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Pigmentation, impression hardness and the presence of melanosomes in bovine claw tissue

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SUMMARY

The physical properties of hardness and dry matter and the histological characteristics of dermal and epidermal tissues were investigated in lateral and medial claws obtained post mortem from mature crossbred female cattle. Claws containing pigmented ($n=22$) or non-pigmented ($n=22$) coronary wall horn were studied. Impression hardness, measured on the dorsal border of claws, proximo-distal at intervals of 5 mm from the lower perioplic line was shown to increase to the 20 mm lower site. Similarly, in comparison with values for pigmented horn, pooled measurements for non-pigmented horn showed greater hardness from the lower perioplic line to the 40 mm lower site but were not different for subsequent measurements towards the distal edge. Differences in values recorded for sole and heel and for coronary wall horn measured under the distal edge were not different for claws varying in coronary horn pigmentation. The absence of differences in dry matter at measurement sites suggested that differences in impression hardness values were not caused by variation in water content of horn. Histological examination of pigmented claw wall horn and underlying soft tissue showed pigment-containing cells in the coronary epidermis but not coronary dermis or in dermis or epidermis of laminae. Pigmentation appeared concentrated in melanosomes in cells along the basement membrane and was present uniformly throughout the epidermis and in cells lining horn tubules. There was no pigmentation detected in the soft tissue epidermis of claws selected visually for non-pigmented wall horn. The presence of melanosomes in epidermal cells from the pigmented coronary region was demonstrated by electron microscopy. The results suggest that pigmentation in the coronary region is associated with reduced initial development of hardness. The possible antagonism between antioxidant properties of melanins and requirement for an oxidative environment for disulphide bond formation in the cytoskeleton of horn cells is discussed.

INTRODUCTION

Lameness is increasingly recognized as an endemic production disease that may affect up to 0·6 of cattle in dairy herds (Vermunt 2004). It may derive from microbial infection or be non-infectious in origin and caused by lesions affecting the horn capsule of the claw. Risk factors have been described as extrinsic and related to external environment or intrinsic and associated with the biology of the foot and other tissues within the animal. Among such intrinsic factors is the 'quality' of horn at individual sites within the claw (Kempson & Logue 1993; Galbraith *et al.* 2006).

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Claw horn is produced by proliferation and differentiation of specialized cells of the epidermis, which is the outermost layer of the claw integument (Budras *et al.* 1998; Galbraith *et al.* 2006). The epidermis is non-vascular and its basal horn-forming cells are located on the basement membrane adjacent to the underlying vascularized dermis. The composition and properties of horn vary according to site of production in, for example, coronary, laminar, sole and heel regions. The dermis supplies nutrients and regulatory molecules to support synthetic processes of horn production in the epidermis (Tomlinson *et al.* 2004).

Good quality horn is characterized by the presence of cornified epidermal cells containing a well-synthesized cytoskeleton and effective intercellular adhesion. A range of biomechanical properties

Table 1. *Mean values and pooled mean values (± S.E.M.) for impression hardness of the external surface of horn for anatomical sites shown in Fig. 1 a, b*

Sites of testing	Claws with non-pigmented horn	Claws with pigmented horn	Significance
C–G	47 ± 1·8	40 ± 1·6	$P < 0{\cdot}05$
H–K	68 ± 0·5	65 ± 0·7	$P < 0{\cdot}001$
L–S	71 ± 0·4	70 ± 0·4	n.s.
UDE	43 ± 2·6	37 ± 2·8	n.s.
Sole	28 ± 2·3	27 ± 2·3	n.s.
Heel	27 ± 1·5	25 ± 1·9	n.s.

determines the effectiveness of claw components in body weight-bearing and protection of underlying soft tissue. These include connective tissue strength (Tarlton *et al*. 2002) and response to material stresses (Hinterhofer *et al*. 2004). The use of impression hardness measurements has also contributed to an understanding of 'hardness' as an important physical property of claw horn (Vermunt & Greenough 1995). It has been shown to vary according to the concentration of sulphur amino acids and location in the horn capsule (Galbraith *et al*. 2006). The greater impression hardness values of the wall region are associated with greater rigidity required for suspension of body weight, whereas sole and heel horn provide greater flexibility and softness required for locomotion.

