Involvement of the Digital Cushion and the Distal Phalanx in the Development and Reoccurrence of Claw Horn Disruption Lesions in Dairy Cattle

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Abstract

Claw horn disruption lesions (**CHDL**s: sole haemorrhage, sole ulcer and white line disease) cause a large proportion of lameness in dairy cattle and have a high rate of recurrence, yet their aetiopathogenesis remains poorly understood. Untreated CHDLs appear to be associated with trauma within and damage to the internal anatomy of the foot. Chapter 2 explored associations between abnormal bone modelling on the flexor tuberosity of the distal phalanx of cull cows and lameness during life, using a retrospective cohort study design. The hind claws of 72 Holstein dairy cows culled from a research herd were imaged using μ-computed tomography (**CT**) and lameness and lesion incidence data were available on cows throughout life. Four measures of bone modelling were taken from CT images from the flexor tuberosity of each distal phalanx, in plantar, distal and dorsal planes, and combined within claw. Bone modelling was greater in older cows, in cows with history of CHDL and in cows that had been lame at an increased proportion of locomotion scores during the 12 months preceding slaughter. Further, histological study demonstrated that the bone modelling resembled heterotopic ossification, also termed osteoma, which could have been due to either inappropriate force transfer through the distal phalanx or pathology in the soft tissues with lesion presence. Anatomical damage within the foot does appear to be associated with lameness and CHDLs, and may further predispose lameness.

Preventing lameness constitutes a critical component of lameness control, and prophylactic foot trimming is a common management strategy for maintaining claw structure and function. However, over-trimming can cause damage to the foot architecture and lameness. Step 1 of the widely used Dutch Method of foot trimming states to cut the dorsal wall of the hoof to 75 mm. A vertical 5 mm step is left at the toe, therefore based on these recommendations, dorsal wall length would be 82 mm if the toe were trimmed to a point and the dorsal wall extended to the floor (at a toe angle of 50°). Chapter 3 used the CT data to assess the *minimum* dorsal wall length that would be suitable for trimming each claw. The median length was 76 mm (83 if the toe were trimmed to a point) and ranged from 59 to 86 mm; trimming all claws to 75 mm would have over-trimmed 55 % of claws. In a linear regression model, *minimum* dorsal wall length increased with age and carcass weight; older and larger cows had bigger claws. However, the vast majority of variation in claw length remained unexplained (only 22 % of the null variance was explained). In order to minimise the number of claws that are over-trimmed, recommendations for foot trimming dimensions should be based on the proportion of claws for which a measurement is suitable, rather than on population means. The *minimum* lengths that would have been suitable for all claws were 93 mm for cows aged ≥4 years and 86 for cows aged <4 years; 7 mm could be taken from these measurements if a step is left at the toe.

CHDLs appear to initially occur through trauma to the germinal epithelium of the sole, and Chapters 4, 5 and 6 present a longitudinal study of how the sole soft tissues (**SST**; i.e. the digital cushion and corium) alter throughout lactation. The digital cushion is a modified layer of the subcutis that is situated beneath the plantar and distal aspects of the distal phalanx and is considered to be important in dissipating forces during foot strike and to protect the germinal epithelium. The digital cushion contains depots of adipose tissue and recent work has identified that body condition loss is a risk factor for lameness. Previous work found that fatter cows had thicker SST and suggested that fat could be mobilized from the digital cushion and causes it to have decreased biomechanical function. The prospective cohort study assessed the SST of 179 parity 1, 2 3 or 4 cows at 5 assessment points, between 8 weeks pre- and 29 weeks post-calving of one lactation. Lesions present on claws and measures of body fat were recorded at each assessment point, and mobility scoring was performed fortnightly from calving. SST thickness at two sites beneath the distal phalanx were used as outcomes in 4-level mixed effects linear regression models (Chapter 5), and was positively correlated with back fat thickness. However, the effect size was much smaller than reported in previous cross-sectional work and only apparent under some circumstance. SST was thicker when a sole ulcer was present on a claw and was thinner immediately after calving (during the 4-10 days post-calving). The final model left 61 % of the null variance unexplained, of which 48 % remained between repeated measures of the same claw at different assessment points.

Chapter 6 presents a series of logistic regression models of survival to first lesion or to first lameness (repeated lameness events were initially tested, but models were discarded due to the high rates of recurrence of both lameness and lesions). Lesion models demonstrate that claws were more likely to develop a lesion if SST was thin, and there was an additional effect of having thin back fat (all animals) or having lost back fat between previous assessment points (parity >1 animals only). Lameness models demonstrated that thin SST on the lateral claw increased the odds of a leg becoming lame, but SST on the medial claw had no effect on lameness. *Change* in SST thickness did not predispose lesions or lameness; only absolute thinness did. The work suggests that whilst loss of body condition loss may be one variable that contributes towards thinning of SST and subsequent claw horn disruption, many other variables also had a large effect on SST thickness, CHDL and lameness.

This thesis presents a sequence of studies of how the anatomy of the foot is related to CHDL incidence, addressing recurrent lameness, mechanisms for the onset of new lameness and the appropriateness of prophylactic foot trimming guidelines as a management tool for lameness. The research literature is deficient in work demonstrating beneficial effects of interventions on lameness, and work throughout this thesis provides novel insights into the aetiopathogenesis of the claw horn disruption lesions. Based on this work, targeted interventions to reduce lameness can be tested.

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BCS	Body condition score
BFT	Back fat thickness
CHDL	Claw horn disruption lesion
CI	95 % confidence interval
CT	Computed tomography
DIM	Days in milk
IQR	Interquartile range
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
SD	Standard deviation
SE	Standard error
SST	Sole soft tissue

1 General Introduction

1.1 Lameness: a costly condition

Lameness can be defined as a disorder of locomotion that is associated with impaired function of at least one limb. Many painful conditions of dairy cattle that affect the locomotor apparatus cause lameness (Whay et al., 1997; O'Callaghan, 2002) and these diseases can principally be divided into two groups: infectious diseases such as digital dermatitis or interdigital necrobacillosis, and the non-infectious "claw horn disruption lesions" (**CHDL**), which include sole haemorrhage, sole ulcer and white line disease (Cramer et al., 2008; Barker et al., 2009; Capion et al., 2009; Bicalho and Oikonomou, 2013). Lameness is regarded as one of the three most important conditions afflicting cows within the dairy industry, alongside mastitis and fertility (Kossaibati and Esslemont, 1997), and affects the Holstein breed more than other dairy breeds (Alban, 1995; Anderson et al., 2007; Hoffman et al., 2013). Despite profound effects on cow health and dairy economics, lameness has historically received little research focus relative to other important diseases of dairy cattle, and this important disease still blights cows in developed dairy systems globally (Bicalho and Oikonomou, 2013).

1.1.1 Prevalence and incidence of lameness

Two metrics commonly used to report lameness levels are incidence and prevalence. Incidence reports the number of cases occurring within a population over a period of time, whilst prevalence reports levels in a population at a given time point, and can be reported as a proportion of the number of cows assessed (Archer *et al.*, 2010a). Based on data collected from 90 UK dairy herds between 1992 and 1993, Esslemont and Kossaibati (1996) reported lameness incidence to be 24 cases per 100 cows-years. Similar estimates have been reported more recently around the world (Bicalho *et al.*, 2008; Olechnowicz and Jaskowski, 2010). However, reporting a reliable incidence of lameness requires the diligence to detect every lame case in a consistent manner and to distinguish between new and repeat lameness cases, and reports vary greatly. A detailed study of five farms where locomotion was monitored intensely and treatments were administered free of charge found lameness incidence to be 68.9 cases per 100 cow-years, and it differed greatly between farms (Hedges *et al.*, 2001). Reporting an accurate lameness incidence can therefore be difficult, but in a review of UK literature published between 1972 and 2010, Archer *et al.* (2010a) estimated that on average, in published literature, 50-100 limb-cases of lameness occurred per 100 cows per year.

On the other hand, prevalence levels might be easier to identify since they can be assessed at a snapshot in time. Barker *et al.* (2010) reported that on average 37 % of cows were lame on the day of assessment across 227 farms in England and Wales, with a range between farms of 0 to 79 %. Another large study has reported lameness prevalence of 21 % (Clarkson *et al.*, 1996). The difference in prevalence reported by these two studies could represent an increase during the intervening years, it could represent a difference in monitoring precision, or it could represent an increase in the prevalence of digital dermatitis (Barker *et al.*, 2007). Studies in other countries have reported similar lameness prevalence to UK studies, for example Hoffman *et al.* (2013) and Espejo *et al.* (2006) reported a lameness prevalence of 36 % and 25 % in Holstein herds in the United States, Dippel *et al.* (2009) reported 34% across Germany and Austria and recently Solano *et al.* (2015) reported 21% in Canada.

1.1.2 Economic implications associated with lameness

Lameness has considerable financial implications. In an economic review, Wilshire and Bell (2009) summarized the main financial costs associated with lameness as loss of milk yield, decreased fertility, increased risk of culling (and subsequent replacement costs) and treatment costs. The first three are hidden costs and constituted 87 % of the financial burden; the main costs are not seen as a direct expense related to treatment.

The highest yielding cows are at the greatest risk of sole ulcer and white line disease (Amory *et al.*, 2008; Oikonomou *et al.*, 2013), and milk production appears to be reduced both before and after a lameness event (Warnick *et al.*, 2001; Green *et al.*, 2002; Reader *et al.*, 2011). One estimate of the milk losses associated with CHDLs stated that 350 kg of milk per 305 day lactation was lost per lesion incidence, with greater losses when lesions persist or recur (Archer *et al.*, 2010b); another estimate found 570 and 370 kg lost specifically associated with sole ulcer and white line disease, respectively (Amory *et al.*, 2008). Further, both studies found that chronically lame cows were associated with the greatest losses. Similar estimates have been reported in other countries, with sole ulcer consistently being considered as associated with the greatest production loss (Cha *et al.*, 2010). In contrast, Haskell *et al.* (2006) did not find production to be a risk factor for lameness, yet with lameness being a disease of the highest yielding cows and production decreasing when lame, the work may have failed to identify milk yields of lame cows as being lower *than expected* for those cows that went lame. Most sources concur that lameness is associated with high milk production.

Lameness has long been considered to have profound effects on fertility, leading to reduced reproductive cyclicity (Garbarino *et al.*, 2004), increased calving to first service and conception intervals and lower pregnancy rates to first service (Collick *et al.*, 1989; Hernandez *et al.*, 2001; Hernandez *et al.*, 2005). A study of 112 herds found that sole

ulcers were associated with increased intervals from calving to both first and last service, and white line lesions were associated with poorer conception (Sogstad *et al.*, 2006). Reduced fertility in turn reduces survival within the herd (Bicalho *et al.*, 2007b). Contrastingly, a recent simulations based study used data from 39 herds and predicted that whilst clinical lameness was linked with poor reproductive performance (a lameness case was associated with a 25% reduction in risk of a cow becoming pregnant), the simulation estimated that when other factors affecting fertility were taken into account, herd-levels of lameness were unlikely to influence fertility overall and this effect even varied little within farm (Hudson *et al.*, 2014). The association between lameness and fertility remains uncertain, with large studies presenting conflicting data. A lameness-associated loss that is consistently agreed upon, however, is that lameness has a major influence on culling decisions, increasing the risk of culling at the end of lactation (Esslemont and Kossaibati, 1997; Booth *et al.*, 2004; Bicalho *et al.*, 2007b).

The exact financial cost of lameness is dependent on many factors, such as the cost of culling, the value of animals and cost of replacement, milk price, specific diseases prevalent on a farm and consequences of lameness on other health parameters such as fertility, to name a few. Cha *et al.* (2010) attempted to calculate the cost of milk production whilst adjusting for milk loss, decreased fertility and treatment cost. The aim was to create a tool for farmers to inform treatment decisions. They estimated that the cost of sole ulcer was US$216 (approx. £150), compared with digital dermatitis $133 (approx. £92), not including further effects on fertility. They built a model to allow many factors to vary including milk price, replacement costs, lameness levels, pregnancy rates. The model is an insightful tool as it demonstrates that as milk prices rise, the value of a high producing cow that does not go lame rises. Similarly, when replacement costs are high or cull-cow value low, there is greater financial incentive to treat lame cows. The model of Cha *et al.* (2010) has limitations, however, and it did not consider factors such as higher incidence of lameness in high producing cows. The economic review of Wilshire and Bell (2009) estimated the cost of a sole ulcer to be much higher: £519. Further, they estimated the cost of a case of white line disease to be £300, and that lameness cost the UK dairy industry £128 million based on the "average" herd at the time (defined 112 Holstein-Friesian cows, producing 6,885l/year). This review might be out of date, and average herd size and average cow production has since increased; in the year April 2014 to March 2015, average herd size was 136 and average cow production was 7,916 L (AHDB, 2015).

In summary, lameness is associated with widespread costs, which ideally would be avoided through prevention of the disease. Particularly in the face of low milk prices and the number of profitable dairy farms decreasing (AHDB, 2015), avoiding financial losses associated with lameness could be a key component of remaining in business.

1.1.3 Poor Welfare associated with lameness

Aside from the financial considerations associated with lameness, these diseases are painful and present a welfare issue (Whay *et al.*, 1997; O'Callaghan, 2002; O'Callaghan *et al.*, 2003; Rushen *et al.*, 2007; Tadich *et al.*, 2013). Poor mobility has been associated with sole ulcer presence (Flower and Weary, 2006) and cows with sole ulcers placed a smaller proportion of their weight on the leg with the lesion (Pastell *et al.*, 2010). Further, Whay *et al.* (1998) demonstrated hyperalgesia (an increased pain response) occurs with both sole ulcer and white line disease; and nociceptive threshold was reduced elsewhere on the cow when and for a prolonged period after either lesion was present (Whay *et al.*, 2005).

In a scientific report to the European Food Safety Authority, lameness was described as "the most severe welfare problem facing the dairy cow and the European dairy industry", partly due to its severe consequences on cow welfare, partly due to associated financial costs and partly due to its high prevalence (Algers *et al.*, 2009). At the end of the last century, the Farm Animal Welfare Council (FAWC, 1997) described the state of lameness in this country to be "at an unacceptably high level", and this is recognised within the industry: in a study that consulted members of the dairy industry with experience in lameness control, over 75% of respondents considered lameness levels to be unacceptable (Whay *et al.*, 2003a). The significance of the disease may also go further than financial costs and welfare. Regulation (EC) No 853/2004 states: "Raw milk must come from animals that are in a good general state of health". Interpreting this, the Netherlands government banned milk from severely lame cows from being put into the bulk tank, through their national quality assurance schemes. The UK government could choose to do the same (FAWC, 2009). Further, milk retailers are increasingly setting standards above national minimums, and a time where penalties exist for farmers having lame cows on farm is not unforeseeable.

Even a conservative estimate of prevalence of lameness in intensively managed dairy herds would state figures upwards of 25 %. Whether it is the single most important disease in dairy farming (Algers *et al.*, 2009) or specifically the second most costly (Kossaibati and Esslemont, 1997), its financial consequences and welfare implications demonstrate that it is a highly significant disease to the industry.

1.1.4 Diseases causing lameness

The majority of lameness originates in the foot, and further, the majority of lesions occur on the hind feet, whether infectious cause of lameness or a claw horn disruption lesion; one 37-herd study estimated that 92% of foot lesions associated with episodes of lameness occurred on hind feet (Murray *et al.*, 1996). The most common infectious disease causing lameness appears to be digital dermatitis, which was first reported in

1974 (Cheli and Mortellaro, 1974) and has become widespread (Murray *et al.*, 1996; Barker *et al.*, 2007). Digital dermatitis primarily affects the skin of the heel bulbs of cattle and *Treponema spp.* bacteria appear to be the causative bacteria (Gomez *et al.*, 2012).

The focus of this thesis, however, is the non-infectious claw horn disruption lesions (reasons for the use of this term are discussed throughout this chapter). These lesions, and specifically sole ulcer, appear to be associated with both the greatest economic impact (Amory *et al.*, 2008; Wilshire and Bell, 2009) and the greatest pain response (Whay *et al.*, 1998; Pastell *et al.*, 2010). These common lesions are described briefly in Table 1.1, with alternative names for the lesions used in the research literature. Further, where lesions are specific to zones of the foot, the foot map (Figure 1.1) demonstrates the locations of the zones.

Table 1.1: List of common foot lesions of dairy cattle.

Lesion name, abbreviation	Other terms used in literature	Description
Sole haemorrhage, SH*	Pododermatitis aseptica diffusa Sole bruising	Diffuse haemorrhage visible in the sole horn. Zones 4, 5 and 6.
Sole ulcer, SU*	Pododermatitis circumscriptica Rusterholz disease Solar ulcer	Full depth penetration of the sole horn in zone 4, though the basement membrane.
Heel ulcer, HU*	Bulb ulcer, bulbar ulcer, sole fracture	Full depth penetration of the sole horn in zone 6, though the basement membrane.
Toe ulcer, TU*	Thin-sole induced toe ulcer	Full depth penetration of the sole horn in zone 5, though the basement membrane.
White line disease, WLD*	White line lesion *N.B.* in Chapters 4, 5 and 6, white line lesions are referred to as either: - White line separation (WLS) - White line haemorrhage (WLH)	Lesions (haemorrhage or separation) of the white line, sometimes impacted. Bacteria can harbor and pus can build up under the sole. Zones 1, 2 and 3.
Digital dermatitis, DD	Mortellaro disease Hairy heel warts	Cutaneous *Treponema spp.* infection at the junction between the haired skin and the horn; Zone 10.
Interdigital necrobacillosis, IDN	Foul in the foot Foot rot Phlegmon	Necrotizing inflammation of interdigital skin caused by Fusobacterium necrophorum infection. Zone 0.
Heel horn erosion, HHE	Slurry heel	Erosion of the heel, with pit-like depressions. Plantar aspects of and plantar to zone 6.

*denotes CHDL

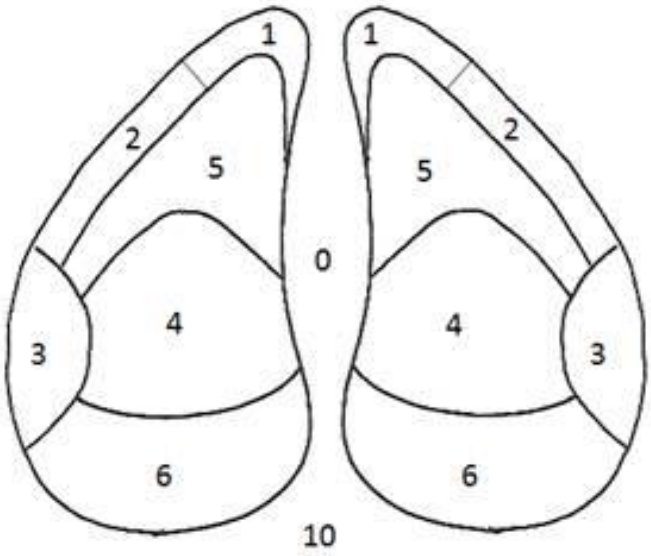

Figure 1.1: The foot map. Zones of the distal surface of the claw, as recommended at the 6th Symposium on Diseases of the Ruminant Digit, Liverpool 1990. Zones 1, 2 and 3 are regions of the white line, zone 4 is considered to be the sole region, 5 the toe and 6 the heel. Zone 0 is the interdigital space and 10 the heel bulbs.

The claw horn disruption lesions occur more frequently on the lateral claw than the medial; in a study of 37 UK farms, Murray *et al.* (1996) found 82% of hind limb CHDLs occurred on the lateral claw and 18% on the medial. The high incidence of lesions seen on the lateral claw could in part be due to load bearing differences; the lateral claw of the hind limb was found to bear more weight than the medial (van der Tol *et al.*, 2002). Additionally, during foot placement, the lateral claw of the hind limb bore almost all of the force of the leg, which evened out and became approximately equal before the leg left the ground. Pressure bearing was much closer to equal between claws of the fore limbs (van der Tol *et al.*, 2003). Further, studies of the pressure differences on the base of the foot could explain lesion locations. During standing, the abaxial aspect of the lateral claw (zone 3) bore most weight (i.e. a region in which white line lesions are commonly seen), but during walking, peak pressures occurred at the axial aspect of the sole (van der Tol *et al.*, 2002), in the region of the typical sole ulcer site (Rusterholz, 1920).

Sole haemorrhage lesions can frequently be seen before sole ulceration and the diseases appear to be part of a similar disease process, although sole haemorrhage does not always lead to ulceration (Leach *et al.*, 1997; Whay *et al.*, 1997). Similarly, two components of the white line disease process have been identified as haemorrhage and separation, and it is thought that haemorrhage can precede separation and impaction. Additionally, white line separation can be a result of direct trauma, as it is already a weak point on the sole of the foot (Kempson and Logue, 1993; Leach *et al.*, 1997; Le

Fevre *et al.*, 2001; Sogstad *et al.*, 2007). Sole and white line lesions additionally appear to be different presentations of a similar disease process, which primarily occurs through insult to the germinal epithelium of the sole (Le Fevre *et al.*, 2001; Vermunt, 2007; Bicalho and Oikonomou, 2013), although they have some different risk factors and are at greatest incidence at slightly different stages of lactation; white line lesions appear to occur a little earlier, peaking at 9 weeks post-calving as opposed to 14 for sole lesions (Leach *et al.*, 1997; Le Fevre *et al.*, 2001). Authors have explained such differences suggesting that differences in wear rates at different sites result in lesions being displayed at different times.

The claw horn lesions are frequently associated with lameness, yet lesions can occur when lameness has not been detected and lameness can occur in the absence of lesions (e.g. Whay *et al.*, 1997; Manske *et al.*, 2002b; Tarlton *et al.*, 2002; Knott *et al.*, 2007; Maxwell *et al.*, 2015). The poor association between lesion presence and lameness could be for a variety of reasons: some lesions appear not to be painful, lesions on both hind feet might make the cow equally lame on both hind legs which can be difficult to detect, and the time between insult occurring in the sole horn and being seen on the surface of the horn can be long, therefore associations with lameness missed. Additionally, cows are stoic animals that hide pain where possible; given their ancestral heritage as prey animals, displaying signs of pain is an outward sign of weakness and could identify them as an easier target for predation (Whay *et al.*, 1997; Lin, 2014). Nonetheless, the lesions do appear to be associated with pain, and studies have consistently shown that ease of walking improves when analgesia was administered to the claws of lame cows (Whay *et al.*, 1997; Rushen *et al.*, 2007; Pastell *et al.*, 2010).

1.1.5 Visual lameness detection

Lameness is detected by alterations in gait, and can be done visually or using automated methods, and a review of the commonly reported methods for visual locomotion scoring is provided by Whay (2002). Briefly, Manson and Leaver (1988) described a 9-point scale (1-5 with half-point intervals), providing descriptions such as 1= even gait and not lame, 2 = uneven gait, 3 = lameness that does not affect behavior, 4 = "obvious" lameness and behavior affected, 5 = extreme difficulty walking. Sprecher *et al.* (1997) described a 5-point system which puts emphasis on the shape of the spine: arched back posture was one component of the lame categories (≥3). These systems are widely referred to as "locomotion" scoring systems in the research literature. Alternatively, the UK Industry standard and that endorsed by AHDB Dairy (the UK milk levy board) is referred to as the "mobility" scoring system. This is a 4-point scale ranging from 0-3, where 0 = sound, 1 = abnormal locomotion and possibly tender-footed, 2 = visibly lame on a leg and 3 = severely lame (Whay, 2002; Whay *et al.*, 2003b). More detailed

descriptions of these categories are provided by Thomas *et al.* (2015a) and later in this thesis (4.3.4.1 and Table 4.4).

1.1.6 Lesion classification

A great array of lesion classification systems have been described, some of which are reviewed in 4.3.2.1. Recently, an attempt to standardize the nomenclature of the lesions of cow feet has been made; ICAR (2015) provides a pictorial guide to many lesions. This resource displays a great array of lesions described in the research literature. However, lesion terminology can become complex, for example digital dermatitis can complicate the classical claw horn lesions and create lesions that are much more difficult to treat and heal; these have been described as "non-healing" or "DD-associated" lesions (Evans *et al.*, 2011). However, the vast majority of lesions associated with lameness can be linked back to the primary claw horn disruption lesions, which are the primary focus of this work: sole ulcer, sole haemorrhage and white line disease.

