffraction studies of the cornea, with implications for stromal hydration. *Biophysical Journal* **60**, 467–474.

ORGEL, J. P. R. O., IRVING, T. C., MILLER, A. & WESS, T. J. (2006). Microfibrillar structure of type I collagen *in situ*. *Proceedings of the National Academy of Sciences USA* **103**, 9001–9005.

OSSENT, P., LISCHER, CH. J., RABER, M. & GEYER, H. (2000). The significance of the suspensory mechanism of the third phalanx in the pathogenesis of sole ulcers in cattle. Part II: microscopic findings. In *XI International Symposium on Disorders of the Ruminant Digit* (Eds C. M. Mortellaro, L. de Vecchis de L. & A. Brizzi), pp. 226–229. Parma, Italy.

PARRY, D. A. D. (1996). Hard alpha-keratin intermediate filaments: An alternative interpretation of the low-angle equatorial X-ray diffraction pattern, and the axial disposition of putative disulphide bonds in the intra- and inter-protofilamentous networks. *International Journal of Biological Macromolecules* **19**, 45–50.

PARRY, D. A. D., SMITH, T. A., ROGERS, M. A. & SCHWEIZER, J. (2006). Human hair keratin-associated proteins: sequence regularities and structural implications. *Journal of Structural Biology* **155**, 361–369.

PLOWMAN, J. E. (2003). Proteomic database of wool components. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **787**, 63–76.

PURSLOW, P. P., WESS, T. J. & HUKINS, D. W. L. (1998). Collagen orientation and molecular spacing during creep and stress – reaction in soft connective tissues. *The Journal of Experimental Biology* **201**, 135–142.

QUANTOCK, A. J., KINOSHITA, S., CAPEL, M. S. & SCHANZLIN, D. J. (1998). A synchrotron x-ray diffraction study of developing chick corneas. *Biophysical Journal* **74**, 995–998.

TARLTON, J. F., HOLAH, D. E., EVANS, K. M., JONES, S., PEARSON, G. R. & WEBSTER, A. J. F. (2002) Biomechanical and histopathological changes in the support structures of bovine hooves around the time of first calving. *The Veterinary Journal* **163**, 196–204.

TOMLINSON, D. J., MUELLING, C. H. & FAKLER, T. M. (2004). Invited review: formation of keratins in the bovine claw: roles of hormones, minerals and vitamins in functional claw integrity. *Journal of Dairy Science* **87**, 797–809.

VAN AMSTEL, S. R., PALIN, F. L. & SHEARER, J. K. (2004*a*). Measurement of the thickness of the corium and subcutaneous tissue of the hind claws of dairy cattle by ultrasound. *The Veterinary Record* **155**, 630–633.

VAN AMSTEL, S. R., SHEARER, J. K. & PALIN, F. L. (2004*b*). Moisture content, thickness and lesions of sole horn associated with thin soles in dairy cattle. *Journal of Dairy Science* **87**, 757–763.

VERMUNT, J. J. (2004). Herd lameness – a review, major causal factors and guidelines for prevention and control. In *13th International Symposium on Lameness in Ruminants* (Ed. B. Zemljic), pp. 3–18. Maribor, Slovenia.

VERMUNT, J. J. & GREENOUGH, P. R. (1995). Structural characteristics of the bovine claw: horn growth and wear, horn hardness and claw conformation. *British Veterinary Journal* **151**, 157–180.

WEBSTER, A. J. F. (2002). Effects of housing practices on the development of foot lesions in dairy heifers in early lactation. *The Veterinary Record* **151**, 9–12.

WESS, T. J. & CAIRNS, D. E. (2005). Nanoarchitectures of the animal extracellular matrix: opportunities for synchrotron radiation studies on collagen and fibrillin. *Journal of Synchrotron Radiation* **12**, 751–757.

WESTERFIELD, I., MÜLLING, C. K. W. & BUDRAS, K. D. (2000) Suspensory apparatus of the distal phalanx (PH111) in the bovine hoof. In *XI International Conference on Disorders of the Ruminant Digit* (Eds C. M. Mortellaro, L. de Vecchis & A. Brizzi), pp. 103–108. Parma, Italy.

WILKINSON, S. J. & HUKINS, D. W. L. (1999). Determination of collagen fibril structure and orientation in connective tissues by X-ray diffraction. *Radiation Physics and Chemistry* **56**, 197–204.