An additional compositional property of horn epidermis is the variable presence of pigmentation in the wall region. The presence of such pigmentation has been associated with greater resistance to lameness and with perceptions of better quality horn. However, results published in the literature are not consistent (Vermunt & Greenough 1995). Work carried out on equine feet showed that hoof colouring did not affect the material properties of the hoof (Landeau *et al*. 1983; Douglas *et al*. 1996) although Runciman *et al*. (2004) presented evidence showing that peak extraction force and energy required for removal of nails were more variable in hooves with dark-coloured compared with light-coloured horn.

Pigmentation in integumental tissues, such as skin and hair follicles, is produced by melanocytes (Renieri 1994; Guibert *et al*. 2004). These cells are neurodermal in origin and derive from melanoblasts, which migrate during development from the embryonic neural crest into a range of tissues. Melanocytes transfer melanosome organelles into surrounding keratinocytes. These synthesize, from the common precursor L-tyrosine, the major 'melanin' pigments comprising the black-brown eumelanin and red-yellow pheomelanin. Production of melanins is regulated by melanocyte-stimulating hormone from the pituitary gland. These pigments provide photoprotective properties on tissues, particularly in response to ultraviolet radiation. Melanins are considered to be antioxidant but also under certain conditions may convert to pro-oxidant in action (Meyskens *et al*. 2004). The effects of the presence of melanosomes and deposited pigments on cytoskeleton and other structures in keratinocytes and on physical properties of bovine horn are poorly understood.

The aims of the present study were to test the hypotheses (i) that variations in pigmentation affected physical properties of dry matter and impression hardness in terminally differentiating keratinocytes in the horn capsule and (ii) that pigmentation was derived from the presence of intracellular melanosomes, which may be detected by techniques of conventional histology and electron microscopy.

MATERIALS AND METHODS

Colour classification, hardness, dry matter and electron microscopy of claws

Right hind medial and lateral claws were collected from female crossbred cattle from a commercial abattoir and transported to the laboratory on ice. The claws were wiped dry on the external surface and then graded by colour. Both claws from 11 heifers with very dark brown or black ('pigmented'), and those from 11 heifers with light brown ('non-pigmented'), wall horn were used in the study.

Impression hardness was determined, by the same operator, using a duropenetrometer (Type D model B101; Durotech, Northampton, UK). Measurements were made on coronary wall horn of the dorsal border (Budras & Habel 2003), at 5 mm intervals, proximo-distal, from the lower perioplic horn line to the distal edge (DE), under the distal edge (UDE) and on the sole (sole–bulb junction) and mid-heel at consistent locations (Table 1, Fig. 1). Readings were recorded in arbitrary units from 0 (very soft) and 100 (very hard).

Measurements of dry matter were made to test the hypothesis that water content may vary according to level of pigmentation and so affect the hardness of claw horn. Blocks (approximately 150 mm^2 measured on the external surface) of horn were taken in duplicate from randomly selected claws from heifers with either pigmented ($n = 5$) or non-pigmented wall horn ($n = 5$). The sites sampled were: uppermost coronary horn from immediately below the lower perioplic line; mid-wall; wall at the DE; sole at the sole–bulb junction; and heel. Non-cornified soft tissue was removed and the blocks of horn were dissected into smaller blocks (approximately 50 mm^2) and fresh weights recorded. They were then dried at 80 °C to

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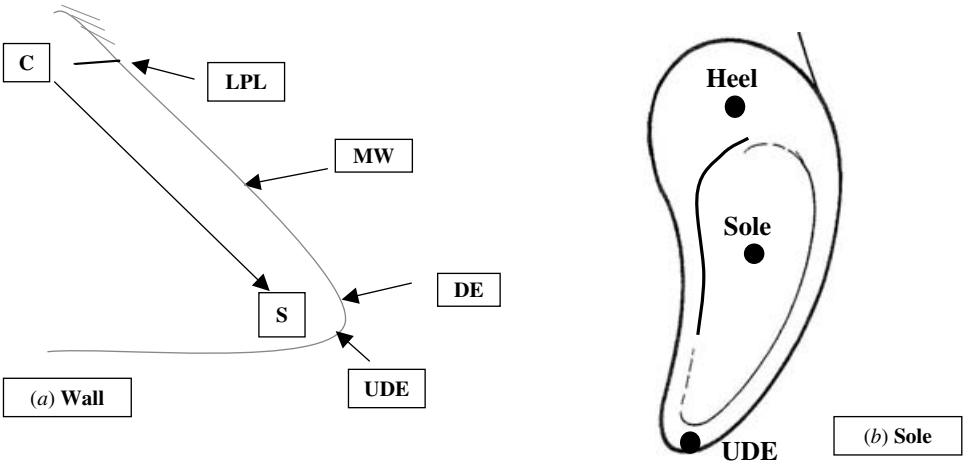

Fig. 1. Sites of measurement of impression hardness and collection of horn for determination of dry matter: (*a*) measurements were made on wall horn at 5 mm intervals from point C at the lower perioplic line (LPL) to S towards the distal edge (DE) and under the distal edge (UDE) and (*b*) (solear perspective). At UDE, sole and heel sites (MW = mid-wall).

constant weight and the content of dry matter calculated.