1.2 Pathogenesis of CHDLs

1.2.1 Anatomy of the foot

The distal phalanx is the distal-most bone of the limb and sits within the hoof capsule (Figure 1.2), which consists of integument layers that are modified to transfer weight into the ground and manage concussive and rotational forces during locomotion (Mulling and Budras, 2003). The distal phalanx is suspended from the hoof wall by laminae (the "laminar" region) and supported above the base of the foot by cushioning structures; these systems can be considered the "suspensory apparatus" and the "supportive apparatus" respectively (Lischer *et al.*, 2002). Together, these apparatuses provide a useful model by which to consider the roles of different aspects of the anatomy, and how forces are transferred throughout the foot.

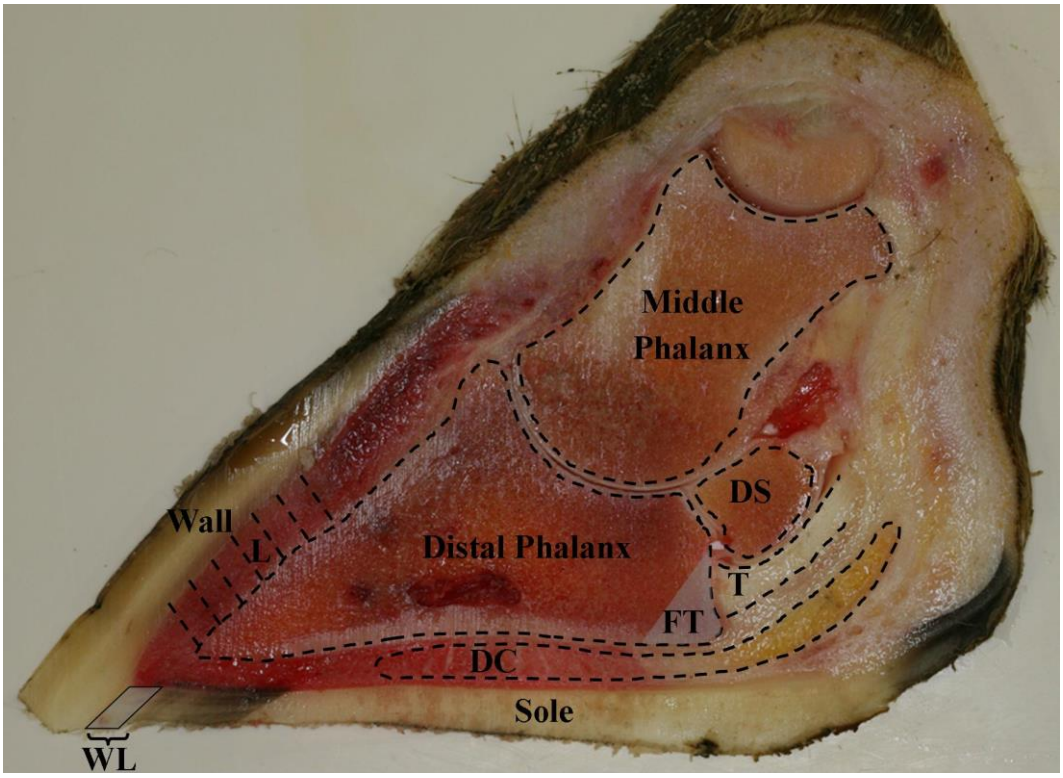

Figure 1.2: Prosection of a bovine claw. The following structures are marked: the middle phalanx, the distal phalanx, **DS** the distal sesamoid bone, **T** the deep digital flexor tendon, **FT** the flexor tuberosity (the shaded area shows the inexact region that can be described as the flexor tuberosity; it is the tuberosity/protrusion of the distal phalanx onto which the deep digital flexor tendon inserts), **DC** the digital cushion, **L** shows the laminar region of the suspensory apparatus, **Wall** is the wall horn, **Sole** is the sole horn and **WL** is the region of the white line. The typical sole ulcer occurs in the region of the sole beneath the flexor tuberosity. The laminar region of the hoof extends around the distal phalanx, which is not shown in the current image.

The distal phalanx is suspended from the wall by laminar attachments that run from the surface of the distal phalanx, upwards towards the wall horn; this apparatus extends around the axial and abaxial surfaces of the distal phalanx. The wall consists of the hardest hoof horn (Ossent and Lischer, 1998) and is attached to the sole and bulbar regions on the base of the foot by the white line (Kempson and Logue, 1993). The laminar attachments in the cow extend over the distal half of the dorsal wall of the distal phalanx, and appear to be better developed on the abaxial aspect of the distal phalanx than on the axial; the axial aspect of the distal phalanx is additionally suspended by longer interdigital ligaments. These longer ligaments appear to allow greater depression of the axial aspect of the distal phalanx during load bearing, and the depression of the flexor tuberosity in this site corresponds with the sole ulcer site over the axial aspect of zone 4 of the foot map (Rusterholz, 1920; Lischer *et al.*, 2002). These anatomical descriptions tie in with van der Tol *et al.* (2002) and van der Tol *et al.* (2003), where weight was borne on the abaxial aspect of the lateral claw when standing, but transferred

to the axial aspect during walking, with greater depression of the axial aspect over the sole ulcer site when walking (1.1.4).

On the base of the foot, the skin layers consist of the subcutis, dermis and epidermis. The subcutis is present along the plantar two thirds of the distal phalanx and extends in a plantar direction, adjacent to the deep digital flexor tendon. Superficial to the subcutis, or directly attaching to the bone towards the distal tip of the distal phalanx, only dermis and epidermis are present (Räber *et al.*, 2004). The dermis interlocks with the epidermis in this site and the deepest layer of the epidermis is the stratum basale (Hendry *et al.*, 1997), also termed the stratum germinativum or the "germinal epithelium". This layer of basal cells that makes up the germinal epithelium is the site of cell proliferation and horn production. Keratinocytes progress distally from this germinal epithelium undergoing keratinization to form tubular and inter-tubular horn; this process of keratinization is reviewed thoroughly by Hendry *et al.* (1997). The base of the foot can be divided into sole and bulbar regions, approximately occupying zone 5 and zones 4 and 6 of the foot map, respectively. Size and density of tubules varies across regions of the sole, too, and the biomechanical properties of the sole horn differ with regions of the sole; the bulbar horn is softer and has greater elasticity than the sole horn (Mulling and Budras, 2003; Winkler, 2005). For simplicity in this thesis, and in line with much of the research literature, the two regions will be collectively referred to as the sole, in reference to their location on the base of the foot rather than differences in mechanical properties (and the typical sole ulcer technically occurs through bulbar horn, not sole horn). Further, the dermis will be referred to as the corium, in line with much of the published literature.

Sources assessing hoof horn growth rate (and wear rate) are reviewed by Winkler (2005) in a PhD thesis on the mechanical properties of hoof horn. Many factors appear to influence growth rate, including season, claw (lateral or medial), floor surface, lactation number and possible hormonal influences (Hendry *et al.*, 1997). Similarly, many factors can influence wear rates, such as management system (e.g. extensive versus intensive) and flooring surface. The net effect of these influences is thickness of the sole horn.

The supportive apparatus of the foot describes the anatomical structures that appear to be adapted to dissipating forces exerted down by the distal phalanx. In addition to the flexible properties of the horn on the base of the foot, the digital cushion appears to be adapted to bear load transferred down through the distal phalanx. It appears to play a role in dissipating forces across the base of the foot and into the structures that are designed to bear weight, such as the wall (Räber *et al.*, 2004).

1.2.2 Pathology of hoof horn

The bovine foot consists of a vast array of modified tissue layers that act in synchrony to dissipate forces; how these break down during the pathophysiology of claw horn diseases has been described by Mulling and Greenough (2006). Sole haemorrhage appears to originate via disruption in the dermal layer that supports proliferation within the germinal epithelium (Hoblet and Weiss, 2001), sole ulcer is thought to be an extreme case of this disease process, in the region of the sole that bears greatest weight (van der Tol *et al.*, 2002). Hoblet and Weiss (2001) describe how this could have occurred through mechanical forces or vascular compromise, both of which could compromise the quality of horn production. Haemorrhage in the area could then be seen in the sole horn or the white line as it grows out. Additionally, haemorrhage in the hoof horn influences its structural capacity, with decreased puncture resistance found in horn with haemorrhages (Winkler and Margerison, 2012); weaknesses in the sole horn as a result of prior pathology could further predispose lesion formation.

The white line is a weak point on the base of the foot and is a predilection site for the entry of debris (Kempson and Logue, 1993; Leach *et al.*, 1997; Blowey, 2008). Haemorrhage in the white line could further predispose it as a site for impaction, causing separation and the harboring of bacteria that occurs with white line disease. Haemorrhage in this area could be a result of compression of the soft tissues around the edge of the foot (Kempson and Logue, 1993), hence the aetiopathogenesis of white line lesions and sole lesions might have similar origins.

Hoblet and Weiss (2001) describe the claw horn lesions as occurring through the production of poor quality horn that can be a result of inappropriate forces on the germinal epithelium of the sole, which prevents appropriate growth and keratinization of hoof horn. Both suspensory and supportive apparatuses might have effects on how the distal phalanx is positioned within the hoof and how much it depresses towards the germinal epithelium of the sole during foot strike. Any factor that influences the forces through the foot, and therefore on the germinal epithelium, could cause haemorrhage in the dermis or affect normal cell division in the germinal epithelium. With haemorrhage in the dermis being associated with impaired horn production and the production of poorer quality horn, this appears to predispose ulceration. Inadequacies in the suspensory apparatus could cause the distal phalanx to sit lower in the foot and place greater forces on the germinal epithelium of the sole, and inadequacies in the supportive apparatus may insufficiently dissipate shock during foot strike. The next section explores the risk factors for the claw horn disruption lesions, in light of these mechanisms surrounding their aetiopathogenesis.

1.2.3 Risk factors for CHDLs

Many risk factors have been identified as predisposing claw horn disruption lesions and it appears that they can be related to contusions within the corium of the sole. This section briefly reviews the risk factors and how they appear to be related to claw horn lesions, and for more exhaustive sources of the specific risk factors, the reader is referred to recent comprehensive publications (Bell *et al.*, 2009; Bicalho and Oikonomou, 2013; Chapinal *et al.*, 2013; Solano *et al.*, 2015; Foditsch *et al.*, 2016). In particular, Solano *et al.* (2015) recently outlined a "causal web of risk factors", which illustrated risk factors found to be associated with lameness and how they are thought to play a role in the onset of lameness.

It can be helpful to consider the risk factors associated with lameness as belonging to one of two categories: extrinsic and intrinsic factors (Mulling and Greenough, 2006). Extrinsic factors can be considered as external to the cow and tend to result in the foot encountering greater or abnormal forces. These include traumatic or abrasive flooring surfaces, factors that result in decreased lying times such as heat stress or increased waiting times to be milked, insufficient feed space and increased social competition (Bicalho and Oikonomou, 2013). These factors that alter the forces applied to the foot challenge its ability to cope with forces.

On the other hand, intrinsic factors can be considered as those that affect the transfer of forces through the foot, making forces that are appropriate under certain conditions become too great. Examples are as follows. Sole haemorrhages are commonly visible on the base of the foot after calving, and comparing the suspensory apparatus of the foot of calving heifers to maiden (non-calving) heifers, the suspensory apparatus appears to become weakened with calving (Tarlton *et al.*, 2002; Knott *et al.*, 2007). This work found that the connective tissue of the suspensory apparatus displayed higher levels of catabolic enzymes (metalloproteinases) that were associated with non-inflammatory degradation, and weakening, of the laminae. The authors suggested that this could be a result of relaxin around calving, which facilitates degradation of collagen within the reproductive tract to aid parturition. Subsequent sole haemorrhages were higher in the heifers that calved, and the authors suggested that this could be a result of the distal phalanx sitting lower in the hoof capsule around calving and placing greater forces on the dermis and germinal epithelium of the sole, causing greater contusion within it (Tarlton *et al.*, 2002; Knott *et al.*, 2007). This complements many other studies that found parturition to be a risk factor for sole and white line lesions (e.g. Bergsten and Herlin, 1996; Leach *et al.*, 1997; Livesey *et al.*, 1998; Chaplin *et al.*, 2000; Offer *et al.*, 2000).

Body condition could be described as another intrinsic factor: thinner cows and those that have undergone body condition loss have been found to be at higher risk of claw horn disruption lesions, which could be a result of fat mobilization from the digital cushion and subsequent poorer force dissipating capacity, resulting in greater contusion within the dermis and greater forces on the germinal epithelium of the sole (Bicalho *et al.*, 2009; Machado *et al.*, 2011; Green *et al.*, 2014; Lim *et al.*, 2015; Randall *et al.*, 2015; Solano *et al.*, 2015). Additionally, foot trimming can also fall into this intrinsic category, as it can both promote or impede the appropriate transfer of forces through the foot (Kofler, 1999; Amory *et al.*, 2006; Espejo and Endres, 2007).

One particular description that encapsulates recent thinking on the pathophysiology of specifically sole ulcer was given by Nuss (2014), who stated: "*Continuous displacement [of the distal phalanx] leads to compression of the solar corium, which in turn initiates the cascade of vascular compromise, ischemia caused by congestion, oedema and thrombosis, interrupted keratogenesis and finally sole ulcer*." This describes the lesions of claw horn disruption not as a one off event, but more a progressive process that leads to inadequacies in and failure of horn production. Further, it highlights that a vast range of risk factors can interact and culminate, perhaps resulting in greater forces on the germinal epithelium of the sole. The different lesions (i.e. sole or white line) appear to have some similar and some different risk factors (Barker *et al.*, 2009; Oikonomou *et al.*, 2013), but authors have explained the differences in lesions as damage at different sites of the germinal epithelium and being a result of rotational or shearing forces (Leach *et al.*, 1997; Le Fevre *et al.*, 2001). Throughout this chapter, more risk factors for lameness will be discussed and it is likely that they can be related to the above pathophysiology; that is, that the lesions of claw horn disruption are largely a result of inappropriate forces being placed on the sensitive soft tissues within the base of the foot.

1.3 The suspensory and supportive apparatuses and sole horn production

1.3.1 The laminitis theory

Historically, laminitis has been thought to be the predominant disease underlying white line and sole lesions, and has been defined as "inflammation of the laminae within the claw" (Greenough *et al.*, 1981; Vermunt and Greenough, 1994). In their review of the predisposing factors of laminitis, Vermunt and Greenough (1994) point out that the disease process was poorly understood, despite extensive study both experimentally and through clinical observation, and refer to the theories of laminitis that predominated the understanding of equine laminitis.

In horses, laminitis leads to sinking and rotation of the distal phalanx within the hoof capsule, through loss of integrity of the suspensory laminae. Originally, this was thought to be purely a result of a systemic inflammatory response following carbohydrate overload, acidosis within the hindgut and breakdown of caecal lining, which resulted in metalloproteinase activation and leucocyte infiltration into the laminae. This mechanism has been supported by the induction of laminitis following carbohydrate overload (Hood *et al.*, 1993; van Eps and Pollitt, 2006) or toxin administration such as black walnut (Loftus *et al.*, 2007), and has been referred to as the "inflammatory" mechanism of laminitis. In this model of laminitis, early histological alterations in the lamellae include separation of the basement membrane which connects dermal and epidermal aspects of the suspensory apparatus, followed by secondary infiltration of red blood cells, leukocytes and an inflammatory cascade that results in enzymatic degradation of the basement membrane (Pollitt, 1996; Katz and Bailey, 2012). In cattle, it appears that laminitis can also be induced with carbohydrate overload. Thoefner *et al.* (2005) administered 17g/ kg bodyweight of oligofructose to heifers, and euthanized them 48 or 72 hours after oligofructose administration, observing histological changes in the laminar region including stretching of lamellae, dermal oedema and haemorrhage. These were considered to be signs of acute laminitis, similar to that in the horse. The oligofructose administration caused heifers to develop clinical sickness associated with severe ruminal acidosis and have difficulty walking in this and in other work (Danscher *et al.*, 2009), yet hoof pain was not detected in either study; the animals appeared to have difficulty walking as a result of clinical sickness rather than hoof pain. Danscher *et al.* (2010) reported inconsistent associations between oligofructose overload and hoof pain, which they assumed to be a sign of laminitis; these studies cast doubt over whether oligofructose overload or acute laminitis affected the hoof clinically. Further, Danscher *et al.* (2010) tested strength of the suspensory apparatus post-slaughter and no difference in either the force required to displace tissue by 1 mm or the force needed to break tissue were observed between the treatment and control groups. Another study that fed large volumes of readily fermentable diets failed to detect any signs of laminitis, despite causing severe rumen acidosis and making animals sick (Momcilovic *et al.*, 2000).

It does appear that some form of laminitis has been induced experimentally in cattle following the alimentary administration of large amounts of readily available carbohydrates, but laminitis has not been demonstrated following engorgement of high concentrate diets. Further, whilst laminitis has been displayed histologically, it was not observed to progress to the claw horn disruption lesions. Claw horn lesions are very common diseases of dairy cattle (Barker *et al.*, 2009) and if carbohydrate overload were to induce laminitis and CHDLs, it seems likely that studies such as those described above would have detected CHDLs associated with grain overload. The absence of such a finding in these studies suggests that there might be alternative mechanisms to the

feeding of high volumes of readily fermentable carbohydrates in the development of claw horn disruption lesions.

More recently in the equine literature, a second mechanism for the development of equine laminitis has come to predominate the research field, with reports that hyperinsulinaemia has major effects on the suspensory laminae. Insulin appears to be a risk factor for laminitis; induction of hyperinsulinaemia through dietary manipulation or intravenous glucose infusion (de Laat *et al.*, 2012; Selim *et al.*, 2015), or by direct infusion of insulin (Asplin *et al.*, 2007; de Laat *et al.*, 2010), have been found to induce changes in lamellar morphology. Hyperinsulinaemia in the horse can occur through pituitary pars intermedia dysfunction (PPID), diet, obesity and equine metabolic syndrome, and in all cases, it appears that hyperinsulinaemia precedes laminitis (Karikoski *et al.*, 2015).

The mechanism by which insulin might induce laminitis is unknown, and the lack of insulin-sensitive glucose transporters within the lamellae suggest that laminitis is not a result of altered glucose metabolism (Katz and Bailey, 2012). However, insulin may act on lamellar epithelial cells via insulin-like growth factor 1 receptors and have a proliferative effect on these cells (Burns *et al.*, 2013), causing weakening during growth and leading to lamellar pathology. Other histological studies have described early lamellar changes associated with insulin resistance to include increased length and widening of lamellae, abnormal keratinization and evidence of increased mitotic activity in the basement membrane (de Laat *et al.*, 2010; de Laat *et al.*, 2012; Karikoski *et al.*, 2014; Karikoski *et al.*, 2015). Unlike previous reports of inflammatory laminitis, inflammation in the lamellae appeared to be secondary to these changes.

Laminitis in horses remains not fully understood, but in a simple sense can be seen as having two routes of pathogenesis: inflammatory and metabolic (Katz and Bailey, 2012; Morgan *et al.*, 2015). Even inflammatory mechanisms that involve carbohydrate engorgement could induce metabolic laminitis through hyperinsulinaemia, and Katz and Bailey (2012) acknowledge that there could be important crossovers between the different routes. To the author's knowledge, theories of the endocrinopathic laminitis have not been explored as being implicated in the bovine claw horn disruption lesions, but this could be a possible mechanism. For example, changes in the laminae observed by Thoefner *et al.* (2005) following carbohydrate overload could have been mediated by hyperinsulinaemia. This remains a possible area for future research.

The term "laminitis" implies an inflammatory process is occurring in the laminar region of the foot (the suffix "itis" pertains to an inflammatory process). However, in the bovine literature, the lesions of claw horn disruption have been demonstrated in the absence of inflammation in the laminae: Lischer *et al.* (2002) found both sole

haemorrhage and white line haemorrhage without evidence of pathology within the laminae, and the work of Knott *et al.* (2007) described previously demonstrated that distension and weakening of the suspensory apparatus occurred in the absence of inflammation. Additionally in the equine literature, it has been demonstrated that distension of the laminae can occur without inflammation, and Katz and Bailey (2012) suggested the term "laminopathy" would be more appropriate for changes in the laminae associated with metabolic factors in the horse. Some authors of bovine literature acknowledge that acute laminitis may not be the cause of claw horn lesions and use the term "subclinical laminitis" to describe both sole ulcer and sole haemorrhage (Smilie *et al.*, 1999). However, "subclinical laminitis" still refers to an inflammatory disease process. As reviewed by Vermunt (2007), the term claw horn disruption has become accepted as the mechanism for the claw horn disruption lesions, as it is more specific to the disorders seen in hoof growth that define this set of diseases and avoids confusing the disease process. Whilst some form of laminopathy (that is not fully understood) might constitute a component that leads to claw horn disruption, the current work will refer to the disease process as being via the mechanism of claw horn disruption.

1.3.2 The digital cushion

1.3.2.1 Structure and composition of the digital cushion

The digital cushion is considered to be a key component of the supportive apparatus (Räber *et al.*, 2004). It is a modification of the subcutis layer of the integument that is thought to dissipate ground reaction forces during foot strike into the structures that are designed to bear weight, and reduce peak loads on the germinal epithelium of the sole (Dietz and Heyden, 1990; Lischer *et al.*, 2002; Räber *et al.*, 2004). The digital cushion is composed of connective tissue, containing three cylindrical parallel fat depots. Proximally, the pads are situated in the heel, parallel to the deep digital flexor tendon, and extend dorsally beneath the plantar two thirds of the flexor tuberosity of the distal phalanx (Lischer *et al.*, 2002; Räber *et al.*, 2004). Figure 1.3 displays the location of the fat components of the digital cushion in relation to the distal phalanx.

Räber *et al.* (2004) presented descriptive anatomical work of the digital cushions of 54 cows (breeds: 23 Brown Swiss, 22 Simmental × Red-Holstein, 9 Holstein). They reported subjective assessments of fat content and suggested that the pads of the front claws contained more fat than the hind claws, and both the lateral front and medial hind claws contained more fat than the other claw of the same foot. Within each claw, the pads appeared to contain less fat in sites subject to high pressure. Further, they reported that the total tissue thickness beneath the distal phalanx appeared to be thinner in older animals.

In a subsequent publication, Räber *et al.* (2006) presented fat analysis of the digital cushion from a subset of the same cohort, consisting of 6 heifers (2.3 years) and 6 parity >1 (mean age: 8 years) Brown Swiss cows. They reported that heifers (parity 1 animals) had less triglyceride as a proportion of tissue mass than parity >1 cows (26.4 g/100g tissue in heifers versus 36.7 in cows, $p<0.001$), and the fat pads of parity >1 animals appeared to be better developed. Further, fat in the digital cushion was higher in mono-unsaturated fats (MUFAs) than fat from elsewhere in the body (77% [units: g/100g of fatty acid methyl ester] MUFAs in the digital cushion versus 52% in peri-renal fat and 33% in subcutaneous fat; the precise location of the "subcutaneous" fat was not stated). Further, heifers reportedly had higher saturated fatty acid and lower concentrations of some unsaturated fatty acids, although there was no overall difference between MUFA content between heifers and cows: fat from the digital cushion of heifers and cows consisted of 79.5 % and 80.1 % MUFA, respectively.

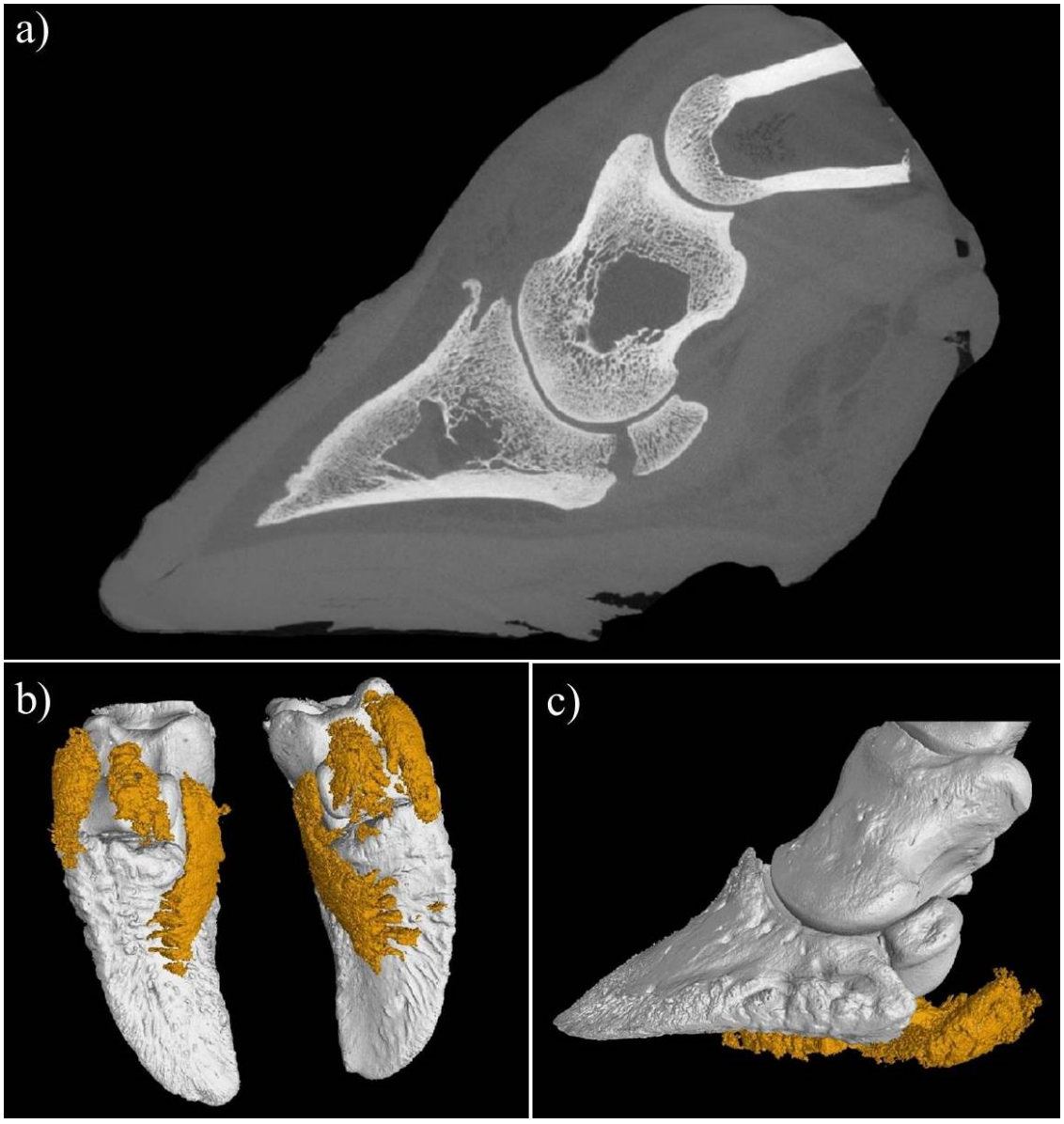

Figure 1.3: Computed tomography images of a bovine foot. a) A mid-sagittal section through a claw, similar to the prosection displayed in Figure 1.2. b) and c) Three dimensional graphics displaying the bones (white) and fat depots (yellow) within the digital cushion, viewed from plantar and abaxial perspectives, respectively. b) and c) were constructed using VGStudio Max 2.2 Software (Volume Graphics GmbH, Heidelberg, Germany) and applying rendering thresholds for bone and lipids.

This is important work, highlighting that there are differences in the fats of the digital cushion compared with other sites in the body. However, they frequently state highly significant differences in fat content or fatty acid concentrations between study groups, and there could have been some oversights in their statistical analysis. Importantly, the study compared the lipid content and fatty acid profiles of cows and heifers, using 16 samples of digital cushion from each cow. Oleic acid (C18:1) was the most abundant fatty acid, comprising 65.8% of fat in the digital cushion of heifers, and 67.4% in cows;

the difference was reported as significant at $p<0.001$. However, from the description of the statistical analysis, it appears that the samples were treated as independent rather than as measures clustered within an animal; this could have inappropriately added statistical power such that differences they reported as significant were not. Standard deviations were not provided in the work and, based on a t-test, to acquire this significance level with these means and sample sizes of 6 animals, the standard deviation of each group would have to have been <0.6. Whilst it remains possible that these differences were truly significant, these results perhaps ought to be treated with some caution due to poor clarity surrounding the statistical analysis. Further, the differences in lipid content of the digital cushions could have been due to differences in fatness of the animals, which was not reported. This work could be improved with further work where factors that might influence fat content and composition are controlled, and samples from a larger number of cows are used.

Räber *et al.* (2006) also reported that arachidonic acid was reduced in older cows and highlighted that it is a precursor to inflammation (Williams and Higgs, 1988). Specifically, arachidonic acid belongs to a wider group of 20-carbon fatty acids that are easily conjugated into 20-carbon inflammatory mediators: prostaglandins and leukotrienes. Collectively, these 20-carbon molecules in the inflammatory cascade are termed eicosanoids (Dennis and Norris, 2015). Arachidonic acid could be lower in older animals as a results of prior inflammation during active claw horn disruption lesions (Räber *et al.*, 2006), although similarly this ought to be confirmed with further work and greater sample sizes.

Studies have assessed the effect of feeding regimes on fatty acid composition of the digital cushion. Baird *et al.* (2010) analysed the effect of four feeding regimes with different linoleic acid content on the fatty acid composition of the digital cushions of 47 bulls; the breeds were Holsteins-Friesians, Jerseys, Norwegians, and crosses between these breeds. They found no difference in the linoleic acid content of the digital cushion, although ratios of other fats did differ with dietary differences. Additionally, Räber *et al.* (2015) compared the composition of the digital cushions from animals fed on different diets. The main significant findings were that the digital cushions of cows fed higher roughage were higher in omega-3 fatty acids, whilst those fed higher concentrates had higher omega-6 fatty acids. Interestingly, the inverse was seen in subcutaneous fat. However, different breeds were assigned to each feeding regime with no crossover, therefore it is not possible to determine whether the differences were due to differences in breed or due to differences in diet. Further, the differences in fat profiles of the digital cushion reported by both Baird *et al.* (2010) and Räber *et al.* (2015) were small despite large differences in diet, and neither work demonstrated whether these differences related to differences in either digital cushion function or claw horn lesion formation. Therefore, despite suggesting that diet might influence digital cushion function and

assist in preventing CHDLs, no strong evidence has been presented to support this hypothesis.

In a different type of intervention study to the feed trials, Gard *et al.* (2015) tested whether raising dairy bull calves on different terrains with different amounts of exercise affected the structure of the digital cushion. Between 2 and 6 months of age, calves were either kept in grass paddocks (control group) or on a 0.8 km lane of dirt, stones and grass, and fed at alternate ends of the lane to entice exercise (exercise group). The control calves walked a mean of 1.1 km daily on grass, compared with the exercise group of 3.2 km daily on rougher terrain, and at 6 months old the digital cushion had a volume 37 % greater than the control group. Whilst both Baird *et al.* (2010) and Räber *et al.* (2015) demonstrated that feeding could influence composition of the digital cushion and suggested this could alter its biomechanical properties and aid in lameness prevention, the differences reported between feed groups were small. Contrastingly, Gard *et al.* (2015) demonstrated large differences in volume of the digital cushion with different management systems before 6 months of age, and this might be a factor that is more easy to manipulate than diet, giving life-long preventive effects on lameness.

1.3.2.2 Summary of the structure and composition of the digital cushion

From these descriptive studies of the digital cushion, it appears that the digital cushion is higher in mono-unsaturated fatty acids than normal adipose tissue. The mono-unsaturated fatty acids are less viscous than saturated fatty acids (Glaser *et al.*, 2004) and resemble a non-compressible fluid, bound by cell walls and a collagen structure. The mono-unsaturated fats might better dissipate forces during foot-strike than saturated fatty acids, which has led to the postulation that their presence in the digital cushion is an adaptation to aid the mechanics of force dissipation (Räber *et al.*, 2006; Baird *et al.*, 2010). It might be possible to manipulate the content of the digital cushion with feeds, but whether this improves the functional capacity of the digital cushion has not been demonstrated. It appears possible to alter the size of the digital cushion in calves using different rearing systems, and it would be interesting to see whether this relates to decreased lameness incidence during adult life.

1.4 Body fat and the digital cushion

1.4.1 *In vivo* studies of the digital cushion

It has been suggested that fat is mobilized from the digital cushion during negative energy balance, decreasing its capacity to dissipate shock and predisposing the germinal epithelium of the sole to contusions (Räber *et al.*, 2004; Bicalho *et al.*, 2009). Bicalho *et al.* (2009) reported that digital cushion thickness was positively correlated with body

condition score. This work was a cross-sectional study and measured digital cushion thickness beneath the flexor tuberosity of the distal phalanx on 501 cows at any stage of lactation (up to and including the 10th month after calving). They reported that the digital cushion was thinner in thin cows, in parity 1 cows and if a lesion (sole ulcer or white line disease) were present. The nadir of body condition score (**BCS**) and digital cushion thickness were both at around 120 days in milk (**DIM**). Cows with a thin digital cushion were more likely to develop a claw horn disruption lesion during the lactation, but body condition score did not appear to be associated with lesion incidence. The authors suggested that the difference in diameter of the digital cushion between cows in first and second months of lactation was a result of thinning of the digital cushion with body condition loss and lead to primary damage to the corium by the distal phalanx, which was then exacerbated by further fat mobilisation until the nadir of digital cushion thickness, and lesions were detected visually later. An important limitation of the study is that temporal associations between change of body condition, change in digital cushion thickness and lesion incidence could not be analysed due to one-off measurements of body condition and digital cushion thickness.

In a second study, the same research group used digital cushion thickness, BCS and claw horn lesion presence at drying off as predictors of treatment for claw horn disruption lesions in the subsequent lactation (Machado *et al.*, 2011); low BCS, thin digital cushion thickness and presence of a CHDL all increased the likelihood of developing a lesion in the subsequent lactation. Again, digital cushion thickness correlated with BCS and digital cushion thickness was thinner in cows with CHDLs.

A possible problem with the aforementioned two studies is that the digital cushion has been reported to be thinner in claws that have CHDLs (Lischer *et al.*, 2002; Munk and Capion, 2013). Further, Räber *et al.* (2004) report that the digital cushion and corium was thinner in older cows, and Räber *et al.* (2006) stated that the fat content had changed, possibly as a result of inflammation that had scarred the tissue. In the work of Machado *et al.* (2011), thinner digital cushions at drying off could have been a result of prior CHDL incidence and damage to the internal structures of the foot. Further, they reported that thin cows were more likely to develop lesions, yet since cows could be thin as a result of prior lameness (Espejo *et al.*, 2006; Bicalho *et al.*, 2009; Hoffman *et al.*, 2013), having thinner digital cushion could be a result of either being thin or prior lameness; the work cannot decipher whether prior lesions, body condition score or other factors caused the lameness during the subsequent lactation. In a different cohort study, Toholj *et al.* (2013) measured cushion thickness at 30 DIM and used these data to predict lameness at 70 and 180 DIM, and lesions at 180 DIM. Cows were more likely to go lame and develop a sole ulcer if they had a thin digital cushions at 30 DIM. This complements the work of Machado *et al.* (2011) that suggests thin digital cushion

thickness leads to lesion incidence. Unfortunately, Toholj *et al.* (2013) included no measures of body fat in the study, making some aspects of the work difficult to compare.

After linking digital cushion thickness with body condition (Bicalho *et al.*, 2009) and with lameness (Machado *et al.*, 2011), this research group calculated heritability estimates for an ultrasonographic measure of digital cushion thickness, based on 923 dairy cows on one farm (Oikonomou *et al.*, 2014a). They suggested that the phenotype was mildly heritable (heritability estimate: 0.33), although admitted that a larger sample size across a greater range of Holstein genetics was needed to fully explore the heritability of digital cushion thickness. They also used infrared thermography in conjunction with ultrasonography of the digital cushion in cows between 4 and 10 days post-calving, and reported that thinner digital cushions were hotter (Oikonomou *et al.*, 2014b). They suggested that this increase in temperature could be a result of inflammation within the corium of the foot, being an indicator of early lesion formation.

1.4.1.1 Summary of in vivo *digital cushion studies*

From live cow studies of digital cushion thickness, it appears that having a thin digital cushion increases risk of subsequent CHDLs and lameness. Further, thin digital cushions shortly after calving appear to be hotter, which could represent inflammation within the soft tissue layers of the foot. A thin digital cushion may also occur after a CHDL, and this could be because fatty acids in the digital cushion are used during the inflammatory process, causing the digital cushion to become depleted, or because the digital cushion and corium become scarred and thinner. Body condition score and digital cushion thickness have been shown to be correlated. These works support the hypothesis that the claw horn disruption lesions could be a result of forces on the germinal epithelium of the sole. Further, work has suggested that digital cushion thickness is a heritable trait and therefore could be bred for.

1.4.2 Low body fat is a risk factor for CHDLs and lameness

Being thin has been identified as a risk factor for lameness. Hoedemaker *et al.* (2009) observed that lameness post-partum was associated with low body condition in the first 10 weeks after calving, and found the greatest prevalence of sole ulcers to be around peak lactation: 60-100DIM. Low BCS at calving or during early lactation greatly increased the risk of lameness from CHDLs in that lactation, supporting previous work by Gearhart *et al.* (1990) who reported cows under-conditioned at calving experienced more foot lesions after calving.

More recently, several epidemiological studies have demonstrated that body condition loss preceded lameness events, whether lameness was defined by visual detection of impaired mobility (Lim *et al.*, 2015; Randall *et al.*, 2015) or treatment incidence of

lesions (Green *et al.*, 2014). Green *et al.* (2014) reported that being thin (BCS ≤2.25) increased the risk of being treated for claw horn disruption lesions but not digital dermatitis: thinness in the 0-2 or the <2-4 months prior to treatment increased the likelihood of sole ulcer or white line disease treatment, yet only thinness in the 0-2 months prior to a lesion increased the likelihood of a sole haemorrhage. Lim *et al.* (2015) reported that cows with BCS ≤2.25 at calving were more likely to go lame (identified by mobility score), as well as cows following body condition loss.

Randall *et al.* (2015) used the outcome lame (on locomotion score) and reported that cows with body condition score <2 were at the greatest risk of lameness. Additionally, loss of body condition in the first four weeks following calving (≥0.25 points) increased the likelihood of becoming lame, and that being thin 8-16 weeks prior to a locomotion score increased the risk of lameness. Since lameness in parity >1 animals could have been a result of lameness in previous lactations (Hirst *et al.*, 2002), the heifer group was also analysed independently from parity >1 cows. Whilst they found that the thinnest heifers were more likely to go lame, loss of body condition did not predispose to lameness in heifers. However, in cows that experienced their first lifetime lameness event in second lactation, loss of body condition increased the likelihood of becoming lame, suggesting that loss of body condition is a risk factor for lameness and not just a sign of prior lameness. The authors postulated that the difference between body fat loss and lameness in heifers compared with cows could be due to a difference in metabolism of fat from the digital cushion in heifers, since the digital cushion in heifers has been reported as being immature (Räber *et al.*, 2006). Finally, given the number of publications that suggest body condition loss is a risk factor for lameness, Randall *et al.* (2015) suggested that managing body condition could be a control point for lameness.

These works could also help explain why the highest producing cows go lame. The highest producing cows mobilise more body fat during early lactation, and Bicalho *et al.* (2008) reported that cows who went lame produced more milk in the first three weeks of lactation went lame, which was associated with a drop in milk production, in line with other work reviewed earlier (1.1.2). It could be that these cows are mobilizing excessive amounts of body condition at the detriment of their own health.

1.4.3 Physiology of body fat mobilization during early lactation

A cow's greatest energy reserve is adipose tissue (Bauman and Bruce Currie, 1980; Bell, 1995; Roche *et al.*, 2009). During early lactation (first 40 to 100 DIM), dairy cows mobilise tissue energy reserves in order to feed offspring (Bewley and Schutz, 2008). This is a common feature of mammals, and intense genetic selection of dairy cows for higher milk yields throughout the past 50 years has drastically increased the ability to mobilise energy reserves (Dillon *et al.*, 2003; Chagas *et al.*, 2009; Lucy *et al.*, 2009),

with an estimated 50-60kg of fat being mobilised in early lactation (Bauman and Bruce Currie, 1980; Smith and McNamara, 1990). This occurs through lipolysis and also muscle catabolism in order to provide nutrients for offspring, which are later replenished (Coffey *et al.*, 2004; Friggens *et al.*, 2004; Sumner and McNamara, 2007).

In an energy deficit, mechanisms of the somatotropic axis increase hormone expression and alter tissue responsiveness, decreasing lipogenesis and increasing NEFA (non-esterified fatty acid) mobilisation. This can occur purely through homeostasis during energy deficit, which governs metabolic equilibrium; additionally, the process driving prolonged lipolysis and muscle catabolism in early lactation is also governed by non-dietary mechanisms, driven at the genome level. The metabolic state of the cow shifts in order to prioritize partitioning nutrients to the mammary gland and support the neonate; this shift in metabolic state is termed homeorhesis (Roche *et al.*, 2006; Roche, 2007; Chagas *et al.*, 2009; Delaby *et al.*, 2009; Roche *et al.*, 2009). This early lactation body fat mobilization is in large part governed by insulin resistance and increasing energy intake in early lactation does not abolish lipolysis from tissues. The mammary gland has insulin-independent glucose uptake transporters that allow it to take up glucose from the blood stream even in the presence of insulin resistance, and therefore to maintain milk production even when food is scarce (Bauman and Bruce Currie, 1980).

Loss of body condition has been shown to be a risk factor for lameness. However, the highest producing cows go lame and it is these cows that lose most condition in early lactation. It would be interesting to determine whether fat from the digital cushion is mobilized during body condition loss, in order to assess whether thinning of the digital cushion in association with body condition loss could be a mechanism by which cows go lame.

1.5 Management of lameness

1.5.1 Foot trimming

Foot trimming is used both during treatment of claw horn lesions (Potterton *et al.*, 2012) and in a preventive manner to maintain claw shape, equal weight bearing through the claws and to reduce risk of lameness (Toussaint-Raven, 1985; Shearer and van Amstel, 2001; Manske *et al.*, 2002a). Routine foot trimming is widely recommended by key opinion leaders for preventing lameness on farm (Potterton *et al.*, 2012) and is a common component of prophylactic foot trimming (Bicalho and Oikonomou, 2013). Reports have shown that having better handling facilities is associated with lower lameness prevalence on farm (Amory *et al.*, 2006; Bell *et al.*, 2009).

Various studies have assessed the effect of routine foot trimming on lameness and lesions, using various methods. Manske *et al.* (2002a) demonstrated beneficial effects irrespective of time from calving and Hernandez *et al.* (2007) found mid-lactation trimming reduced new lameness cases in late lactation. Fjeldaas *et al.* (2006) found that foot trimming was beneficial in tie stall housing, but not in free-stalls, and suggested that this could be due to damage caused by inappropriate trimming. However, using production and fertility-based outcomes, Maxwell *et al.* (2015) found that performing a functional trim on heifers at 65 days in milk had no significant effect on either milk production during that lactation or the likelihood of becoming pregnant.

Whilst foot trimming can be a good management tool for maintaining claw shape and foot health, it can also predispose problems in the foot. Even after trimming, high pressures can still occur beneath the sole ulcer site on the base of the foot (van der Tol *et al.,* 2004); this is possibly affected by trimming technique (Ouweltjes *et al.,* 2009). A randomised controlled trial of treatments for claw horn disruption lesions in cattle used a foot trim as a positive control and found that the success of treatment varied depending on the operator. This suggests that the technique of the treatments differed between operators and had a clinically detectable effect (Thomas *et al.,* 2015a). Additionally, Kofler (1999) studied claws with toe necrosis and suggested that necrosis was a result of over-trimming; the author highlighted that the ease with which horn can be removed when using power tools could be detrimental to the structure and function of the foot. Bell (2015) also discussed this iatrogenic damage that can occur with foot trimming and Van Hertem *et al.* (2014) found that foot trimming sound cows increased locomotion scores. There have also been suggestions that toe ulcer is primarily a result of thin soles, which could be caused by over-trimming (Tsuka *et al.*, 2014).

Whilst foot trimming can be an essential management technique, both the specific technique and preservation of certain structures in the foot appears essential. Toussaint-Raven (1985) described the Dutch Method of foot trimming that has been widely adopted by foot trimmers (e.g. the UK NACFT: National Association of Cattle Foot Trimmers). This original text stated that the dorsal wall of the hoof should be cut to 75 mm, and that this was appropriate for most Friesian cows, although larger cows should be allowed a greater length. Some authors have moved away from using a set length, instead trimming to claw angles to try and promote appropriate weight bearing (Manske *et al.,* 2002a), and Blowey and Inman (2014) highlighted the great variation in length of the distal phalanx within the hoof capsule, suggesting that a set length cannot possibly accommodate all the internal anatomical variation. The appropriateness of foot trimming practices has been assessed little, despite being a common, time consuming and expensive practice on farm. For the health of cows' feet, this could be a vital area of work to address.

1.5.2 Early detection and treatment

A major risk factor for lameness has been mentioned throughout this chapter, but not yet highlighted: previous lameness. Cows that go lame once are more likely to go lame in subsequent lactations, particularly if the lesions associated with lameness were the claw horn disruption lesions (Alban *et al.*, 1996; Hirst *et al.*, 2002; Machado *et al.*, 2011; Reader *et al.*, 2011; Oikonomou *et al.*, 2013; Green *et al.*, 2014). In a fascinating piece of work that might in part explain this, Bell *et al.* (2009) designed a lameness control program and tested it on 60 UK dairy farms. The work found that poor detection and late treatment of lameness was a risk factor for more severe lameness. Specifically, the work considered poor lameness detection, no claw trimmer training, poor treatment facilities and lack of monitoring treatment responses. For the analysis, these were aggregated into one category, and they found that poor lameness management was a risk factor for both mildly and severely lame cows.

Further, early detection and treatment of lame cows is associated with reduced lameness prevalence (Reader *et al.*, 2011) and detecting and treating lameness promptly can reduce subsequent mobility scores compared with a control group (Leach *et al.*, 2012). Groenevelt *et al.* (2014) reported that fortnightly mobility scoring and treatment of lameness resulted in improved mobility compared with a control group. Further, two recent randomised clinical trials for treatment of claw horn disruption lesions highlight the importance of early and effective treatment. When treatment was early (within two days of being detected lame on fortnightly mobility score) a treatment involving a block being applied to the non-lame claw to relieve weight from the lame claw, in addition to the administration of a three-day course of non-steroidal anti-inflammatory drugs (NSAIDs), improved cure rates compared with a positive control of a functional trim only (Thomas *et al.*, 2015a). However, when treatment was delayed (cows were treated if they had been mobility score >1 at two out of three fortnightly mobility scores), there was no difference in recovery between treatment groups (Thomas *et al.*, 2015b). Unlike the work of Groenevelt *et al.* (2014), however, neither of these trials had a negative control, therefore the effect of the foot trim could not be assessed.

1.5.3 Degeneration of foot anatomy with lameness

The treatment group that had the best recovery rate in the work of Thomas *et al.* (2015a) was a combination of a functional foot trim (Toussaint-Raven, 1985), the application of a block to the lesion-free claw and a three-day treatment with an NSAID: ketoprofen. The work of Räber *et al.* (2006) described previously highlighted that the structure of the digital cushion may become damaged with trauma and inflammation; the combination of anti-inflammatory therapy and pressure relief that provided the best cure rates found by Thomas *et al.* (2015a) could be because this treatment allowed the digital cushion to heal and regain functional capacity. On the other hand, if treatment was not early, the digital

cushion may become damaged, which could explain why delayed treatment showed no beneficial effects of NSAIDs or a block (Thomas *et al.*, 2015b). Further, many works demonstrated that lesions lead to more lesions in the subsequent lactation. A good example is the work of Machado *et al.* (2011) where thin digital cushion, that was associated with presence of a CHDL at drying off, predisposed lameness in the next lactation.

These works suggest that pathologic changes within the foot are occurring with lameness. Further, abnormal bone modelling appears on and around the flexor tuberosity with age (Maclean, 1970; Blowey *et al.*, 2000; Lischer *et al.*, 2002; Tsuka *et al.*, 2012) and appears greater in cows with sole ulcers at slaughter (Tsuka *et al.*, 2012). This could be a normal change with age, as risk of sole ulcer also increases with age (Sanders *et al.*, 2009; Oikonomou *et al.*, 2013; Solano *et al.*, 2015), or it could be another sign of degeneration of the internal anatomy within the foot that occurs with lameness; no link between bone modelling (new bone growth on the surface of an already mature flexor tuberosity) and severity of lameness during life has been demonstrated.