Fresh blocks of coronary soft tissue (1–2 mm thick) for transmission electron microscopy (TEM) were fixed in glutaraldehyde (25 g/l in 0·1 M phosphate buffer pH 7·4) for 4 h and then washed overnight in three changes of 0·1 M phosphate buffer pH 7·4. The blocks were post-fixed in osmium tetroxide (10 g/l in distilled water) for 1 h and then dehydrated through an ethanol series; 500, 700, 900 and 950 ml/l in distilled water for 15 min each, ending with 4×15 min in undiluted ethanol. The samples were then placed into propylene oxide for 2×10 min and then a propylene oxide:resin (1:1) mixture for 2 h. Blocks were left in resin overnight on a rotary mixer at 5 rpm and subsequently embedded in mould and polymerized in an oven at 60 °C for 24 h. Semi-thin sections of each block were prepared and stained using methylene blue and observed under a light microscope to determine whether the tissue sections were correctly cut. Ultrathin (80–90 nm) sections were then conventionally prepared and stained with heavy metals and viewed by electron microscopy (CM10, Philips).

The Fontana-Masson technique was used to detect pigment and pigment-containing structures in cells (Barrnett & Seligman 1954; Duval *et al*. 2002). Tissue sample blocks (approx. $6 \times 5 \times 4$ mm) were taken from four pigmented and four non-pigmented claws. Vertical sections (10 μm) of soft tissue dermis and epidermis and cornified horn were prepared from the coronary and mid-wall regions using a cryostat (2800 Frigocut E, Reichart-Jung) and mounted onto acid-washed slides. Sections were fixed with paraformaldehyde (40 g/l in phosphate-buffered saline) for 4 min and then phosphate-buffered saline for 4 min. The slides were then placed through a series of solutions prepared in distilled water: silver nitrate (110 g/l) at 56 °C for 60 min; gold chloride (1·0 g/l) for 10 min; sodium thiosulphate (50 g/l) for 5 min. Finally, sections were counterstained with Eosin Y Phyloxine stain (1·0 g/l) and viewed under a light microscope (BX-UCB, Olympus) with conventional image capture.

Data analysis

Data were examined statistically by one-way ANOVA (Minitab v 13.3, General Linear Model) followed by Tukey's method for pairwise comparisons of means. Differences in means were considered significant at $P < 0{\cdot}05$ for all comparisons made.

RESULTS

Impression hardness and dry matter concentration

The results for impression hardness for both pigmented and non-pigmented claw wall showed a progressive increase from the uppermost measurement site to the 20 mm lower site at point G (Fig. 2). The non-pigmented horn showed, on average for pooled values, significantly greater values for hardness in measurements taken from the uppermost site to the 40 mm lower site (points C–G ($P < 0{\cdot}05$) and H–K ($P > 0{\cdot}01$)) with no differences recorded thereafter (Fig. 2 and Table 1). There were no significant differences in values for hardness between pigmented and non-pigmented horn at sites UDE of the dorsal wall and for sole and heel of the same claws. Similarly, there were no significant differences in

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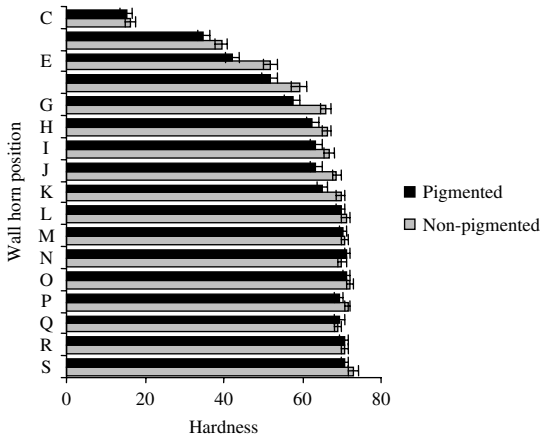


Fig. 2. Mean values (±S.E.M.) for impression hardness for pigmented and non-pigmented horn are shown for the external surface of wall horn from the coronary region at the lower perioplic line (LPL, site C) towards the distal edge (DE, site S).