1.6 Summary and aims

This review covers theories surrounding the pathogenesis of the claw horn disruption lesions and highlights the complexity of the disease processes. There appears to be benefit in better understanding the anatomy of the foot and how it changes during periods of risk for lameness. The digital cushion appears to play a role in lesion formation and lameness; it seems plausible that mobilization of lipids from the digital cushion during negative energy balance results in thinning of the digital cushion, which reduces the cushioning capacity and leads to lesions and lameness. Further, it would be useful to further explore how the internal anatomy of the foot becomes damaged during lameness, which could help inform and understand lameness prevention strategies. Finally, the most frequent manual management intervention on the claw health, foot trimming, may in fact be doing damage to foot anatomy if performed incorrectly.

Aims of the current thesis were to:

- Assess how the digital cushion changes throughout lactation with changes in measures of body fat, and whether changes in digital cushion thickness in association with changes in body fat measures lead to lameness and lesions.
- Assess the appropriateness of commonly quoted dimensions for foot trimming.
- Determine whether bone modelling is associated with lameness during life, or whether it is merely an effect of age.

2 Linking Bone Modelling on the Flexor Tuberosity of the Distal Phalanx with Lameness during Life

2.1 Introduction

Claw horn disruption lesions (CHDLs) constitute a non-infectious subset of the lameness-causing diseases and include sole ulcers, sole haemorrhage and white line disease (Offer *et al.*, 2003; Bicalho and Oikonomou, 2013). CHDLs have a high rate of reoccurrence (Enevoldsen *et al.*, 1991; Green *et al.*, 2014; Foditsch *et al.*, 2016), delayed detection of lameness increases the risk of more severe lameness (Bell *et al.*, 2009) and the risk of CHDLs increases as a cow ages (Sanders *et al.*, 2009). Given that CHDLs are associated with production losses, reproductive inefficiency and poor welfare (Sprecher *et al.*, 1997; Dyer *et al.*, 2007; Algers *et al.*, 2009), preventing the disease would be ideal (Potterton *et al.*, 2012). However, their aetiopathogenesis remains poorly understood; better understanding of the disease process may inform targeted prevention strategies (Algers *et al.*, 2009; Potterton *et al.*, 2012).

Within the hoof capsule, the distal phalanx is suspended from the wall through laminar attachments and supported above the sole by the digital cushion (Lischer *et al.*, 2002). The 'typical' sole ulcer (one of the most severe manifestations of claw horn disruption) develops beneath the axial aspect of the flexor tuberosity of the distal phalanx (Rusterholz, 1920); sole haemorrhage is considered a precursor (Whay *et al.*, 1997). Short ligaments attach the abaxial aspect of the distal phalanx to the abaxial hoof wall, whilst longer interdigital ligaments supporting the axial side of the distal phalanx allow greater depression of the axial aspect of the flexor tuberosity during foot-strike, perhaps leading to greater compression of the germinal epithelium at the sole ulcer site (Lischer *et al.*, 2002). The digital cushion dissipates concussive forces transferred through the flexor tuberosity of the distal phalanx during foot-strike and loading, and is thought to aid CHDL prevention by reducing peak forces on the germinal epithelium of the sole (Räber *et al.*, 2004; Bicalho *et al.*, 2009; Gard *et al.*, 2015).

New bone modelling appears on and around the flexor tuberosity with age (Tsuka *et al.*, 2012) and has been termed "exostosis" (Maclean, 1970; Blowey *et al.*, 2000; Lischer *et al.*, 2002), indicating growth of new bone from the surface of a bone, or "enthesopathy" (Tsuka *et al.*, 2012), indicating the inclusion of an enthesis (the insertion of a tendon or ligament onto bone). The new bone modelling may be an exacerbating factor for ulceration (Rusterholz, 1920; Maclean, 1970; Tsuka *et al.*, 2012), and appears greater in cows with sole ulcers at slaughter (Tsuka *et al.*, 2012), yet a link between lifetime history of lameness and lesions has not been demonstrated. No single term is

unanimously used for this new bone modelling, but it could be described as "modelling of the flexor tuberosity", and will be referred to from here in as "bone modelling".

The primary aim of this study was to discern whether bone modelling on and around the flexor tuberosity of the distal phalanx was associated with lameness from CHDLs throughout a cow's life ("Section 1" in both Materials and Methods, and Results). Bone modelling was defined as new bone growth on the surface of a mature flexor tuberosity. The secondary aim was to define the structural composition of bone modelling, with a view to understanding the pathologic process behind their formation ("Section 2" in both Materials and Methods, and Results).

2.2 Materials and Methods, Section 1: Measurement of bone modelling and exploring a link with lameness history

2.2.1 Study Design and Hypothesis

A retrospective cohort study investigated whether lameness and other variables recorded during life were associated with bone modelling on and around the flexor tuberosity of the distal phalanx at slaughter. The null hypothesis was that a lifetime history of poor locomotion or occurrence of CHDLs (assessed using locomotion score and treatment data, respectively) was not associated with greater bone modelling on and around the flexor tuberosity of the distal phalanx at slaughter.

2.2.2 Study Herd

The study population consisted of cows culled from the Crichton Royal Herd at the SRUC Dairy Research and Innovation Centre, Dumfries, UK, between November 2013 and August 2014. The centre was comprised of two units, 'Langhill' and 'Acrehead', where cows were milked three times daily.

As heifers, all animals calved into the Langhill herd. The Langhill herd runs a long term 2 x 2 factorial design trial, with genetic line by management system. Cows were between 75 and 99% pure Holstein and split into two genetic lines "Control" and "Select". Control line sires had predicted transmitting abilities for fat plus protein yield representative of the UK average at time of breeding, whereas Select line sires had the highest available within the UK (Pryce *et al.*, 1999). Management systems were (1) Home-grown: cows were managed less intensively with access to pasture where possible (typically between April and October) and fed a high forage diet of entirely farm-grown produce, and (2) By-product: cows were housed year-round and fed a low forage diet consisting of straw and bought-in distillery by-products, molasses and soya (Pryce *et al.*,

1999; Chagunda *et al.*, 2009). Cows at Langhill were locomotion scored weekly by trained, experienced assessors following standard protocols, on a five point scale based on Manson and Leaver (1988). Cows given a score of 4 or 5 (defined as 'obvious lameness on any leg, where behaviour is affected', or more severe) on a single visit or a score of 3 ('slight lameness detectable') on two consecutive weeks were considered lame and received veterinary treatment. A professional foot trimmer attended both herds bi-annually to trim feet deemed to be overgrown.

Acrehead is primarily a commercial unit and locomotion score data was not routinely captured. Cows were moved from Langhill to Acrehead at the end of their fourth lactation, although they could have been moved earlier due to incidence of mastitis, poor fertility or requirements of experimental protocols in the Langhill herd. Consequently, locomotion data immediately preceding slaughter was only available for cows that had recently been at Langhill. Culling occurred from both herds based on commercial or health and welfare grounds.

2.2.3 Sample Collection

The hind feet of all cows culled from either herd between the specified dates were collected *post mortem* at an abattoir, uniquely identified, and transported on ice to the University of Nottingham for storage at -20 °C. Feet were thawed overnight at room temperature prior to CT scanning, then packaged in containers, in pairs, using radiolucent foam and polystyrene to minimise movement within the container during the scan.

2.2.4 Computed Tomography Imaging of Claws

2.2.4.1 Scan optimisation

The device used was a cone beam X-ray micro Computed Tomography (X-ray µCT) industrial scanner: Phoenix v|tome|x m (GE Sensing and Inspection Technologies GmbH, Wunstorf, Germany) and the sample container was clamped to the sample stand. Scans were optimized using non-study feet collected from the same herd at the abattoir, altering current, voltage and number of projection images taken in order to best visualize the flexor tuberosity of the distal phalanx. Scatter could occur around the region of interest if the X-ray beam passed through the distal surface of the distal phalanx at a shallow oblique angle, since the bone was particularly dense in this region. Feet were orientated such that the distal surface of the distal phalanx was perpendicular to the X-ray beam in order to avoid this artefact. Also, the area of interest did not overlap with any part of the other sample.

2.2.4.2 Final scanning protocol

For the final scanning protocol, the CT scanner was set at 125 kilovolts and 320 microamps. A 0.5 mm copper filter was placed near the X-ray tube to reduce detector saturation and samples were orientated to minimize scatter at the site of measurement. The distance between the X-ray source and the sample and the X-ray source and the detector was 450.29 mm and 818.69 mm, respectively, resulting in a magnification of ×1.82 and a spatial resolution of 110 µm. Each scan acquired 2160 projection images over a 360˚ rotation of the sample using a detector exposure time of 333 ms, integrated over three averaged images, resulting in a total scan time of 48 min. Data were reconstructed using an inline median smoothing filter in datos|x software (GE Sensing), and exported in a volume file (.vgl) for image analysis using VGStudio MAX 2.2 (Volume Graphics GmbH, Heidelberg, Germany).

2.2.5 Measurement of Bone Modelling

Image files of each foot were assessed to measure the extent of bone modelling extending from the region of the flexor tuberosity of the distal phalanx of each claw (Figures 2.1 and 2.2). Linear measurements were taken of the maximum extent of bone modelling at four locations, A-D, extending in the following directions: (A) distally from the flexor tuberosity, (B) dorsally (towards the toe) along the base of the distal phalanx, (C) in a plantar direction from the axial aspect of the flexor tuberosity and (D) in a plantar direction from the abaxial aspect of the flexor tuberosity. Prior to all measurements being taken, each claw was orientated in sagittal, transverse and frontal cross-sectional views simultaneously following a standard protocol, to ensure landmarks and direction of measurements were consistent (Figure 2.1).

Measurement A was the greatest extremity of bone modelling vertically from the contour of normal bone, on the distal aspect of the flexor tuberosity. The plantar-most aspect of the intercondylar eminence ("eminentia intercondylaris") of the distal phalanx in the distal interphalangeal joint (located as "X" in Figures 2.1 and 2.2) was identified as a consistent landmark that did not alter with bone modelling. A line drawn vertically down from X was visible in all views and became the origin of measurements B, C and D. Measurement B was taken in the sagittal plane and extended perpendicular to the line vertically down from X to the dorsal-most tip (towards the toe) of bone modelling on the distal aspect of the distal phalanx. Measurements C and D were taken in the transverse plane and extended from the vertical line down from X to the plantar-most tip of the greatest bone modelling axial to and abaxial to location X respectively (Figure 2.2). Scrolling through the µCT image slices of 0.11 mm thickness enabled identification of the greatest protrusion in each case. The height (measured vertically from location X to the origin of measurement A) and width (measured across the widest point) of the plantar aspect of the distal phalanx were also recorded.

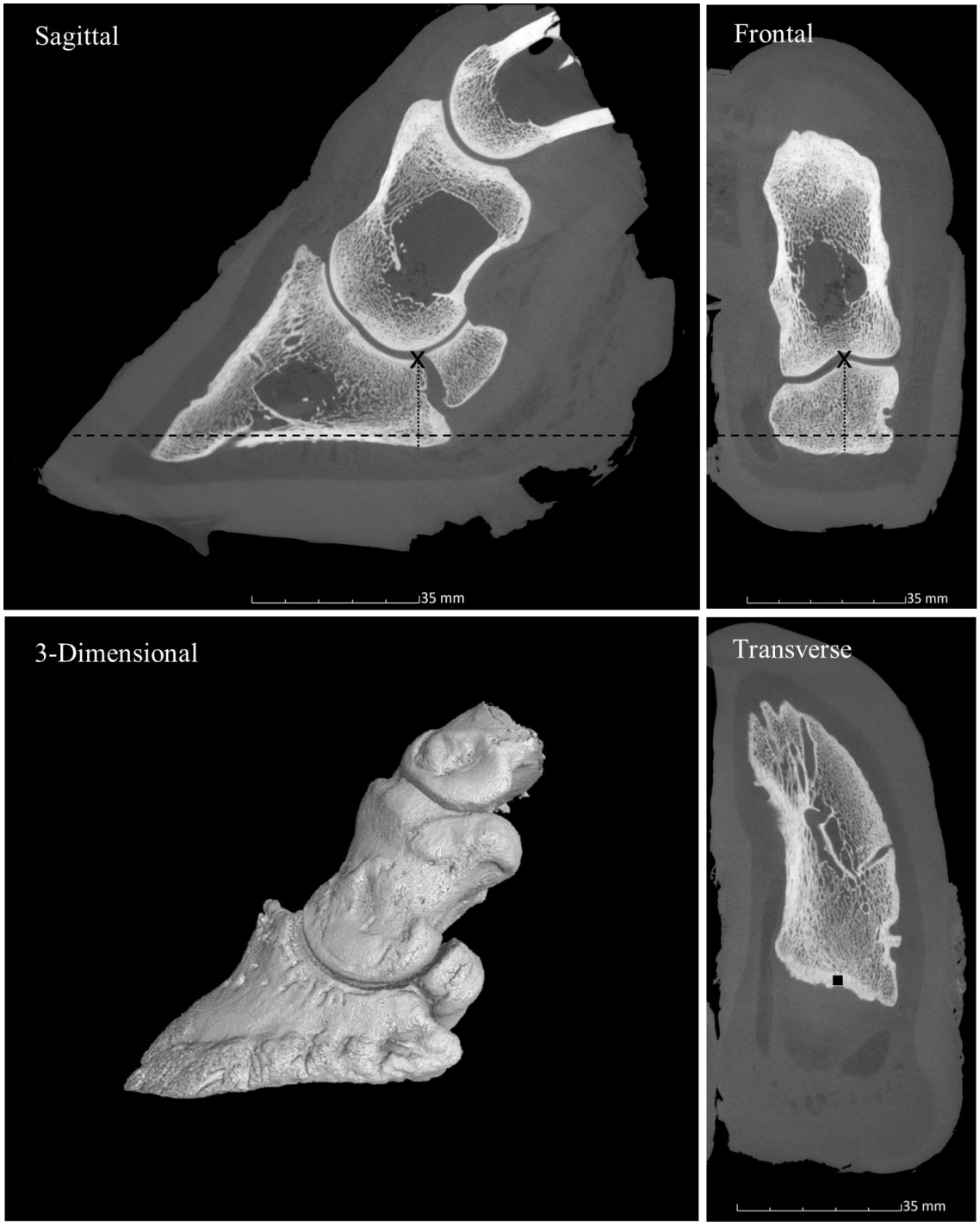

Figure 2.1: Computed tomography images of a normal foot, orientated for measurement of bone modelling. Sagittal, frontal and transverse cross sectional views are shown, and a three-dimensional image to demonstrate the normal bone contour. The plantar-most aspect (identified in the sagittal view) of the intercondylar eminence (identified in the frontal view) of the distal interphalangeal joint is located as "X". Dashed lines in the sagittal and frontal sections demonstrate the plane of the transverse image, within which a square dot (■) marks the intersection with the dotted line drawn vertically down from X.

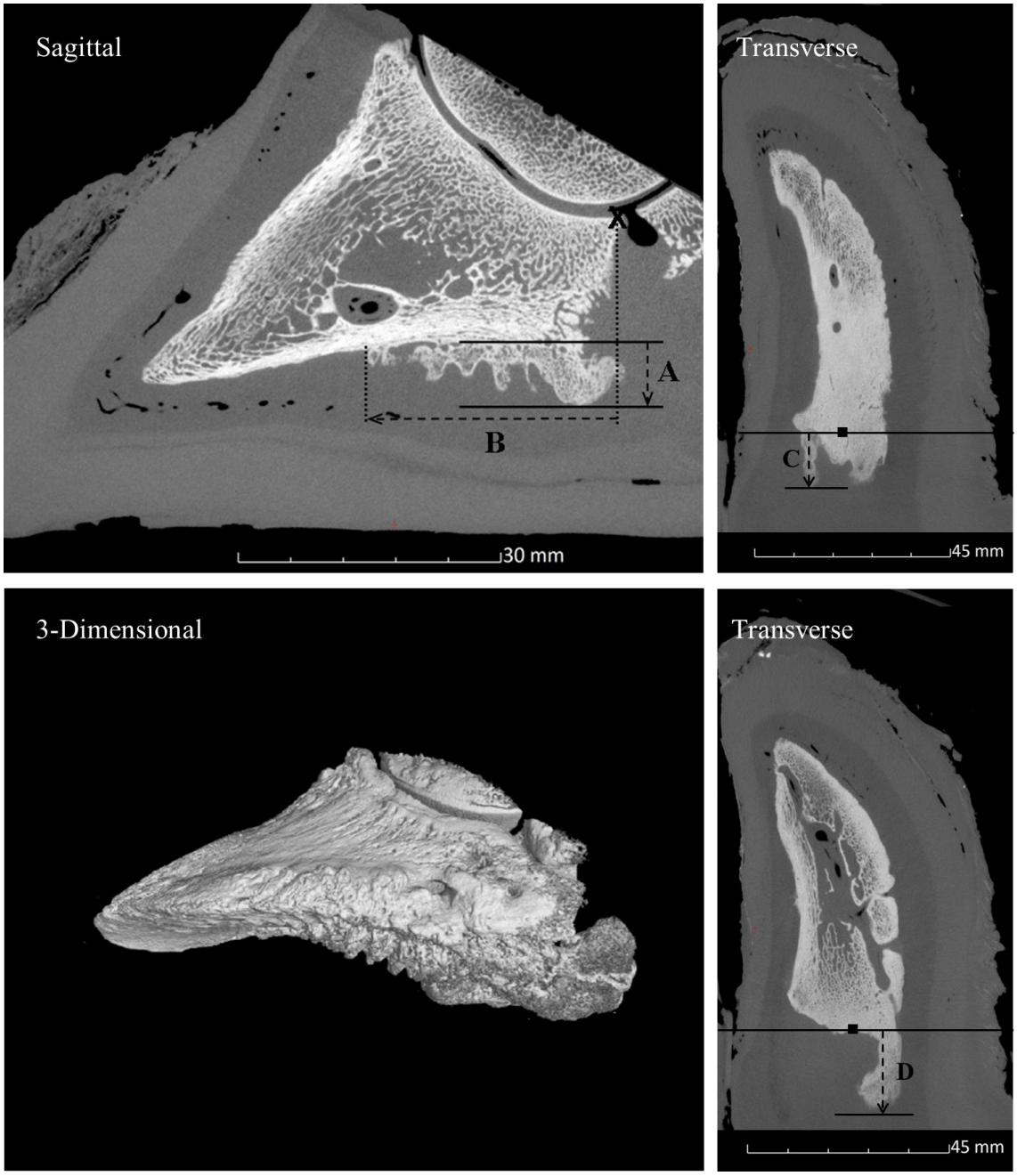

Figure 2.2: Computed tomography images of a foot displaying bone modelling. Measurement A was a linear measurement to the greatest vertical extremity of bone modelling from the contour of the normal bone, on the distal-most aspect of the flexor tuberosity. Site X was a consistent landmark (the plantar-most aspect of the intercondylar eminence of the distal phalanx in the distal interphalangeal joint) that did not alter with bone modelling, and a line drawn vertically down from X was visible in all views and became the origin of measurements B, C and D. Measurement B was taken in the sagittal plane and extended horizontally to the dorsal-most tip (towards the toe) of bone modelling on the distal aspect of the distal phalanx. Measurements C and D were taken in the transverse plane and extended to the plantar-most tip of bone modelling axial to and abaxial to location X respectively.

2.2.6 Statistical Analysis: Descriptive Statistics

Associations of bone modelling between feet within cow and between claws within foot were explored using scatterplots and Spearman rank correlation coefficients or coefficients of determination, and compared with non-parametric Mann-Whitney U tests.

2.2.7 Statistical Analysis: Modelling

To evaluate the relationship between cow lameness history and bone modelling, linear regression models were constructed with outcome variables quantifying bone modelling and explanatory variables describing cow factors, including age and lameness data. Two variables calculated from measurements A to D were tested individually as outcomes in a linear regression model, using ordinary least squares algorithms and a forward stepwise procedure. To obtain the two outcome variables, measurements A to D were summed within each claw; (1) BM-Max was the greatest individual claw value (representing the most severely affected claw) and (2) BM-Ave was the mean value across all hind claws.

Explanatory variables describing a cow's lameness history were constructed to evaluate associations between bone modelling and lameness, and were based on either lesion treatment or locomotion score data. For lesion treatment, binary variables denoted whether a cow had been treated for (i) a CHDL or (ii) an infectious cause of lameness, since first calving. The impact of locomotion score on bone modelling at slaughter was explored using descriptions of locomotion score as follows. The percentage of scores at which a cow was lame (scores 3-5) or severely lame (4-5) was calculated for lactation 1, lactation 2, the 6, 12, 18 and 24 months subsequent to first calving and periods preceding slaughter of between 2 and 12 months. Mean and median locomotion scores were tested during the same periods. Individual animals could only be included in a model if locomotion score data were available within the period defined for that description of the locomotion score variable. Finally, to test the effect of cows with missing data on model parameters, the locomotion score variable was categorised to allow the inclusion of cows with no locomotion score data within the period defined by the model. The category thresholds specified were "severely lame at <2% of locomotion scores", "severely lame at 2-50% of scores" and "severely lame at >50% of scores", and a missing data category contained cows where no locomotion score data was available. Other explanatory variables included were: age (years) at slaughter, genetic line, management system, proportion of life spent in each management system and culling reason. Polynomial terms of all continuous variables were also tested in the models.

Models were constructed in Minitab 17 Statistical Software (2010) following principles outlined by Dohoo *et al.* (2009) and took the format:

$$Y_i = \beta 0_i + \beta^1 X^1_i + \beta^2 X^2_i + \ldots + e0i$$
$$[e0i] \sim \text{Normal}\ (0, \sigma^2_e)$$

where the outcome Y was a cow-level variable of bone modelling (either BM-Max or BM-Ave in separate models), X_i were exposure variables for the i^{th} cow, β were the relevant coefficients of these exposures, $\beta 0$ the intercept value and e0i the residual error term, with mean 0 and variance σ^2_e. Biologically plausible interactions were tested and significant terms were retained in the model at $P < 0.05$.

Model fit was assessed as follows. Data points with large leverage or influence were identified and their impact on model parameters was evaluated using the DFITS function in Minitab®, which quantifies the difference in model parameters both with and without each data point. Model fit was deemed to be adequate if model parameters remained biologically and statistically similar (i.e. coefficients remained significant) when the model was re-fit excluding data points with high leverage or influence. Both outcome variables were log-transformed to determine whether model fit or interpretation changed with the transformed outcome, but since this did not occur, non-transformed data were used for the final models.

2.3 Materials and Methods, Section 2: further study of bone modelling

2.3.1 Selection of a subset of flexor tuberosity samples for further study

A subset of samples were selected in order to explore the tissue architecture of bone modelling, and to visualize micro-architecture of the flexor tuberosity and bone modelling. Nine distal phalanges were selected using a stratified random sampling technique and bone samples were cut from the region of the flexor tuberosity (Figure 2.3). The subset consisted of 3 normal samples, 3 samples with bone modelling from cows where lameness history was available and 3 of the most severely affected samples, which were from old cows and lameness history was unavailable but CT scans had shown severe bone modelling. These samples underwent further micro-CT to a higher resolution followed immediately by processing for histology, and assessed using two staining protocols in order to investigate the tissue architecture and cellular composition.

The bone samples were cut out of the frozen claws to 5 mm x 10 mm x 10 mm cuboids using a band saw (Figure 2.3). Three sagittal cuts through distal phalanx split it into 4 slices. A frontal and a transverse cut then removed samples of the flexor tuberosity, with bone modelling attached where present, for analysis. Once thawed, bone samples were

immediately packed for CT scanning, three per 60 ml rigid plastic pot, and separated and surrounded with radiolucent foam, to minimize movement during scanning.

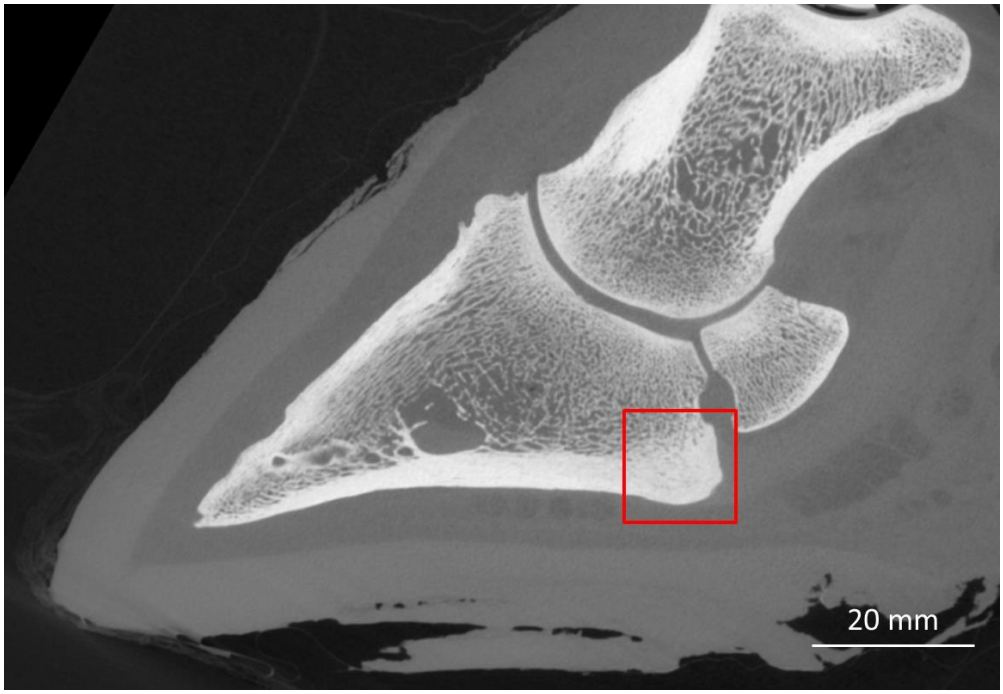

Figure 2.3: Computed tomography image (scan resolution: 110 μm) of a bovine foot, with a red square to mark the region sampled for histology and CT scanning at a higher resolution.

2.3.2 Computed tomography imaging at a higher resolution

The same CT scanner (Phoenix v|tome|x m) was used and the scanning protocol was optimized (as described in 2.2.4.1) using samples of flexor tuberosity displaying similar bone modelling that were not later used for the final analysis. The CT scanner was set at 140 kilovolts and 120 microamps. The distances from the X-ray source to the sample and to the detector were 81.87 mm and 818.69 mm, respectively, resulting in a magnification of ×10.0 and a spatial resolution of 20 μm. Each scan acquired four projection images in each of 540 positions throughout a 360˚ rotation of the sample, using a detector exposure time of 200 ms, resulting in a scan time of 29 min. The first of each four images was skipped to eliminate detector memory and the subsequent 3 were averaged. Data were reconstructed using the techniques described in 2.2.4.2 and analysed using the VGStudio Max 2.2 software. Volume files of individual bone segments were later extracted and exported as image stacks for analysis in the open-source software Fiji (Schindelin *et al.*, 2012).

2.3.3 Processing of bone samples for histology

Immediately following CT scanning, samples were immersed in phosphate-buffered saline for 12 hours, to wash in preparation for histology. The following describes how the histology preparation process was optimized, and the final histology process is presented afterwards.

2.3.3.1 Optimisation of histology processing

The optimization process had used 16 trial samples of flexor tuberosity that were not used for analyses. Two decalcifying agents were tested: 0.79 molar concentration of nitric acid ("5%" nitric acid) and Calci-Clear (National Diagnostics, Atlanta, U.S.), a mildly acidic, slow-acting blend of chelating and sequestering agents. Nitric acid was observed to decalcify the tissue rapidly, but within 2 hours the tissue architecture was severely distorted and the tissue was not completely decalcified, therefore use of nitric acid as a decalcifying agent was terminated. In order to optimize the protocol for decalcification with Calci-Clear, samples were placed in 10× the tissue volume of neat Calci-Clear, which was replaced every two hours. The solution was tested for the end-point of decalcification hourly, which was near when no precipitate ($Ca(OH)_2$) formed after the addition of ammonium hydroxide to 5 ml of the used Calci-Clear solution removed from the sample. This typically took 28 hours. The samples were then left in Calci-Clear for a further 6 hours to complete the decalcification, which made cutting for histology easier without distorting the tissue architecture upon microscopy. Visualization under the microscope showed that tissue morphology was not compromised by decalcification and the bone samples were easily cut, again allowing for good visualization of the tissue.

After decalcification, tissues were processed manually in glass scintillation vials (Fisher Scientific Ltd., Loughborough, UK). Tissues were dehydrated in 70%, 90% then 100% ethanol. The temperature and duration that samples spent in 70% and 90% solutions was optimized using 8 samples in a 2×2 crossover design: samples were placed in 70% ethanol either for 1.5 hours at room temperature or for 72 hours at 5 degrees Celsius, after which samples were transferred to 90% ethanol for either 1.5 hours at room temperature or for 72 hours at 5 degrees Celsius. Samples then went through the rest of the procedure described below (2.3.3.2). The protocol that performed best was 70% ethanol for 1.5 hours at room temperature followed by 72 hours in 90% ethanol at 5 degrees Celsius.

2.3.3.2 Final histology processing protocol

Samples were: (1) decalcified in Calci-Clear at room temperature, which was replaced every 2 hours until the endpoint of decalcification was reached. The endpoint was

identified when no precipitate ($Ca(OH)_2$) formed after the addition of ammonium hydroxide to a sample of the Calci-Clear solution, and typically took 28 hours. The samples were replaced in Calci-Clear for a further 6 hours beyond the endpoint since in preliminary work this had improved cutting without affecting the histology; (2) dehydrated in increasing concentrations of ethanol up to 100% (in the order: 70 % ethanol for 1.5 hours at room temperature, 90 % for 72 hours at 5 °C, 100 % for 3 hours at room temperature [×2]); (3) the ethanol was cleared with xylene for 3 hours [×2]; (4) embedded in paraffin wax at 60 degrees Celsius; (5) cut in the sagittal plane at a thickness of 9 microns using a microtome (Leica Microsystems (UK) Ltd., Milton Keynes, UK) with a "N35" long duration stainless steel microtome blade (FEATHER®, Osaka, Japan); (6) mounted onto polysilinated glass microscope slides (Fisher Scientific Ltd., Loughborough, UK); (7) stained using one of two protocols described below.

2.3.4 Histology staining protocols

2.3.4.1 Haematoxylin and eosin

The protocol for haematoxylin and eosin staining was as follows. Sections were (1) deparaffinised in xylene for 4 mins, (2) rehydrated through an ethanol series (100%, 90% then 70%; 4 minutes each) and rinsed with distilled water (dH_2O), (3) placed into haematoxylin (Sigma-Aldrich, UK) for 2.5 minutes and rinsed with dH_2O, (4) immersed in 1% industrial methylated spirit and 10% ammoniated water for 15 seconds each, rinsed and (5) placed in eosin (Sigma-Aldrich, UK) for 4 minutes, rinsed, (6) dehydrated through an ethanol series (70%, 90% then 100%; 4 minutes each), then (7) placed in xylene for 4 minutes and mounted with DPX mountant and a glass coverslip.

2.3.4.2 Masson's trichrome stain

The protocol used a Masson's trichrome kit (Sigma-Aldrich, UK) and was optimised based on the manufacturer's guidelines; the protocol used was as follows. Sample slides were (1) deparaffinised in xylene for 4 minutes and rehydrated through an ethanol series: 100%, 90% and 70%, 4 minutes per solution, (2) washed in phosphate-buffered saline for 5 minutes, (3) put through a mordant in Bouin's solution for 60 mins at 60 °C, then washed in running tap water for 5 mins to remove picric acid and rinsed in dH_2O, (4) placed in a humidifying tray and covered with 200 µl of haematoxylin, and left for 2 minutes in order to stain the nuclei, (5) washed in running tap water for 5 mins and rinsed, (6) incubated with 25% Biebrich Scarlet for 30 seconds and rinsed twice, (7) treated with 5% phosphotungstic acid for 10 minutes to remove the red colouring from the collagen, (8) treated directly with 200 µl of Light Green for 15 minutes and rinsed, (9) treated with 1% acetic acid for 1 minute and rinsed, (10) dehydrated through ethanol series (70%, 90% and 100%; 4 minutes each), and (11) placed in xylene for 4 minutes and mounted with a coverslip using DPX mountant.

2.3.5 Microscopy of histological slides

Stained histology sections were analysed and photomicrographs taken using a light microscope (DM5000 B, Leica Microsystems Ltd.) and a digital colour camera (DFC420, Leica Microsystems Ltd.) with Leica Application Suite software. Analysis was carried out in a blinded manner.

2.4 Results, Section 1: Measurement of bone modelling and exploring a link with lameness history

2.4.1 Animal data

Within the sample collection period, 142 hind feet from 72 cows were collected; two feet from two different cows were irretrievable at the abattoir. Mean cow age at slaughter was 71 months (median: 69, range: 30 to 139) and reasons cited for culling were grouped as fertility (n = 35), mastitis (n = 14), lameness (n = 8) and 'other' (n = 15).

2.4.2 Descriptive Statistics

Table 2.1 describes data for measurements of bone modelling A to D for the entire data set of 142 hind feet. The distribution for each measurement was right skewed, and A to C had a high count of zero values. Lateral claw measurements were greater than medial when all measurements A to D were tested together ($p = 0.024$); however, when each measurement (A, B, C or D) was tested individually, differences between lateral and medial claw measurement within foot were not significant. Measurements A to C were >0 more frequently in the lateral than the medial claw; poor correlations existed between lateral and medial measurements within foot; and Figure 2.4 illustrates correlations within individual measurements (A to D) between contralateral claws. Within claw, each measurement A to D was significantly correlated with every other measurement: Spearman Rank correlation coefficients (rho) ranged from 0.98 between A and B to 0.39 between C and D ($p < 0.001$ in each case).

The claw with the greatest bone modelling measurement for each cow (which became the variable BM-Max for that cow) was a lateral claw in 44 animals and a medial claw in 28, as broken down: right lateral (n = 22), left lateral (22), right medial (20) and left medial claw (8). BM-Max and BM-Ave were highly correlated ($R^2 = 0.93$; Figure 2.5) with a cow's BM-Max being on average 1.7 times greater than BM-Ave.

Table 2.1: Descriptive data of bone modelling measures at the claw level (A to D) and at the cow-level measures (BM-Max and BM-Ave), for all cows. Measurements A and B quantify bone modelling in plantar and dorsal directions from the flexor tuberosity of the distal phalanx of each claw respectively, and C and D describe the length of plantar protrusions on the medial and lateral aspects of the distal phalanx. Measurements A to D were combined within claw, and the greatest claw value for each cow (BM-Max) and the average value across the hind claws (BM-Ave) are shown.

Measure (mm)	Claw	Count	Mean	Min	Lower quartile	Median	Upper quartile	Max
A[1]	Lateral	142	0.83	0	0	0	1.04	8.31
	Medial	142	0.43	0	0	0	0.79	4.92
B[1]	Lateral	142	5.89	0	0	0	7.83	49.0
	Medial	142	3.45	0	0	0	4.77	42.2
C[1]	Lateral	142	1.41	0	0	0	0	30.4
	Medial	142	0.29	0	0	0	0	17.7
D[1]	Lateral	141	6.47	1.31	4.01	5.14	7.39	28.2
	Medial	140	5.49	0.33	3.34	5.26	6.50	20.9
BM-Max		72	19.4	2.55	5.63	9.62	21.0	96.7
BM-Ave		72	12.2	2.28	4.47	6.94	12.3	69.0

[1]Lateral > Medial when all measurements A to D were tested together, $p = 0.024$. Differences between lateral and medial measurements of A to D when tested individually were not significant ($p = 0.32, 0.30, 0.07, 0.11$ respectively).

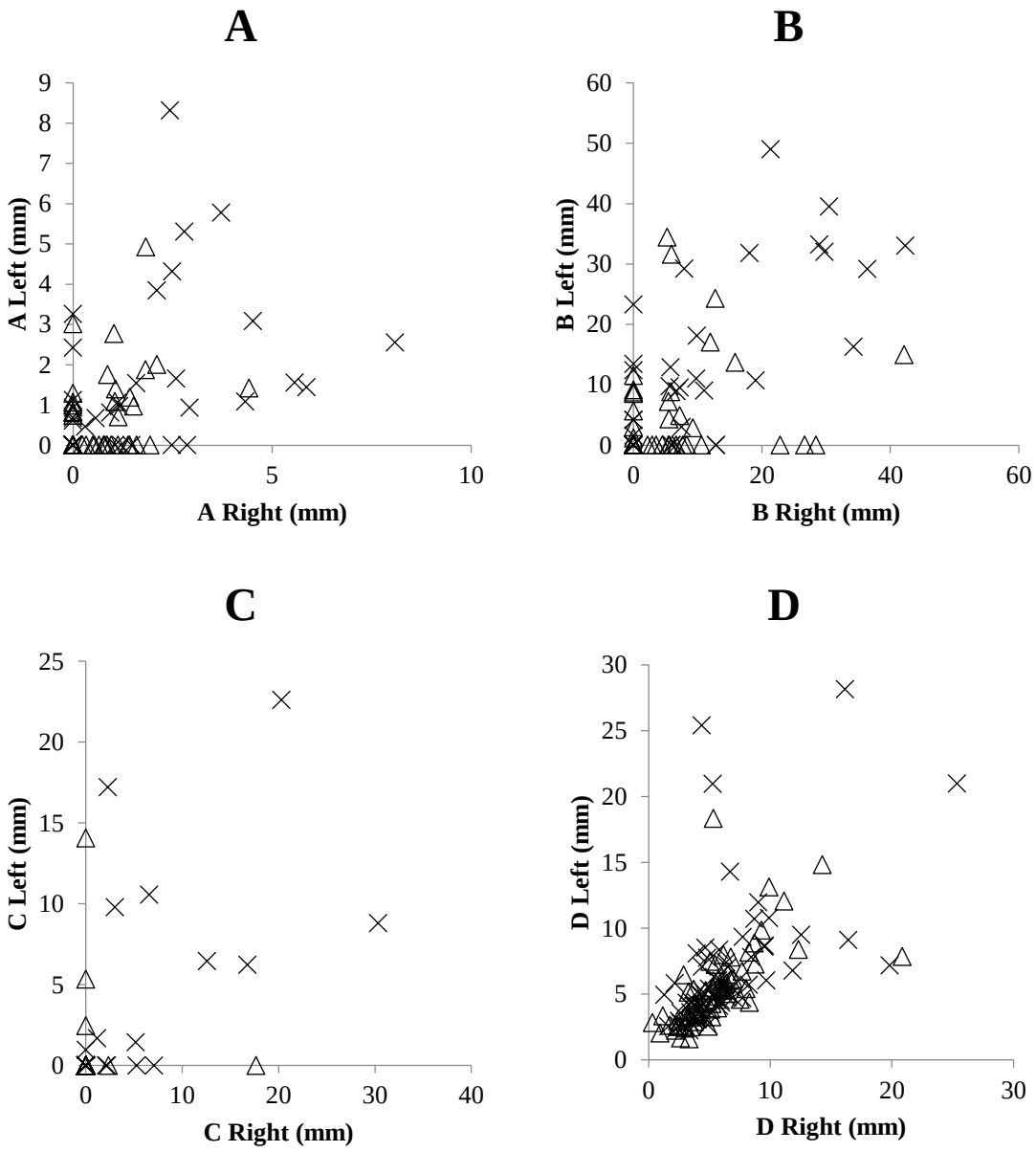

Figure 2.4: Scatterplots of four measures of bone modelling, A to D, from lateral (×) and medial (Δ) claws, between contralateral hind feet within cow. Data collected during a post mortem study investigating the association between bone modelling on the flexor tuberosity of the distal phalanx bone and lameness history.

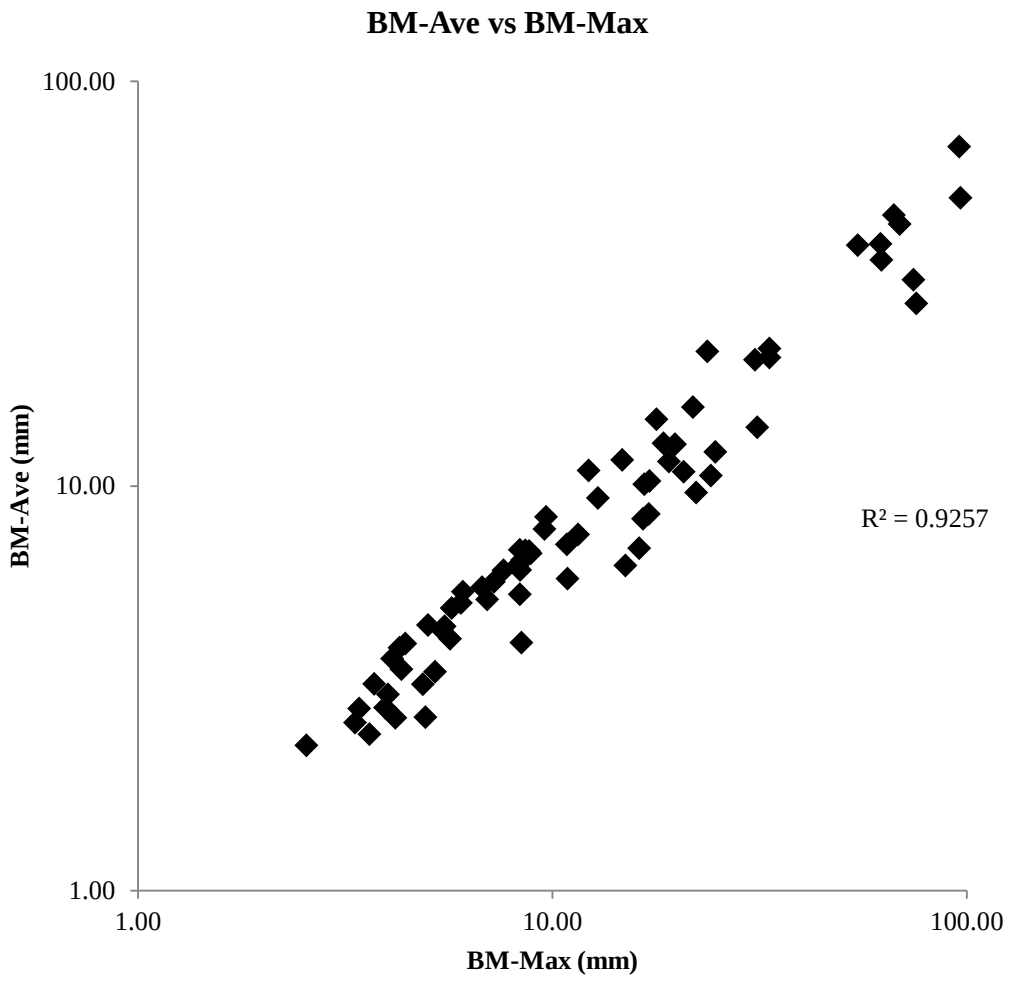

Figure 2.5: Scatterplot showing correlation between two cow level measures that describe the average length and the maximum length of bone modelling on the flexor tuberosity of the distal phalanges of the hind feet (BM-Ave and BM-Max respectively), in a post mortem study investigating the link between bone modelling at slaughter and lameness history.

2.4.3 Statistical Modelling

Locomotion score data were available during the 6, 12, 18 and 24 months following first calving for 60, 57, 58 and 53 cows respectively and during the 6, 12, 18 and 24 months preceding slaughter for 34, 38, 39 and 45 cows. Of the 72 cows, 28 were recorded as having been treated for a CHDL at some point during life.

All final models were based on the 38 cows that had locomotion score data available during the 12 months preceding slaughter. These models are presented in Table 2.2 and include the explanatory variables age and either the percentage of locomotion scores at

which a cow was lame during the 12 months preceding slaughter (Table 2.2a and b) or occurrence of CHDLs during life (Table 2.2c), for each outcome measure (BM-Max and BM-Ave). Locomotion score variables were significant when they described the percentage of lame scores between 2 (n = 24 cows) and 12 (n = 38) months pre-slaughter. Model parameters did not differ substantively when the locomotion score variable included data from different periods pre-slaughter; the models in Table 2.2a are examples of models with good fit that were based on more of the data. These models estimate that a cow that had been scored lame at all locomotion scores during the 12 months pre-slaughter would have had a BM-Max of 9.7 mm (SE: 4.8 mm) greater than a cow that was sound at all locomotion scores (Table 2.2a); the effect of locomotion on BM-Ave was not significant (P = 0.08). These 38 cows had a mean age of 60 months (range: 31 to 85 months) and were lame at 20 % (range: 0 to 93 %) of locomotion scores within the 12 months preceding slaughter; mean age of the remaining cows was 83 months.

Models presented in Table 2.2b had the explanatory variables age and categories of the locomotion score variable; categorising the locomotion score variable enabled the effect of cows with missing data on model parameters to be tested. As cows with missing data were introduced into these categories, the number of outcome data points increased although there was no new explanatory data. The effect sizes and standard errors remained similar, although model fit deteriorated and ultimately prevented the model from converging. Models with good fit have been reported and contain no cows with missing data. These models estimate that cows that had been lame at >50 % of locomotion scores had BM-Max and BM-Ave 9.8 mm (SE: 3.9) and 5.0 mm (SE: 2.4) greater than cows that had been lame at <2 % of scores, respectively (Table 2.2b). Cows that had been lame at between 2 and 50 % of scores had a smaller but non-significant effect in the same direction.

Table 2.2c presents models that contain CHDL occurrence as an explanatory variable, which were based on the same 38 cows as the previous models. BM-Max and BM-Ave were greater in cows that had experienced a CHDL compared with those who had not, regardless of the period from which data were taken. In the reported model, the 12 cows that had received treatment for a CHDL had a BM-Max value 7.0 mm (SE: 2.2) greater than the 26 cows that had not; the effect size for BM-Ave was 3.6 mm (SE: 1.3).

Table 2.2: Linear regression models with outcome either BM-Max (the sum of bone modelling measures A-D on the most severely affected hind claw, for each cow) or BM-Ave (the average of the sum of bone modelling measures on each hind claw of each cow), based on 38 cows with locomotion score data within the 12 months preceding slaughter. Explanatory variables were age at slaughter (years) and either:

(a) Locomotion score: the percentage of scores at which a cow was severely lame (locomotion score 4 or 5) during the 12 months preceding slaughter.
(b) Locomotion score: the locomotion score variable described in (a), but categorised as severely lame at <2% of scores, at 2-50% of scores or at >50% of scores.
(c) Occurrence of claw horn disruption lesions (CHDLs) throughout life (0 or ≥1).

	BM-Max (mm)			BM-Ave (mm)		
Variable	Coef	SE	P value	Coef	SE	P value
Model (a)						
Intercept	-6.48			-1.44		
Age	3.04	0.86	<0.001	1.5	0.52	0.004
Locomotion score[1]	9.72	4.75	0.04	5.02	2.85	0.08
Null σ^2 explained (%)	40			32		
Model (b)						
Intercept	-6.63			-1.78		
Age	2.75	0.87	0.002	1.34	0.52	0.01
Locomotion score[2]						
<2% (n=8)	Reference			Reference		
2-50% (n=26)	3.65	2.46	0.14	2.34	1.48	0.11
>50% (n=4)	9.83	3.92	0.01	5	2.36	0.03
Null σ^2 explained (%)	43			34		
Model (c)						
Intercept	-3.88			-0.12		
Age	2.46	0.84	0.003	1.21	0.52	0.02
CHL[2]						
0 (n=26)	Reference			Reference		
≥1 (n=12)	6.98	2.17	0.001	3.55	1.33	0.007
Null σ^2 explained (%)	48			38		

[1]The coefficient demonstrates the difference between 0 and 100 % of scores lame
[2]Numbers of cows in each category are shown in brackets. Categories were used during modelling to allow the inclusion of all cows with missing locomotion or lesion data. Models did not fit when all cows with missing data were included in these categories. Therefore, models with good fit are reported, rather than models with inadequate fit that include missing data.

In all models, age explained the majority of the variation in bone modelling; polynomial terms of age were not significant in the final models. Both locomotion and CHDL occurrence explained additional variation in bone modelling. Since an increasing percentage of locomotion scores lame was positively associated with CHDL occurrence (Figure 2.6), both variables were not included in the same final models.