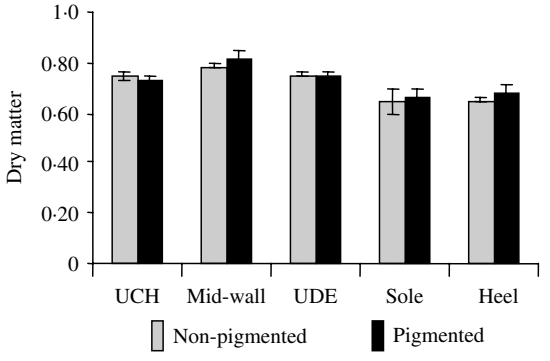
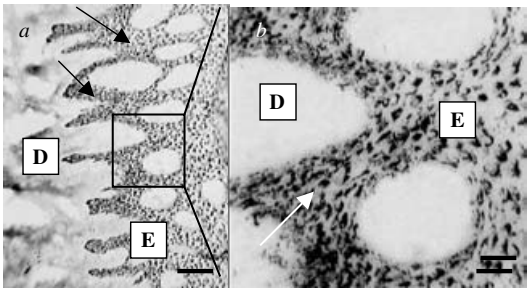


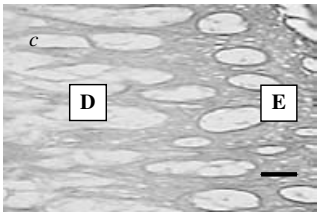
Fig. 3. Dry matter (proportion of fresh weight) in horn samples from claws with pigmented or non-pigmented wall horn. Results are shown as mean ± S.E.M. for $n = 5$ animals (UCH = uppermost coronary horn from below the lower perioplic line; UDE = under the distal edge).

mean values for dry matter between samples of pigmented and non-pigmented horn when compared for the same sites (Fig. 3). As expected, values for hardness were greatest in the order of coronary horn (dorsal aspect) > coronary horn (UDE) > sole ⩾ heel.

Source of pigmentation

Histological examination by Fontana-Masson staining of soft tissue of pigmented claws showed clear evidence of the presence of pigment-containing cells in basal and supra-basal epidermis, of coronary





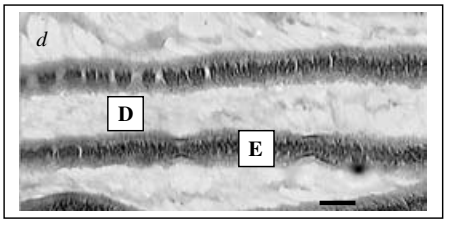


Fig. 4. Fontana-Masson staining of sections of dermal and epidermal soft tissue from claws. (*a*, *b*) Coronary region of a pigmented claw. (*a*) Bar = 100 μm, (*b*) bar = 25 μm (arrows show pigment-containing cells). (*c*) Coronary region of a non-pigmented claw, bar = 100 μm. (*d*) Mid-laminar region of a pigmented claw, bar = 100 μm. In text boxes, D = dermis and E = epidermis.

region soft tissue (Fig. 4*a*, *b*). There was no evidence of positive staining in epidermis or dermis in sections of laminae of the wall or in other non-pigmented regions (Fig. 4*c*, *d*). Similar differences in response to the staining system were recorded in comparing sections of cornified mid-wall horn containing non-pigmented or pigmented horn (Fig. 5*a*, *b* respectively). There was evidence of presence of pigmentation in cornified intertubular horn and in cells lining horn tubules (Fig. 5*b*). Examination of ultrathin sections of coronary epidermis by electron microscopy showed the presence of melanosomes within cells in the sections prepared from claws containing pigmented horn (Fig. 6).

DISCUSSION

Hardness is an important property that develops during differentiation and movement towards the external surface of the horn capsule of basal epidermal

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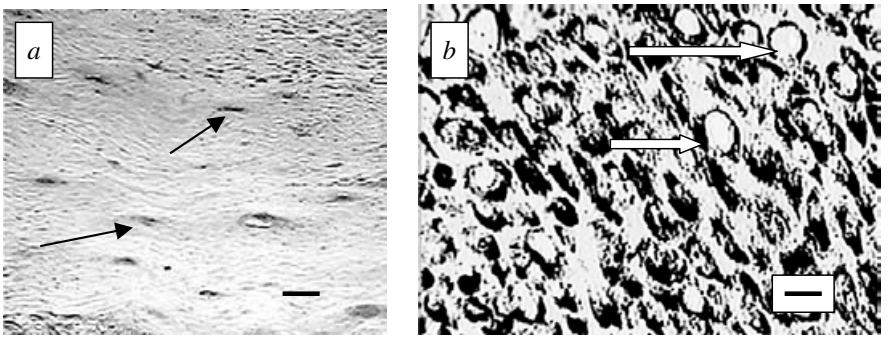

Fig. 5. Fontana-Masson staining of sections of (*a*) non-pigmented mid-wall coronary horn, bar = 100 μm, and (*b*) pigmented mid-wall coronary horn, bar = 20 μm. Arrows show position of tubules.

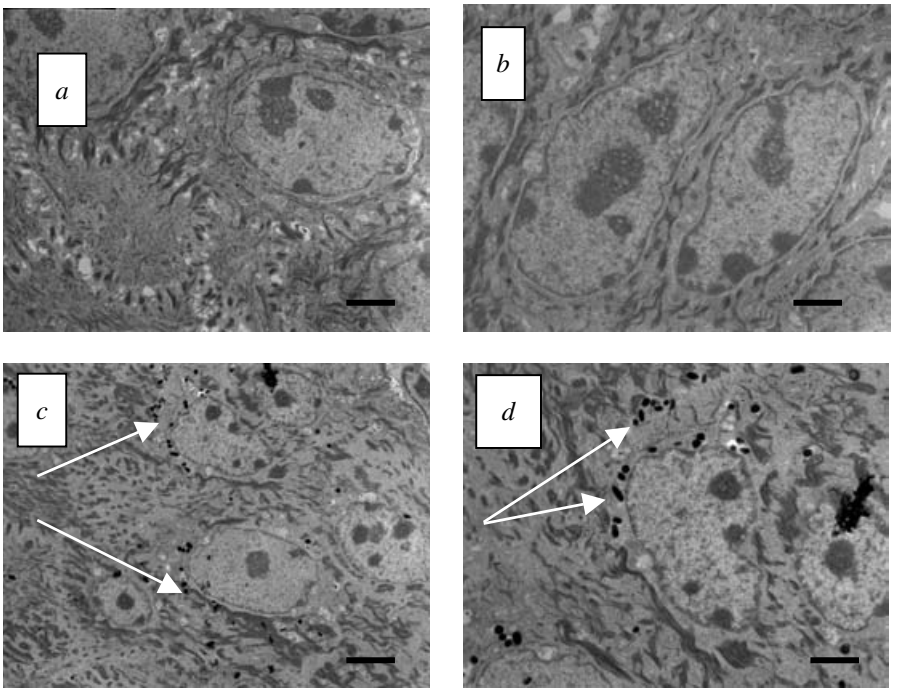

Fig. 6. Electron micrographs of sections of coronary region soft tissue epidermis from claws with pigmented or non-pigmented horn. (*a*) Non-pigmented claw, bar = 3 μm. (*b*) Non-pigmented claw, bar = 2 μm. (*c*) Pigmented claw showing melanosomes (arrows), bar = 6 μm. (*d*) Pigmented claw showing melanosomes (arrows), bar = 3 μm.

cells. The present results suggest that the differentiation process and movement of cornifying cells produced horn of increasing hardness, which maximized, for non-pigmented surface wall horn, at approximately 20 mm below the uppermost measurement. There was also evidence in the present study that, in comparison with non-pigmented horn, the development of hardness was further delayed in pigmented horn until 40 mm below the initial measurement. Subsequent multiple measurements towards the DE and which included those at mid-wall (site L) showed that differences associated with pigmentation were not maintained. These results contrast with those of Clark & Rakes (1982) where measurements on claws of dairy cows, although confined to single sites, did not detect differences in hardness measurements between horn differing in pigmentation, although they did find evidence of differences in hardness between upper and lower sites on dorsal wall.