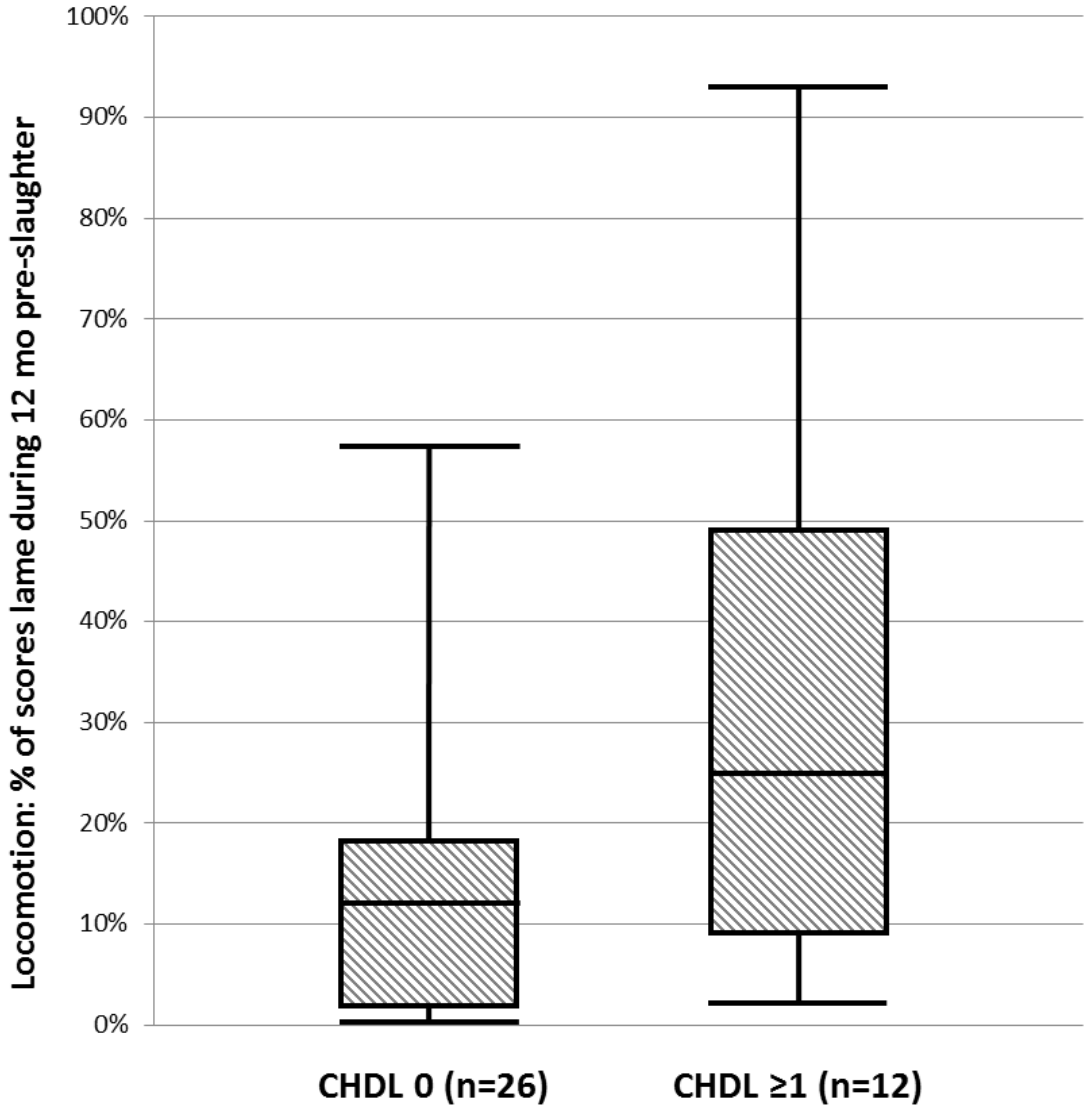

Figure 2.6: Distribution of the locomotion score explanatory variable used in a linear regression model of bone modelling, for cows that had not and that had been recorded as having a claw horn disruption lesion during life; CHDL0 and CHDL1 respectively. Box shows median, 25th and 75th percentiles, and whiskers shown minimum and maximum values of the locomotion score variable.

Locomotion score variables describing lameness in periods subsequent to first calving, or during first or second lactation, did not significantly predict bone modelling at slaughter. Culling reason, genetic line and management system were not significant in the models, nor was occurrence of infectious causes of lameness. Height and width of the flexor tuberosity, as well as variables describing locomotion score data early in life, and mean and median locomotion scores for various periods throughout life, were all non-significant.

2.5 Results, Section 2: further study of bone modelling

All bone samples used in the subset for Section 2 of the analysis were from cows >40 months of age and it was assumed that all cows were skeletally mature.

2.5.1 Computed tomography of bone modelling at 20 µm

On CT, all bone modelling appeared to be calcified. Where new bone modelling extended from the surface of bone, it extended from what appeared to be normal bone within the normal contour of the distal phalanx. The new bone modelling appeared to be less dense than normal bone, although the "normal" contour was difficult to define due to the new bone modelling protruding from it. CT scans of bone modelling are demonstrated in Figures 2.8 to 2.10.

2.5.2 Histology of bone modelling

On histological sections, all 3 normal samples displayed normal mature bone around the surface of the distal phalanx. Additionally, all 6 abnormal samples displayed normal mature bone within the distal phalanx, with osteons visible. All bone that appeared as normal on CT and was within the limits of "normal" bone appeared to be normal on histology. However, in samples with bone modelling, the normal bone was surrounded by newer, loose bone, and in some cases, lysis had occurred into the bone too. Bone modelling was seen in samples from all cows with a lameness history and those without (n = 6 in total). This new bone modelling was confirmed as bone on CT scans and by looking at histological gross morphology photomicrographs. Where bone modelling was small, the surface of the bone had a rough texture with projections extending into the subcutis beneath. Where bone modelling was large, bone modelling was loosely woven (i.e. looser than the normal bone in this region) and represented an expansive mass that had grown from the surface of the mature bone of the surface of the distal phalanx. Figures 2.7 to 2.10 display representative examples of features seen on histological sections, and legends describe observations made.

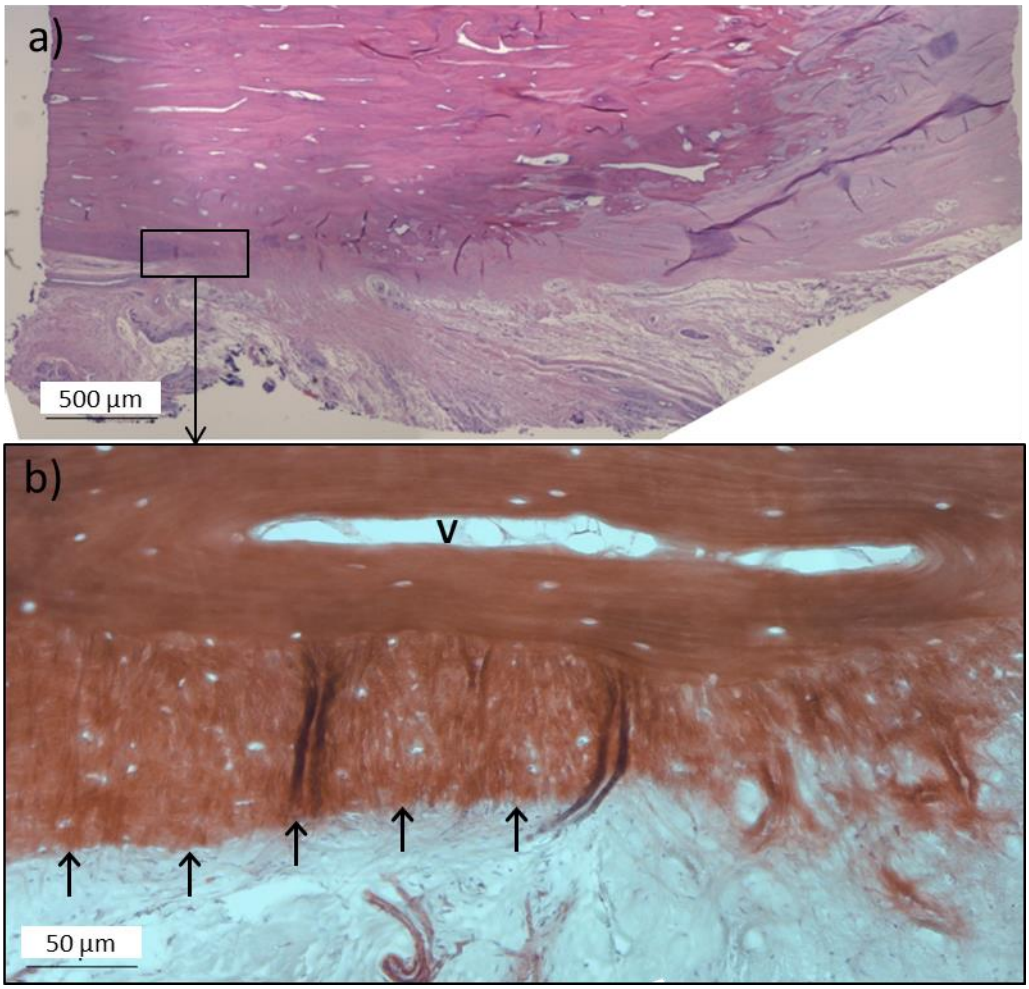

Figure 2.7: Sagittal histology section through a "normal" flexor tuberosity, similar to that shown in Figure 2.3, showing minimal bone modelling on CT.

a) Low magnification image of a slide stained with haematoxylin and eosin. Bone is visible towards the top and left of the image (bright eosinophilic colour), the insertion of the deep digital flexor tendon towards the top right, and connective tissue of the subcutis in the bottom half of the image, containing several small caliber blood vessels.
b) Higher magnification Masson's stain. An oblique section through an osteon is visible at the top, with an elongated clear space that resembles a blood vessel (**V**). Arrows (→) mark the distal border of bone, upon which periosteum may be present, and connective tissue of the subcutis is visible at the bottom, where the Biebrich Scarlet stain has not been taken up. Fibrous strands can be seen running between bone and subcutis.

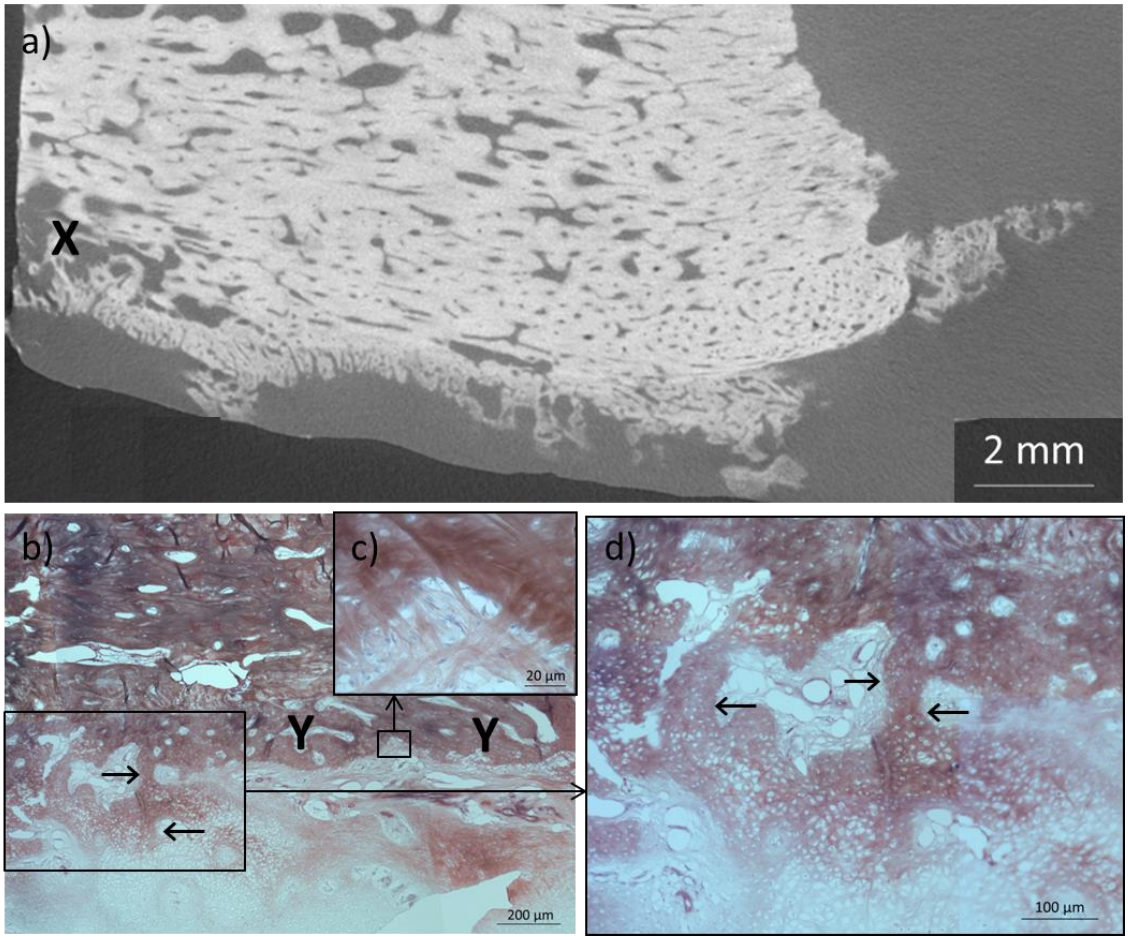

Figure 2.8: Sagittal CT image (resolution: 20 µm) and histology sections (Masson's stain) through the flexor tuberosity of a hoof that displayed bone modelling.

a) On CT, bone within the normal limits of the distal phalanx appeared to be mature bone, predominantly not deformed except for some areas that appears to be lysis, as visible towards the left hand side of the image (**X**). The distal aspect of the bone is rough and less radio-dense, and appears to have proliferated from the normal bone of the distal phalanx.
b) Mature bone is visible towards the top of the photomicrograph, with less structured bone in the centre of the image (**Y**). In the lower half of the image, loose projections of darker tissue extend from the bone into the subcutis (→).
c) Higher magnification image of fibrous strands running between bone and subcutis.
d) The bone/subcutis border is not distinct. Dark, apparently cellular projections appear to be protruding from the surface of the bone into the subcutis (→).

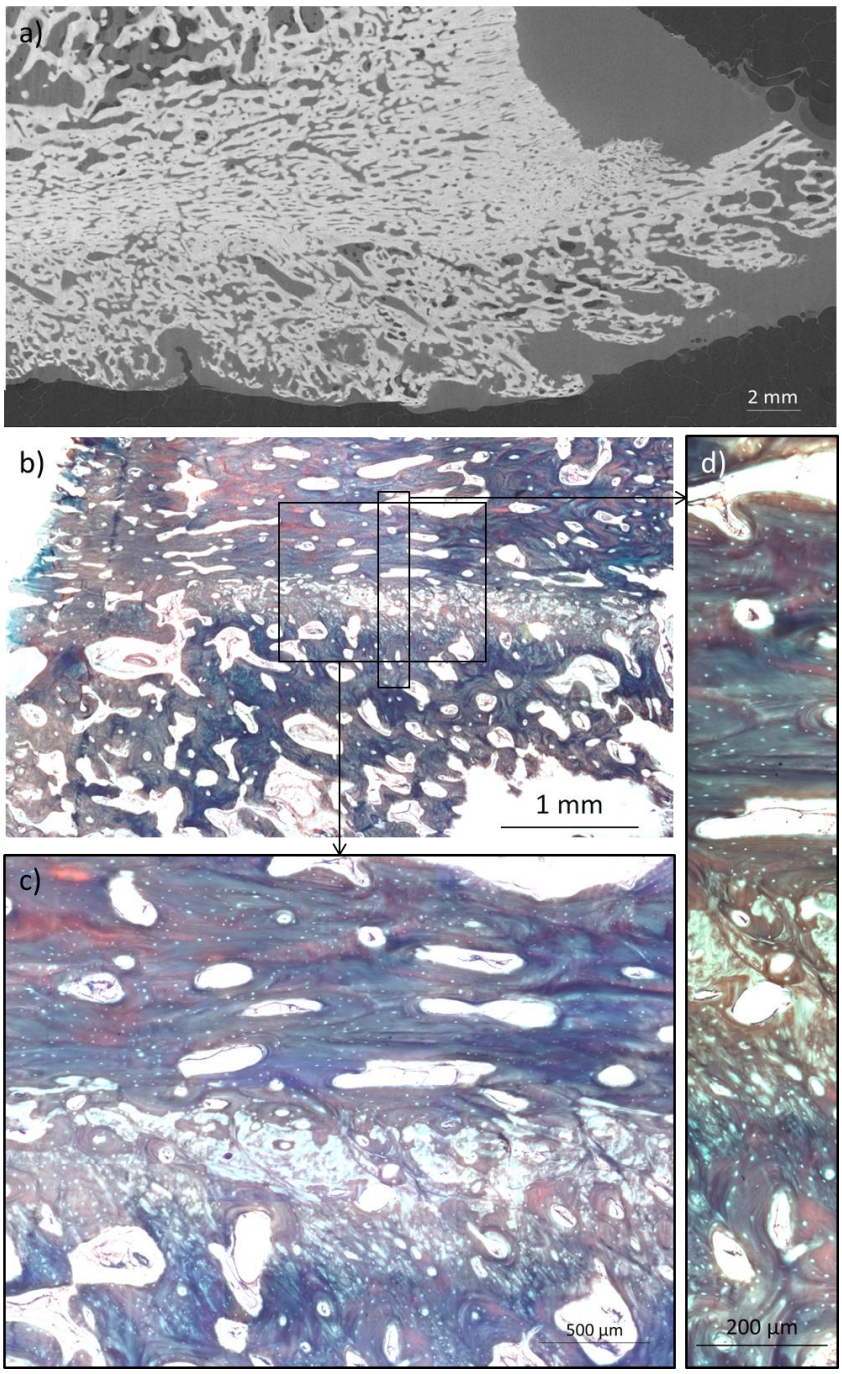

Figure 2.9: CT image (resolution: 20 µm) and histology images (Masson's) of the flexor tuberosity of a distal phalanx displaying a large degree of bone modelling.

a) Normal bone is visible in the top left corner of the image. In the lower half and right hand side of the image, loosely woven bone extends to the edge of the image in distal and plantar directions.
b) Normal bone is visible towards the top of the image. A strip of less deeply stained tissue is visible just above the middle of the image where no osteons are visible. Underneath, a moderate number of osteons are visible in the lower half of the image, where bone has looser structure compatible with new bone growth. These features were observed at higher magnification in "c)" and "d)".

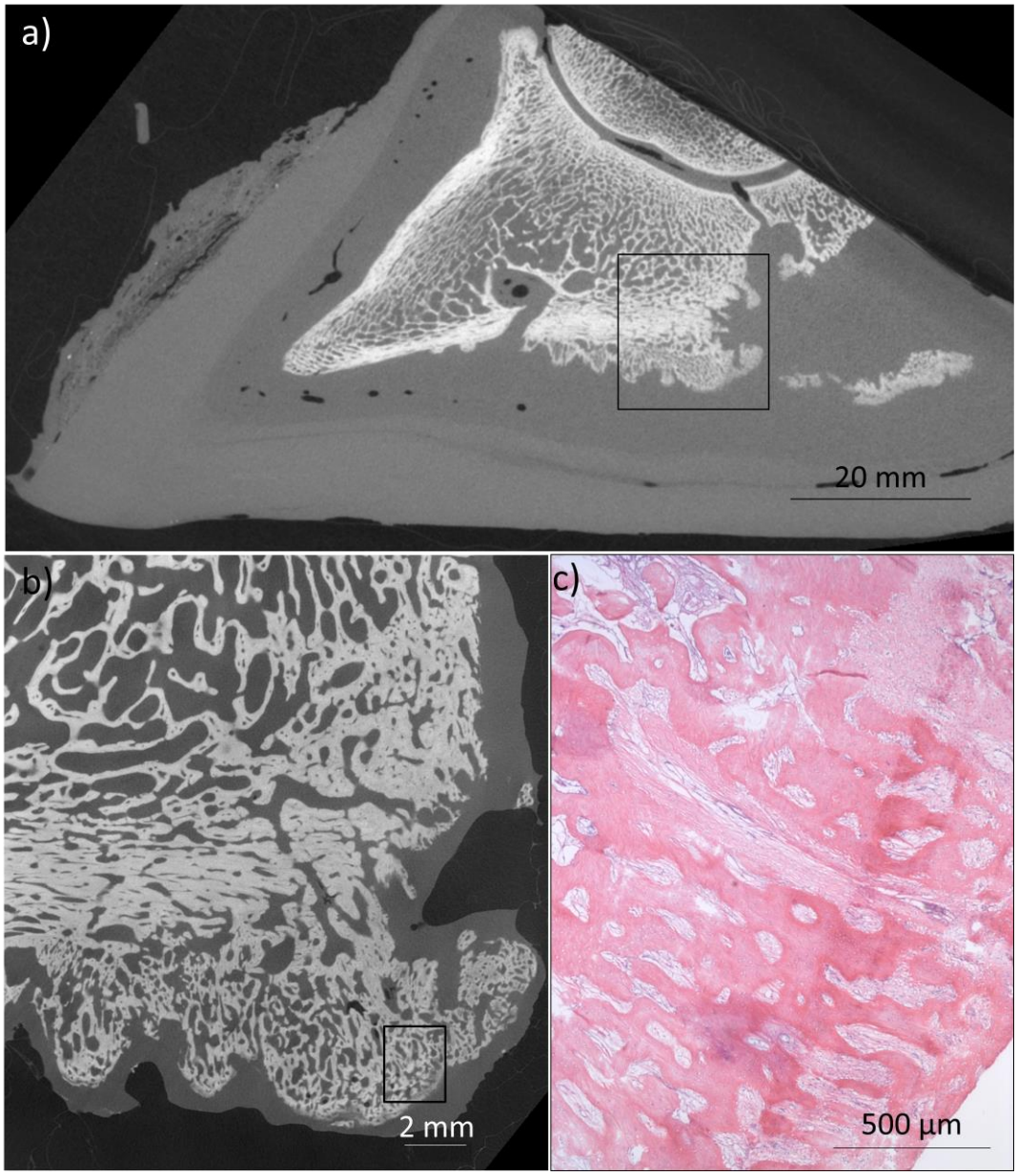

Figure 2.10: Computed tomography (resolution: 110 µm and 20 µm) and histological (stained with haematoxylin and eosin) images of a flexor tuberosity displaying severe bone modelling.

a) CT image (resolution: 110 µm) of a sagittal section through a distal phalanx displaying extensive abnormal new bone growth.
b) Higher magnification image of "a)" (resolution: 20 µm). In the lower third of the image, an expansive mass of loosely woven bone has grown from the surface of mature bone within the distal phalanx.
c) Histological section of the expansive mass imaged in "a)" and "b)", with organised networking of bone trabeculae, appearing as ossified tissue external to the normal bone.

2.6 Discussion

This is the first study to demonstrate that bone modelling on and around the flexor tuberosity of the distal phalanx at slaughter is positively associated with lameness history: after accounting for the effect of age, cows that had experienced more lameness in the 12 months pre-slaughter had greater bone modelling. Lifetime occurrence of CHDLs was associated with bone modelling, whilst the occurrence of infectious lameness diseases was not; this dataset suggests that the bone modelling is specific to CHDLs. Significant locomotion score terms detailed the percentage of scores lame leading up to slaughter, suggesting that bone modelling was associated with chronic lameness. The model indicates that if a cow had been lame at all locomotion scores in the 12 months preceding slaughter, BM-Max would be 9.7 mm greater than if the cow had been sound at all scores.

BM-Max appeared to be the better explained outcome (compared with BM-Ave); leg- or claw-specific locomotion or lesion data were not available, but BM-Max could be the better explained outcome due to bone modelling on the most severely affected claw being associated with lameness or lesions. This work cannot confirm this, but it seems a plausible hypothesis.

Age explained much of the variation in bone modelling, as previously reported on the distal phalanges of cattle (Tsuka *et al.*, 2012) and at a number of anatomical locations in Man (Benjamin *et al.*, 2006; Slobodin *et al.*, 2007). Further, bone modelling was greater on the lateral claw, which bears more weight (van der Tol *et al.*, 2002; van der Tol *et al.*, 2004). This study reports an additional effect of lameness from CHDLs to the effect of age. Extrapolation of the reported models to a greater age range (outside the 31 to 85 months tested) would be inappropriate, yet given that the more severe bone modelling seen in older cattle appeared to be more extreme cases of the bone modelling on claws included in the model dataset, it seems likely that the general inferences regarding the effect of lameness on bone modelling could also exist in older cows.