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Mechanisms responsible for producing the observed differences in hardness values may be considered in terms of chemical composition and physical structure of horn tissue. Chemical composition may be affected by the supply of nutrients, particularly cysteine which is required to provide sulphydril groups in structurally important intermediate filament (IF) and IF associated proteins. These groups undergo oxidation to form intra- and inter-molecular disulphide bonds which, for example, in the more fully described mammalian hair cortex (Wang *et al.* 2000; Fraser & Parry 2003) are known to stabilize the cytoskeleton in differentiating keratinocytes. In horn, such disulphide bond formation, which is known to increase hardness values, has been shown to increase markedly following initial cornification and horn formation (Clark & Rakes 1982; Budras *et al.* 1998; Galbraith *et al.* 2006). One possibility in the claws investigated in the present study is that this process may have continued to the locations in horn where the maximal values were observed.

Mechanisms regulating the oxidation of sulphydril groups required for disulphide bond formation specifically in horn are not known but may include the spontaneous formation of such bonds, perhaps involving molecular oxygen as an electron acceptor or involvement of specific enzyme systems (Tu & Weissman 2004). Although untested, interference with such a mechanism by a more antioxidative environment, such as produced by melanins in other tissues (Meyskens *et al.* 2004; Wang *et al.* 2006), could also provide an explanation for the apparently slower development of hardness in pigmented horn.

Included in the process of late differentiation are reductions in water content as epidermal cells undergo dehydration, which is particularly marked during cornification. The importance of residual water content in determining hardness and other mechanical properties of claw horn has been considered by Vermunt & Greenough (1995) and more recently by van Amstel *et al.* (2004) and Borderas *et al.* (2004). The major conclusion is that, for a given site, water concentration does influence, and is negatively related to, hardness. The results from the present study showed that there were no differences between pigmented and non-pigmented horn samples with respect to water content. It may therefore be concluded that differences in hardness between these types of horn were not due to water content and may be ascribed to other properties of the cornified material.

The effect of pigmentation has also been studied in respect of retention of shoe nails in horn capsule of horse hoof by Runciman *et al.* (2004), who described a common belief that nails were retained more effectively in black-pigmented horn than white horn. Their results, however, suggested that mean values for peak extraction force and energy resistance for extraction of nails were not significantly different, but that variability was greater for dark-coloured hooves. The reasons for the greater variability and its relation to pigmentation were not clear.

In the present study, histological sections were examined by Fontana-Mason staining procedure to investigate the presence and the location of pigmentation in soft tissue and cornified coronary wall horn. Pigment-containing cells, some of which must be melanocytes which produce the original melanosomes, were apparent along the basement membrane and suprabasally. There was evidence also of pigmentation lining the cells of the cortex of horn tubules. The results demonstrated the absence of pigment in dermal tissues and in epidermis of the laminar region. The presence of melanosome-produced pigmentation was evident throughout the whole of the wall horn capsule or only in parts to form a striped pattern in certain claws (not shown). Electron microscopy confirmed the presence of melanosomes in coronary epidermal cells. The melanosomes were electron dense and spherical or oval in shape and were similar in appearance to those reported by Cecchi *et al.* (2004) in melanocytes in skin and hair samples of South American llama and in human skin by Killingsworth & Allman (2004), respectively.

CONCLUSIONS

The physical properties of hardness and dry matter and the histological characteristics of dermal and epidermal tissues were investigated in pigmented and non-pigmented coronary wall horn of crossbred beef heifers. Impression hardness for pigmented and non-pigmented wall horn at the dorsal border increased progressively from the uppermost site at the lower perioplic line, proximo-distal, to a 20 mm lower site. Similarly, in comparison with values for pigmented horn, pooled measurements for non-pigmented horn showed greater hardness from the uppermost to the 40 mm lower site, but were not different for subsequent measurements towards the DE. Differences in values recorded for sole and heel and UDE were not different for claws varying in coronary horn pigmentation. The absence of differences in dry matter at measurement sites suggested that differences in impression hardness values were not caused by variation in water content of horn. Histological examination of pigmented claw wall horn showed pigment-containing cells in the coronary epidermis but not coronary dermis or in dermis or epidermis of laminae. Pigmentation was evident along the basement membrane at the uppermost region of inter-papillary cells and was present uniformly throughout the epidermis and in cells lining horn tubules. There was no pigmentation detected in the epidermis of claws selected for visually non-pigmented wall horn.

The present results suggest that pigmentation in the coronary region is associated with a reduced initial development of hardness which is known to be promoted by intracellular disulphide-bonding which depends on an oxidative environment. It is suggested that the delayed production in hardness may be associated with the presence of a reducing chemical environment produced by antioxidant properties of melanins. Effects on hardness were not maintained beyond mid-wall and so may not be expected to affect contribution to suspension of body weight or wear of horn UDE.

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