2.6.1 Mechanisms of bone modelling

Histologically bone modelling appeared to be new bone growths from the surface of mature bone into soft tissue where they normally would not exist. Bone modelling appeared to be similar to "heterotopic ossification" observed in humans, which is a common sequela to both arthroplasty and arthritis, likely occurring as a result of instability in the joint and inappropriate force transfer through the bone (Romano *et al.*, 2004; Saudan *et al.*, 2007). This bone modelling could also be described as exostosis, and could have occurred through trauma to the bone. The likely mechanism for any such bone modelling is proliferation of osteoprogenitor cells within the periosteum and

periosteal hyperostosis (Long *et al.*, 1993). Using combined dual staining immuno-histochemistry and systematic random sampling, the proliferation and apoptosis rates of each cell type could be quantified in both the mature normal bone and in the bone modelling in order to further understand the mechanism of growth and modelling.

Previous work suggested that similar bone modelling cases observed on the flexor tuberosity of the distal phalanx were lesions of the enthesis and could be termed enthesopathy, rather than being new bone growth directly from the surface of the bone (Tsuka *et al.*, 2012). Within the present study, in some sites, new bone modelling appeared to occur around or within the enthesis; Figure 2.8a displays bone modelling at seemingly separate locations, with one protruding in a plantar direction from the enthesis and some in a distal direction from the distal aspect of the flexor tuberosity. These presentations in different locations may reflect two different pathological processes, and mechanisms for these processes are suggested as follows. Firstly, where bone modelling extended into the tendon, it could have occurred as a physiological response to constant loading in order to increase the surface area of the tendon-bone junction. This has been described in the human literature in the absence of pain, for example at the Achilles tendon insertion in athletes (Benjamin *et al.*, 2000), occurring through vascular invasion into the tendon, followed by osteoblast deposition around capillary surfaces and spur formation, which appears as dense bone (Shaibani *et al.*, 1993; Benjamin *et al.*, 2000). Some of the small plantar bone modellings in the current work could be physiological adaptations to loading, which might more appropriately be termed enthesopathy (Tsuka *et al.*, 2012).

On the other hand, contrary to bone modelling occurring near the enthesis, the majority of new bone modelling was not associated with an enthesis, but resembled heterotopic ossification growing from the surface of the bone, which could occur as a result of periosteal reaction. This periosteal reaction could be stimulated by direct trauma to the osteoprogenitor cells of the periosteum due to insufficiencies in the force-dissipating structures surrounding the flexor tuberosity, such as the digital cushion (Räber *et al.*, 2004; Benjamin *et al.*, 2006); periosteocytes would then likely respond by multiplying and differentiating into osteoblasts to form new bone (Rana *et al.*, 2009). Alternatively, the periosteal reaction could be simulated through macrophage action in surrounding tissues, such as inflammation during an active CHDL (Hasturk *et al.*, 2012). This latter mechanism suggests that a CHDL precedes bone modelling, however a temporal link between lameness and bone modelling cannot be confirmed from this study.

If claw horn disruption lesions occur as a result of greater point-forces on the germinal epithelium of the sole (Bicalho and Oikonomou, 2013), it seems likely that new bone modelling could also cause further contusions within the germinal epithelium of the sole and act in perpetuating lesion formation and lameness. This could be one reason why

lameness predisposes further lameness (Hirst *et al.*, 2002; Randall *et al.*, 2015). NSAIDs have already been found to be useful in resolving claw horn lesions (Thomas *et al.*, 2015a) which was assumed to help recovery through anti-inflammatory action. Perhaps the use of NSAIDs in the treatment of CHDLs associated with lameness could reduce bone modelling too, and have a longer-term effect to help preserve the anatomy of the foot and prevent lameness later in life. Possible mechanisms for this action of NSAIDs are explored in 7.7, and this would be an interesting area for future work.

To summarize the possible mechanisms for bone change, some aspects of bone modelling could be occurring through physiologic mechanisms to increase the surface area of the tendon insertion onto the flexor tuberosity for strength, others through pathologic mechanisms such as trauma to the periosteum; either direct trauma through digital cushion insufficiency, or being stimulated by inflammatory cells in a local lesion. Bone modelling appears to be linked with lameness and claw horn disruption lesions suggesting a pathologic aetiology, and once present, bone modelling could perpetuate trauma within the germinal epithelium and lesions. This cross-sectional study design cannot fully assess the temporal associations between bone modelling and lesions, but this would be an exciting area for further work.

2.6.2 Possible association with white line disease

Whilst the work described here seems most pertinent to the aetiology of sole ulcer and sole haemorrhage, it could also in part help explain the aetiology of other lesions such as white line disease and heel ulcers. White line disease may result from compression of the germinal epithelium when compression occurs beneath the abaxial aspect of the flexor tuberosity (Lischer *et al.*, 2002) and have a similar causal pathway to sole ulcers and haemorrhage. This study and other cross-sectional work (Tsuka *et al.*, 2012) suggests that bone modelling initially occurs on the abaxial aspect of the distal phalanx, since where only small bone modelling was present, they were only observed abaxially. Haemorrhage in the corium beneath this site could elicit or be exacerbated by bone modelling on the abaxial aspect of the distal phalanx, and be visible in the white line as it grows out, becoming a risk area for separation, impaction and infection. This could explain why different CHDLs occur around similar stages of lactation and share similar risk factors (Leach *et al.*, 1997; Machado *et al.*, 2011; Green *et al.*, 2014), possibly pointing to a common underlying disease process.

2.6.3 Possible relevance to heel ulcers

Heel ulcers have been described as related to but distinct from sole ulcers; they occur further plantar to the typical sole ulcer site, appear more prevalent in older animals and recover poorly in comparison (Toussaint-Raven, 1985; Blowey *et al.*, 2000; Haslam and

Roberts, 2011). This study observed extensive bone modelling plantar to the distal phalanx, which extended up to 28.2 mm; the plantar limit of large bone modelling corresponded with the heel ulcer site. It seems possible that during foot-strike, the tips of these plantar bony projections could exert focal pressure on the germinal epithelium in this region, causing contusions that develop into heel ulcers. Further work is required to confirm or refute this mechanism.

2.6.4 Study limitations

This study was based on a convenience sample of the hind feet from 72 Holstein dairy cows culled from one UK research herd. Since locomotion score data were incomplete for cows that had moved from Langhill to the Acrehead unit, it remains possible that the model criteria led to the selection of a biased subset(s) of the study population. The first lameness variable constructed described locomotion during the first two lactations. The sample size was up to 60 cows (depending on the data available within the period tested) and there was no association between locomotion during the first and second lactations and bone modelling. However, in this instance, the period of locomotion data and slaughter were separated by up to 8 years and the lack of an association was not surprising. Next, the locomotion variable described the period immediately preceding slaughter; animals were only eligible for these models if they had locomotion data during the 12 months pre-slaughter. As locomotion data were only collected at the Langhill unit, the cohort was not a randomly selected subset of the population, rather it was a specific cohort constituting younger cows; cows did not remain at Langhill beyond four lactations but could be moved to Acrehead early, largely due to incidence of mastitis, poor fertility or specific experimental protocols. Whilst there is potential for bias, the author has no reason to suspect that early movement of cows to Acrehead influenced the results because the reasons for movement were not directly associated with lameness. Associations were evident and statistically significant in the final models and it is certainly possible that the general inferences reported apply to the wider population of Holstein dairy cows in any lactation, not selected for culling.

In summary, for bias to undermine the central inferences of the results and make them ungeneralizable to the wider population, either the association would need to be specific to the subpopulation studied or the converse of the results would need to be true in the wider population; that is, older cows with bone modelling had better locomotion. This seems unlikely given the previous supporting work in this area (Tsuka *et al.*, 2012) and the biological plausibility of the findings: CHDL occurrence was positively associated with bone modelling (Table 2.2c) and the percentage of lame locomotion scores preceding slaughter was positively associated with CHDL occurrence (Figure 2.6). This suggests an association between lameness measured by locomotion score and bone modelling is plausible. Further studies, either imaging the distal phalanx during life or

prospectively culling randomly selected cows from a herd, are required to confirm or refute the inferences made about the generalizability of these results to the wider population.

Approximately 60% of the variation in bone modelling remained unexplained, depending on the model, and explanatory variables in the statistical models may have been either missed or imprecise. For example, locomotion scoring is known to have relatively poor inter- and intra-observer repeatability (Whay *et al.*, 1997; Channon *et al.*, 2009; Walker *et al.*, 2010) but this is improved by aggregating scores on the 5-point scale to binary 'lame' or 'non-lame' classifications, as was done in this work. If other important variables for these models exist, they were unavailable for analysis. The variables genetic line, management system and culling reason were non-significant, yet given the small dataset, their effect could have gone undetected whilst still being important in the wider population.

2.7 Conclusion

This work describes and quantifies bone modelling on the distal phalanx of cull dairy cows and confirms it as heterotopic ossification, which can also be termed exostosis. Where locomotion data were available, bone modelling was greater in cows that had a history of lameness from lesions of claw horn disruption (as indicated by locomotion score and lesion occurrence) after accounting for the effect of age. A temporal component could not be depicted; it is not clear whether bone modelling occurred prior to or as a result of lesions and lameness, or whether bone modelling further predisposes lesion formation. However, heterotopic ossification is frequently observed in humans as a sequela to trauma and in this study, it could have occurred as a result of trauma to the bone, or as a result of inflammation in the surrounding soft tissues.

3 The appropriateness of 75 mm as a recommended Dorsal Wall Length for Foot Trimming

3.1 Introduction

Foot trimming is used both during treatment of claw horn lesions (Potterton *et al.*, 2012; Bell, 2015) and in a preventive manner to maintain claw shape, equal weight bearing through the claws and reduce risk of lameness (Toussaint-Raven, 1985; Shearer and van Amstel, 2001; Manske *et al.*, 2002a). Literature surrounding foot trimming has been reviewed in 1.5.1. In the UK, the method most widely taught foot trimming method, and that endorsed by the foot trimming profession (NACFT: National Association for Cattle Foot Trimmers) and industry bodies such as AHDB Dairy, is the Dutch Method. This method is based on the recommendations of Toussaint-Raven (1985). In brief, the three steps for preventive trimming are:

1. Cut the dorsal wall of the medial claw to 7.5 cm and make claw level, sparing horn at the heel.
    a. 7.5 cm was considered suitable for a 650 kg Friesian cow and ought to be varied dependent on the size of the cow.
    b. When levelling the claws, leave a 5 mm step at the toe.
2. Match the dorsal wall of lateral claw and make level.
3. 'Model'/ dish out the axial part of the sole, at the typical sole ulcer site (Rusterholz, 1920).

The remaining steps are curative. These involve removing horn from a claw displaying a lesion in order to transfer load onto the non-affected claw, and removing loose or under-run horn.

The original foot trimming method recommended that the dorsal wall should be trimmed to 75 mm, with a step left at the toe for an additional 5 mm of sole thickness. This was deemed suitable for a 650 kg Friesian cow, but ought to be varied with size of cow (Toussaint-Raven, 1985). Little peer-reviewed evidence has been published regarding the suitability of these widely adopted guidelines (Bell, 2015), and a review incorporating non-peer reviewed literature identified three areas of inconsistency: (1) the appropriate length to cut the dorsal wall, (2) the landmark of the proximal limit of the dorsal wall and (3) whether to trim the toe to a point or to leave a step; the second two points must be addressed before the first can be answered. Twelve sources were found relating to claw dimensions; of which, three were peer reviewed (Manske *et al.*, 2002a; Nuss and Paulus, 2006; Tsuka *et al.*, 2014).

Of the non-peer reviewed sources, the most commonly recommended dorsal wall length was 75 to 80 mm (e.g. Blowey, 2004), with a range of 60 (Greenough, 2007) to 85 mm (Blowey, 2008). In the peer-reviewed literature, reports also state that 75 mm is insufficient, finding the *mean* appropriate length to be 77 and 79 mm (Nuss and Paulus, 2006; Tsuka *et al.*, 2014). However, applying population means to recommended foot trimming dimensions could be flawed. Assuming claw length is distributed normally, the consequences of applying mean values to all cows would be to over-trim 50% of claws. The *range* of appropriate claw lengths that central tendencies summarize must be appreciated in order to avoid over-trimming whilst applying a population mean.

In both the peer and non-peer reviewed literature, landmarks for the proximal dorsal wall limit were either not defined (Toussaint-Raven, 1985) or defined inconsistently; examples included the 'proximal end of claw capsule' (Manske *et al.*, 2002a), the 'distal edge of the periople' (Tsuka *et al.*, 2014) or imprecise terms such as coronary band or coronet (e.g. Nuss and Paulus, 2006; Blowey, 2008). Recommended shape of the toe differed between sources (i.e. either trim the distal tip of the claw to a point, or leave a step), and different trimming techniques would require adaptation of dorsal wall length to achieve adequate sole thickness. Recommended toe angle varied from 45° to 55°.

Other methods of foot trimming to the Dutch Method have also been reported. For example, Manske *et al.* (2002a) reported a variation on the Dutch Method that did not involve using set lengths; they shaped the claw to angles, considering a claw trimmed when (1) the toe axis was straight, (2) the toe angle was 50-55 degrees (hind feet; 45-50 degrees in front feet), (3) weight bearing surfaces of the sole were level and (4) the axial part of the sole was dished out. Fjeldaas *et al.* (2006) described what they considered to be the Norwegian approach: "*the sole of the longest claw is trimmed first, and the major aim is to balance the heels of the two claws. Some trimmers dish out the horn of the axial sole*"; this description demonstrates that foot trimming operators may vary the technique, and it does not define stringent guidelines. Other methods include the Kansas modification to the Dutch Method (Ladd, 2005), the "White Line Method" described by Blowey (2008), which aims to prioritise weight bearing on the wall, and a modification that involves taking a greater model out of zone 4, which is becoming known as the "Dairyland" method (Burgi, 2014).

The precise lengths of the dorsal wall applied during the Dutch Method of foot trimming serve as proxies for the dimensions of the dermis within the hoof capsule, which ought to be avoided during foot trimming. Proximity to these soft tissues cannot be assessed in the live animal without imaging equipment, and landmarks that the dimensions are based on appear ambiguous, either because they are not precisely defined (e.g. Toussaint-Raven, 1985) or the literature is inconsistent. The dimensions of the distal phalanx itself can vary greatly. Blowey and Inman (2014) demonstrated this using slaughterhouse

specimens: dorsal wall length of the distal phalanx varied from 40 to 85 mm for 49 claws with lesions, and from 50 to 76 mm for 65 claws without lesions. If distal phalanx dorsal wall length was proportional to that of the wall horn, there would be a great range of appropriate foot trimming lengths: one size would not fit all cows. As with other studies using abattoir material (Nuss and Paulus, 2006; Tsuka *et al.*, 2014), farm and cow level data were unavailable, hindering the authors from associating variation in claw length with a measurable cow-side variable. However, all of these authors highlight that 75 mm may be too short to use as a standard measure during foot trimming.

Mulling and Budras (2003) reviewed the anatomy of the claw. The coronary dermis produces coronary epidermis, which, together with the parietal dermis which sits further distally and creates the parietal epidermis, makes up the majority of the wall of the hoof; together these two layers of epidermis will be termed the wall horn. Proximally, the wall horn is overlaid by the perioplic dermis, which borders the common integument and produces perioplic horn. The perioplic horn flakes off to leave the coronary epidermis as the most superficial layer of the hoof wall. The junction between the hoof horn and the common integument appears to be the most consistently definable anatomical structure on the external aspect of the hoof, and has been described previously as the proximal limit for measuring the dorsal wall during foot trimming (Manske *et al.*, 2002a).

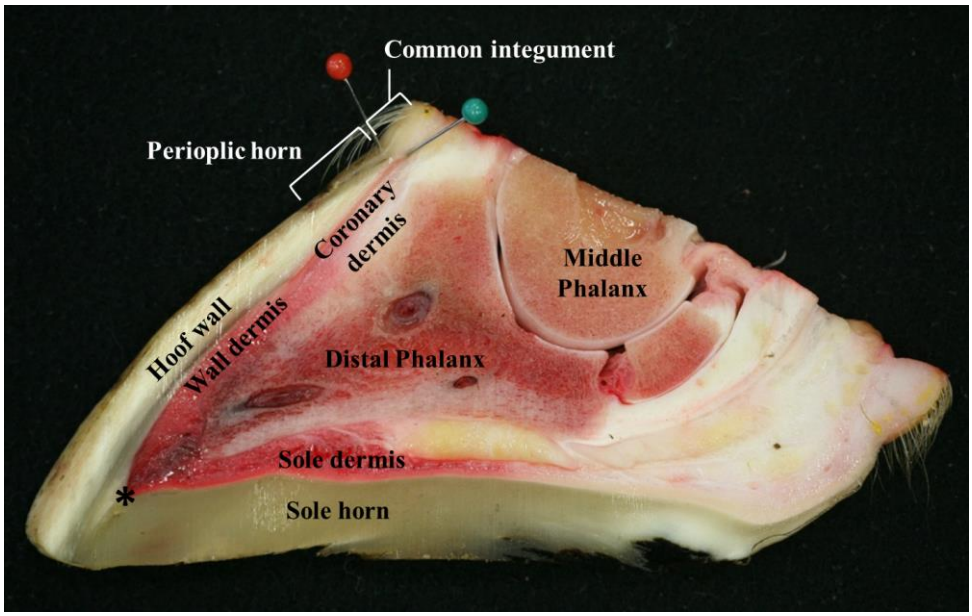

Figure 3.1: Sagittal prosection of a bovine claw demonstrating anatomy relevant to foot trimming claw length. The middle phalanx, distal sesamoid bone, distal phalanx, the surrounding soft tissue, horn of the sole, wall and periople and the common integument are visible. A green pin marks the proximal limit of the wall horn and an asterisk demonstrates the distal tip of the border between wall and dermis. A red pin identifies the proximal limit of the perioplic horn, identifiable from the external aspect of the hoof.

The first step of the Dutch Method sets the dimensions for the entire trim and could be a major control point for over-trimming. Recent literature has suggested that the widely used "75 mm" is not appropriate for the modern Holstein dairy cow, but recommendations for appropriate lengths have been based on population means. Applying a measure of claw length deemed to be appropriate for the average claw could result in over-trimming 50% of claws. Therefore, there is value in describing and understanding the *variation* in claw length. The aims of the current work were to (1) assess whether 75 mm is an appropriate length to be applied to all claws, (2) estimate the *minimum* length that the dorsal wall can be trimmed to and (3) assess how this might vary with "cow-side measures" such as cow age, claw (lateral or medial) and other data that might be available to a foot trimming operator when foot trimming a cow, using cattle from a known source.

3.2 Materials and Methods

3.2.1 Hypothesis

The null hypothesis stated that 75 mm is suitable as a trimming length for all claws.

3.2.2 Study samples

3.2.2.1 Herd of origin

The study sample consisted of the same feet used for the work presented in Chapter 2. This consisted of the hind feet of cows culled from the Crichton Royal Herd at the SRUC Dairy Research and Innovation Centre, Dumfries, UK, between November 2013 and August 2014. Farm information was given in 2.2.2 and the sample size was 142 hind feet from 72 cows (1 foot from each of 2 cows was irretrievable after slaughter; 2.4.1). Cow-level data available for all animals included age at slaughter, parity and carcass weight, genetic line and management system during life. Additionally, milk yield was available for first lactation for 60 cows.

3.2.2.2 Computed tomography imaging

The feet had been stored at -20 °C (2.2.3), then thawed prior to scanning using an industrial computed tomography scanner, to a resolution of 110 μm. Scanning protocol and data reconstruction were described in 2.2.4. As such, computed tomography volume files (.vgl) of each claw were available for the measurement of hoof dimensions.

3.2.3 Measuring internal dorsal wall length

Volume files of each claw were studied to measure the length of the dorsal wall that was in contact with the dermis, internal to the claw capsule. The claw was orientated in a

standard manner. Then, a cross-sectional image in a sagittal plane through the 3-D volume was identified (Figure 3.2a), which corresponded with the highest point of bone on the distal phalanx. This sagittal plane was approximately 10 mm abaxial to the axial aspect of the dorsal ridge of the wall horn, and was considered to correspond with the plane in which foot trimming operators reportedly measure dorsal wall length from. Anatomical landmarks described by foot trimmers were not identifiable on CT due to the radiographic homogeneity of tissues. However, the proximal tip of the wall horn sitting beneath the periople was visible on CT and corresponded well with the reported external landmark (validated in 3.2.4) therefore the proximal tip of the wall horn (Figure 3.2b) in this sagittal section was used as the proximal landmark. A linear measurement was taken from the proximal tip of the wall horn to the distal-most tip of the dermis at the toe, and the measurement was termed "internal dorsal wall length" (Figure 3.2).

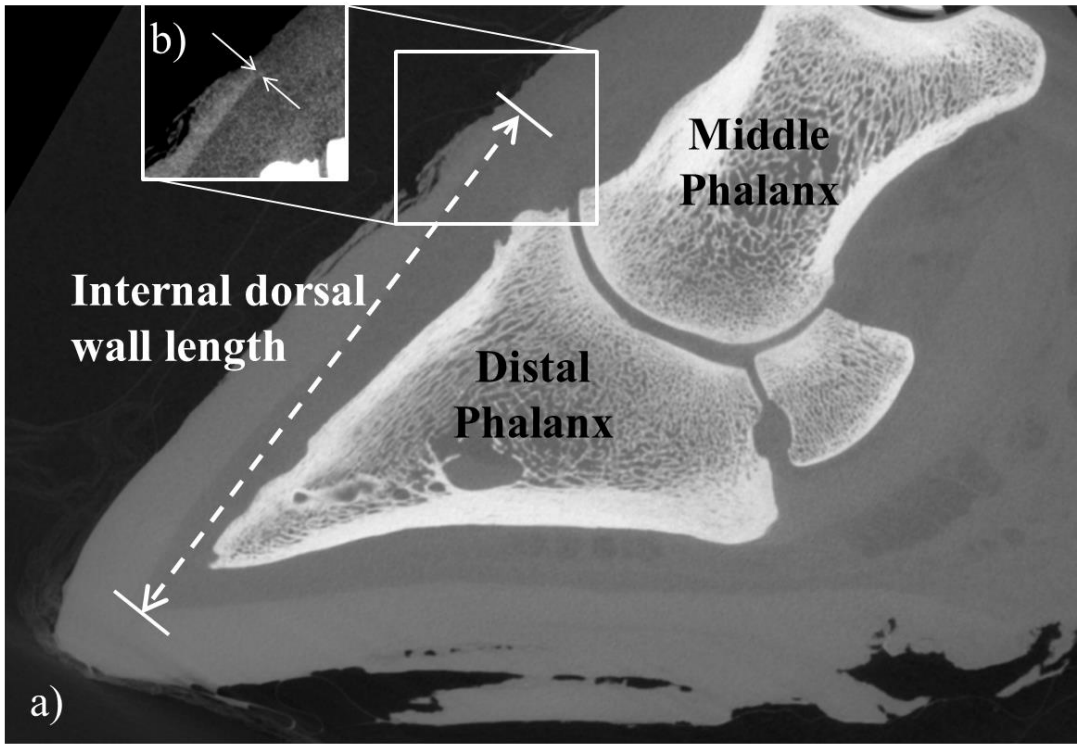

Figure 3.2: Two-dimensional mid-sagittal CT image of a bovine claw demonstrating the landmarks for measurement of internal dorsal wall length, in a study assessing the appropriateness of 75 mm as a set length to be applied during foot trimming. (a) middle phalanx, distal sesamoid bone, distal phalanx, surrounding soft tissue and the horn capsule. Internal dorsal wall length is marked, being the distance from the proximal limit of the wall horn to the tip of the dermis. (b) section of image demonstrating distal tip of the wall segment, being the proximal landmark of internal dorsal wall length. In (b), image contrast is increased to demonstrate differences in tissue layers.

3.2.4 Validation of proximal landmark of internal dorsal wall length

Validation work used *post mortem* specimens to assess the relationship between the proximal limit of the wall horn (i.e. the landmark used in this study, marked by a green pin in Figure 3.1 and arrows in Figure 3.2b) and the overlying proximal limit of the perioplic horn (which has been described the landmark used during foot trimming, marked by a red pin in Figure 3.1). Sagittal sections through the dorsal ridge of the distal phalanx of eight claws were prepared using a band saw, and distances between the proximal limit of both the wall and the overlying perioplic horn were measured. The sites varied by no more than ±2 mm within each claw, and the CT measurement of the limit of the wall horn was deemed to be a suitable indicator of the landmark reportedly used during foot trimming.

3.2.5 Adjustments to internal dorsal wall length

In order to deduce the minimum external dorsal wall length that should be applied to each claw, two adjustments were added to the internal dorsal wall length measured by CT as follows, and are detailed in Figure 3.3.

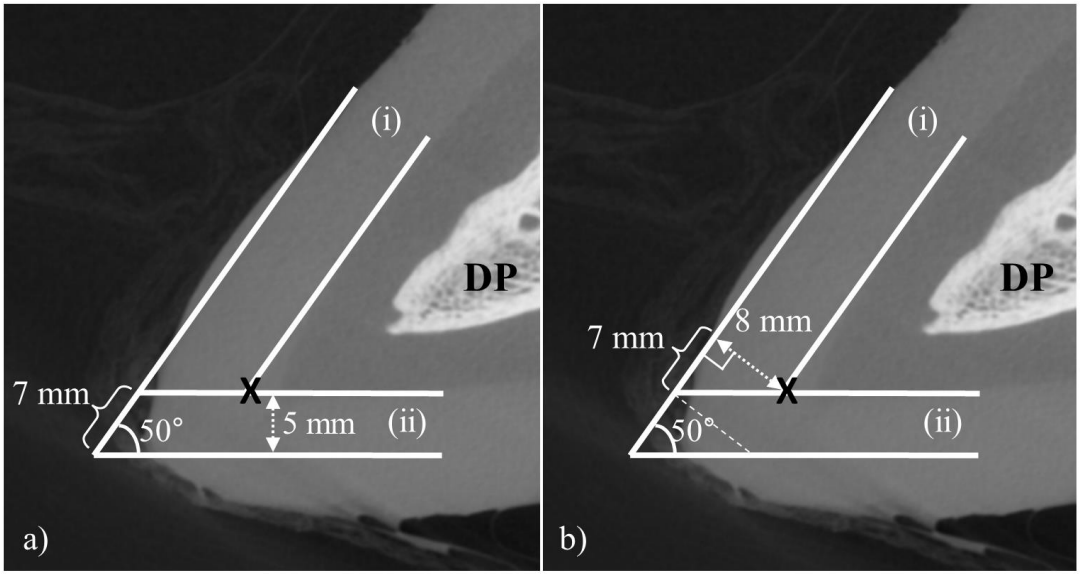

Figure 3.3: Magnifications of the distal tip of a claw (imaged in Figure 3.2), to demonstrate two adjustments made to internal dorsal wall length. a) demonstrates the adjustment made to allow for a minimum of 5 mm sole thickness. b) demonstrates the adjustment made to allow for a wall thickness of 8 mm. In both images, the distal phalanx (DP), soft tissue layers and both the wall and the sole horn of the claw capsule are visible. In both images, parallel lines extrapolate from (i) the inner margin of the sole horn at the toe and 5 mm below this margin, representing the minimum safe sole thickness and (ii) the inner and outer aspect of the wall horn, which is assumed to be 8 mm thick.

3.2.5.1 Adjustment 1: to allow for sole thickness

The first adjustment (Figure 3.3a) allowed for a *minimum* sole thickness and assumed that the toe would be trimmed to a point. Using the sine function of trigonometry, the adjustment for sole thickness was calculated:

$$\text{sine}(\theta) = \text{opposite} / \text{hypotenuse}$$

where θ was the toe angle, "opposite" was the minimum sole thickness and "hypotenuse" was the adjustment for sole thickness. The *minimum* sole thickness that should be left when trimming was taken to be 5 mm (Laven *et al.*, 2012) and the claw angle to be 50° (identified as the most frequently recommended claw angle for hind feet). Given these fixed measures, the adjustment to allow for a 5 mm sole thickness was an addition of 7 mm onto the "internal dorsal wall length". Varying the hoof angle within the recommended ranges of the non-peer reviewed literature (40-55°) changed the adjustment by no more than 1 mm each way.

3.2.5.2 Adjustment 2: to allow for wall thickness

The second adjustment allowed for the fact that the proximal limit of the dorsal wall length measurement was internal to the wall. A perpendicular line drawn to the outer aspect of the wall (Figure 3.3b) demonstrates that an adjustment must be made to account for wall thickness. Validation work assessed the thickness of the wall at the distal tip of the dermis in a subset of 80 hind claws from 20 study cows; mean wall thickness was 8 mm (SD: 0.9 mm). The tangent trigonometric function was applied to calculate the second adjustment, to allow for wall thickness adjustment:

$$\text{tangent}(\theta) = \text{opposite} / \text{adjacent}$$

where θ was the toe angle (50°), opposite was the thickness of the wall (which was fixed at 8 mm) and adjacent was the required adjustment for wall thickness.

This second adjustment was also 7 mm. Consequently, the *minimum* recommended dorsal wall length to be applied during foot trimming was derived by adding 14 mm (7 mm + 7 mm) to the internal dorsal wall length measurements for each claw, which is referred to as "minimum dorsal wall length".

3.2.6 Statistical analysis of minimum dorsal wall length

A mixed effects linear regression model was constructed to explore variation in minimum dorsal wall length. Models were developed in MLwiN 2.32 (Rasbash *et al.*,

2012) using iterative generalized least squares algorithms and a forward stepwise procedure. The model had two levels and took the format:

$$\text{minDWL}_{ij} = \alpha + \beta 1 X_j + \beta 2 X_{ij} + u_j + e_{ij}$$
$$u_{0j} \sim N(0, \sigma^2_u)$$
$$e_{0ij} \sim N(0, \sigma^2_e)$$

where the outcome, "minDWL", was the minimum dorsal wall length of the i^{th} claw of the j^{th} cow, α was the intercept, X_j and X_{ij} were fixed effects variables at the cow and claw levels respectively, $\beta 1$ and $\beta 2$ were vectors of coefficients, u_j and e_{ij} were random effects to account for residual variation between cows and between claws respectively (assumed to be normally distributed, with a mean of 0 and a variance of σ^2). Cow level explanatory variables tested were age at slaughter, lactation number at slaughter, genetic line, management system, cumulative lactation milk yield during parity 1 at 60, 100 and 160 days in milk (available for 60 cows) and carcass weight. The carcass was weighed whilst "hot" (before chilling) after exsanguination, removal of the head and distal limbs, skinning, evisceration and splitting of the carcass (the weight of both halves of the carcass weight were summed). The only claw-level variable tested was a dummy variable to denote "Lateral" or "Medial" claw.

The Wald Test was applied to determine whether a term remained in a model; i.e. a variable was significantly different to zero if it had a coefficient ≥ 1.96 times the standard error (Rasbash *et al.*, 2012). The likelihood ratio test was used to compare subsets of models and to assess whether the additional complexity of using higher model levels improved model fit (Dohoo *et al.*, 2009). Biologically plausible interactions were tested.

Assumptions of linear regression were checked by assessing the normality of residuals at each level and by checking for data points with high influence: by removing a data point and refitting the model, the degree to which a data point pulls the regression line can be calculated (Rasbash *et al.*, 2012). The difference between the fitted data point for the two models can be calculated and is therefore the true influence of a data point. This is visualized in MLwiN by plotting "deletion residuals" and "influence" charts. Models were considered safe if model parameters remained similar with the exclusion of data points with high influence. This can be performed at each level in a linear regression model. The model was also checked for outliers (which do not necessarily have high influence on the model).

3.3 Results

3.3.1 Description of dataset

In total, 224 minimum dorsal wall measurements from the hind feet of 69 cows were available for analysis (54 from the right lateral claw, 55 from the right medial, 52 from the left medial and 63 from the left lateral). Some claws had been excluded from the analysis due to errors when processing the samples: the top of the dorsal wall had been removed during sampling at the abattoir or during band saw use prior to packaging samples for computed tomography scanning.

3.3.2 Descriptive data

Of the 224 claws included in the final analysis, the median minimum dorsal wall length was 83 mm, and the range was from 66 to 93 (IQR: 80 to 85). Table 3.1 demonstrates the proportion of claws that would not be cut too short given a range of fixed trimming lengths, at 5 mm increments. The proportions for a set trimming length of 82 mm are also shown, which would be the length of the dorsal wall used if a set length of 75 mm was applied with a sole thickness of 5 mm left at the toe.

Table 3.1: Descriptive data for minimum dorsal hoof wall length (mm) based on 224 claws from the hind feet of 69 cows.

	Minimum Dorsal Wall Length[1]					
	≤ 75mm	≤ 80mm	≤ *82mm*[2]	≤ 85mm	≤ 90mm	≤ 95mm
All	0.04	0.25	*0.45*	0.73	0.96	1.00
< 4 years	0.04	0.52	*0.84*	0.98	1.00	1.00
≥ 4 years	0.03	0.17	*0.35*	0.66	0.95	1.00
Claw						
Left lateral	0.03	0.20	*0.41*	0.70	0.93	1.00
Left medial	0.04	0.27	*0.47*	0.75	0.96	1.00
Right lateral	0.07	0.22	*0.48*	0.69	0.96	1.00
Right medial	0.04	0.30	*0.43*	0.77	0.98	1.00

[1]Values in table show the proportion of claws that would *not* be cut too short if a particular dorsal wall length were applied and the claw trimmed to a point.
[2]The cut-off at 82 mm is included to demonstrate the proportions associated with trimming to 75 mm and leaving a step at the toe to leave 5 mm sole thickness.

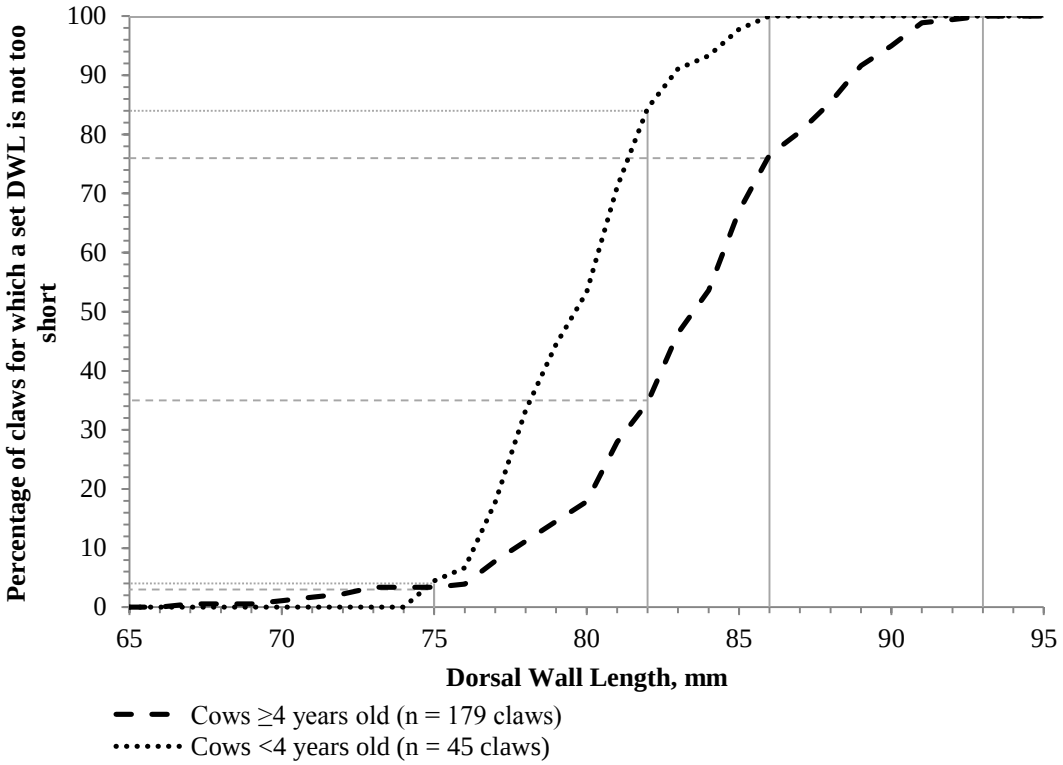

Figure 3.4: Cumulative frequency plot demonstrating the proportion of claws that could have safely been cut to a set dorsal wall length (**DWL**), whilst leaving a 5 mm sole thickness, based on an average wall thickness of 8 mm and when cutting the toe to a point. Two age groups are displayed: a dotted line shows cows <4 years old at slaughter (n = 45) and a dashed line represents cows ≥4 years old (n = 179). Four vertical lines are shown, at DWL lines of 75, 82, 86 and 93 mm, and horizontal lines extend from the intersection with each age-group line to the y axis; this demonstrates the proportion of claws from each age group that would be cut too short given each set trimming length.

Figure 3.4 demonstrates the proportion of claws that would have been cut too short at any set trimming length, for claws of cows <4 years and ≥4 years old. To highlight certain lengths, vertical lines are marked at four dorsal wall lengths, and horizontal lines assist reading the percentage of claws from each age group that would be cut too short if this length were applied during trimming. The line at 75 mm demonstrates that if trimming to this length and trimming the toe to a point, the vast majority of claws would be over-trimmed. If trimming to 82 mm, which would be equivalent to trimming to 75 mm and leaving a 5 mm step at the toe, this would only have been suitable for 35 % of claws of cows ≥4 years old and 84 % of claws of cows <4 years old. The vertical lines at 86 mm and 93 mm demonstrate the minimum lengths that would be suitable for all animals <4 years and ≥4 years old respectively when trimming the toe to a point; if leaving a 5 mm step at the toe, 7 mm can be taken from each of these measurements.

3.3.3 Model results

The final model is presented in Table 3.2. One cow (all four claws) and one additional claw from a separate cow were excluded from the final model due to their high influence at the second and bottom levels respectively. These data were outliers, as demonstrated in Figure 3.5. The single claw that was identified had toe necrosis (and was the only claw identified with toe necrosis). No reason was found for the poor fit of the one cow that was excluded. Model parameters were similar either with the inclusion of all 224 claws or with the exclusion of outliers, although model fit was better with the exclusion of outliers. No variables included in the final model became non-significant with the inclusion of all data; i.e. significance did not depend on the influence of a small number of data points.

Table 3.2: Final model minimum dorsal wall length that would be appropriate for claws during foot trimming. The model was based on 219 claws from 68 cows, after outliers were excluded[1].

Response:			Minimum Dorsal Wall Length (mm)	
	No. of units[2]	Mean (SD)[3]	Coefficient	SE
Fixed Part				
Intercept			73	2.6
Claw				
Medial	105		Reference	
Lateral	114		0.98	0.3
Age, y		5.5 (1.8)	0.92	0.2
Carcass weight, kg		296 (48)	0.016	0.0076
Random Part				
Level:		Variance explained	Remaining variance (SE)	
j: Cow	68	29%	7.4 (1.6)	
i: Claw	219	7%	4.8 (0.55)	
Total variance:		22%		

Variables are significant ($P \leq 0.05$) where the mean effect is ≥ 1.96 x SE (Wald test)
[1]One cow (all four feet) and one foot of a cow were excluded; explained in Figure 3.5.
[2]Number of units in each category, for categorical variables.
[3]Mean and standard deviation of continuous variables.

The final model was based on 219 claws from 68 cows. Age, claw (lateral vs medial) and carcass weight were significant in the final model. Minimum dorsal wall length was 1.0 mm greater (SE: 0.3) on the lateral claw and increased with age by 0.9 mm (SE: 0.2) per year. Minimum dorsal wall length increased with carcass weight by 0.016 mm per kg. Median carcass weight was 298 kg (range: 206 to 415; IQR: 261 to 331), and as estimated by the model, the difference in minimum dorsal wall length between the lower

and upper quartile of carcass weights was 1.1 mm (corresponding to a difference of 70 kg).

The final model explained 22 % of the null model variance; 29 % at the cow-level and 7 % at the claw-level. Of the unexplained variance, 61 % and 39 % remained at the cow and claw level respectively.

3.3.3.1 Inspecting model fit

Model fit was good (Figure 3.5b) and the two-level model structure fit the data better than when the cow-level random effect was excluded. When data from all 224 claws were included in the model, there was one particularly outlying data point at the bottom level and one outlier at the second level. Assessment of residual plots demonstrated that these data had high influence on the model and these data were not included in the final model. The legends for Figure 3.5 explain how the residuals were assessed, using the bottom level outlier as an example (Figure 3.5a) of why an outlying data point might be excluded. Distributions of residuals at each level were improved by excluding these outliers (Figure 3.5b).

Figure 3.5a

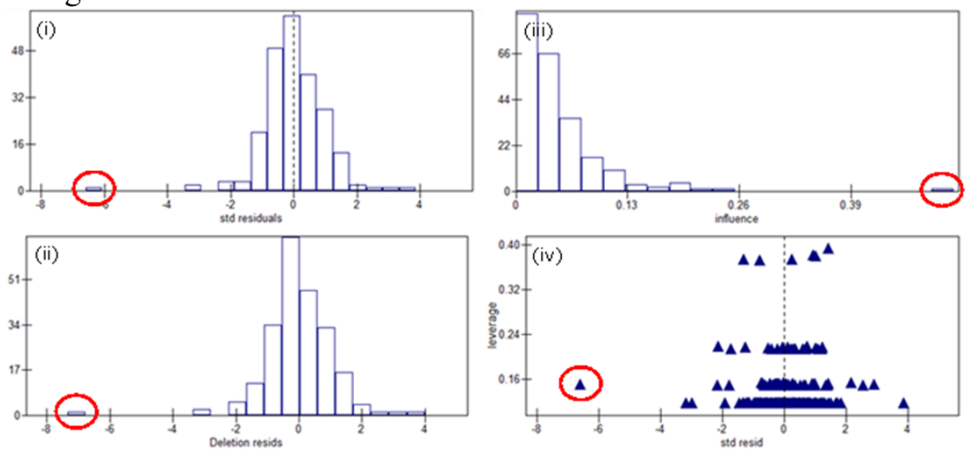

Figure 3.5b

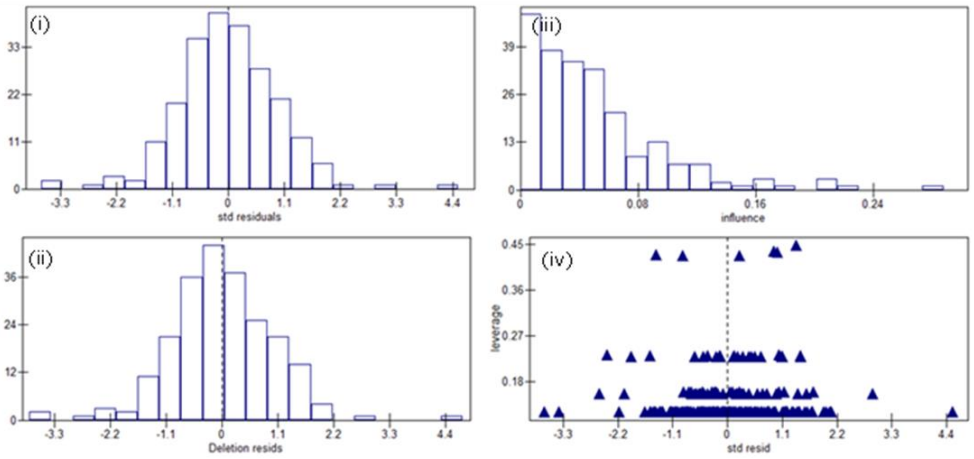

Figure 3.5: Plots of residuals at the bottom level of a 2-level linear regression model with the outcome minimum dorsal wall length (Table 3.2), both with (3.5a) and without (3.5b) outlying data points. Figures presented as an example of why outlying data points might have high influence on a model and be excluded. (i) Frequency plot of the standardized residuals. (ii) Frequency plot of the deletion residuals. (iii) Frequency plot of the influence of values, calculated as a function of the deletion residuals. (iv) Standardized residuals plotted against leverage.

Figure 3.5a: Residual plots of the model when data from all 224 cows were included (model not shown). One data point has a very high standardized residual (i, iv) and is circled in red. This data point has a deletion residual far from 0 (ii) and (iii) confirms that its influence is much greater than that of other data points; i.e. the difference between the predicted value of minDWL when the model was estimated with and without the datum was large. This could be considered an outlier and be excluded from the model, and model parameters should be re-assessed to check that one data point does not change the model parameters. In the model, the level 2 residuals contained a similar outlier from a different cow, and all four claws from that cow were removed from the model (plots not shown).

Figure 3.5b: Residual plots for the final model, where a claw and a cow with high influence had been excluded. Standardized residuals are approximately normal. Model parameters changed little with the exclusion of outlying data points and only the model with outlying data points excluded is reported (Table 3.2).

3.4 Discussion

3.4.1 Descriptive data

This study measured the length of the dorsal wall in contact with the dermis that must be avoided during foot trimming. The proximal landmark was the proximal limit of the wall horn, which was identifiable on computed tomography and was deep to the periople. The distal landmark was the distal-most tip of the dermis. Two constants were then added, (1) to allow a *minimum* sole thickness of 5 mm when trimming to a toe angle of 50 degrees, and (2) to allow for a wall thickness of 8 mm; these adjustments gave the "*minimum* dorsal wall thickness" that each claw should be trimmed to, when trimmed to a point. With this method, applying a dorsal wall length of 75 mm would have been too short for 96% of claws overall. The shortest that the claws of all animals aged <4 years (assumed to be in their first and second lactation) could have been trimmed to without any being too short was 86 mm, whilst this still would have been too short for 24 % of cows aged ≥4 years (Figure 3.4); for a set trimming length to be suitable for *all* claws, 93 mm would have had to have been used. This should not be surprising as median *minimum* recommended dorsal wall length of the 224 claws imaged was 83 mm.

The original Dutch Method of foot trimming stated that a step should be left at the toe to leave a 5 mm sole thickness (Toussaint-Raven, 1985). At a toe angle of 50°, leaving a step at the toe of 5 mm would create a difference between the dorsal wall length described by Toussaint-Raven (1985) and the *minimum* dorsal wall length deduced in this study of 7 mm, thus a trimming length of "75 mm with a 5 mm step at the toe" would equate to a *minimum* dorsal wall length of 82 mm. The proportion of claws for which a *minimum* dorsal wall length of 82 mm would be too short is displayed in both Table 3.1 and Figure 3.4; trimming to this length would have been too short for 55 % of claws overall, and 16 % or 65 % of claws from cows <4 or ≥4 years old, respectively. It appears that 75 mm is no longer suitable as a set length for foot trimming dairy cattle, and that due to the great amount of variation in claw lengths within a relatively small population, there may be no appropriate length to apply to all claws.

3.4.2 Statistical model

Cow age was significantly associated with dorsal wall length in this study, an observation made before for claws of German Simmental cows (Nuss and Paulus, 2006). The current study found a linear association with an effect size of 1 mm per year of age. Kehler and Sohrt (2000) also concluded that 5-8 mm should be added to the existing recommendation of 75 mm in older cattle to account for increasing size with age. The dorsal wall length of the lateral claw was 1 mm greater than the medial.

Carcass weight was the only variable available that might indicate cow size and it was significant in the model. It is not clear how carcass weight related to stature in these cows. The original text describing the Dutch Method recommends varying the trimming length by size of the cow (Toussaint-Raven, 1985) and it has been reported that experienced trimmers do this (Manske *et al.*, 2002a). An outcome of this work that would be useful for foot trimmers could have been to measure the size of cow and adjust trimming length accordingly, in a manner similar to adjusting dorsal wall length for age of cow (86 mm would be suitable for all cows < 4 years old). Pending further investigation, these results suggest that dorsal wall length varies with size of cow.

Where fixed dorsal wall lengths are to be applied at a population level, caution must be taken to minimise the proportion of claws for which that fixed length is too short. Where fixed lengths are used to simplify protocols and to facilitate the training of farm staff and other less experienced operators, these results suggest that the *minimum* recommended length for trimming the external dorsal wall should be at least 90 mm for Holstein cows, to reduce the risk of over-trimming. Applying measures of central tendency for recommendations does *not* allow for the variation in claw length demonstrated, and thresholds based on proportional measures are more appropriate.

In addition to the pain elicited from cutting through sensitive tissue, over-trimming of the toe (even without drawing blood) can lead to a thin sole and has been postulated to predispose to toe ulcers and toe necrosis (Kofler, 1999). Over-trimming can disrupt load bearing, compress the corium and predispose sole haemorrhage and other claw horn lesions (Sanders *et al.*, 2009; Tsuka *et al.*, 2014). Once lameness has occurred, recurrent cases are more likely, leading to severely compromised lifetime productivity (Green *et al.*, 2014).

It should be acknowledged that trimming all claws to 93 mm could leave some claws under-trimmed, which could have implications on toe angle and result in higher forces being transferred through the plantar-distal aspect of the hoof (i.e. the region of the flexor tuberosity). Perhaps a solution would be to trim to toe angle, as described by Manske *et al.* (2002a); these authors described a technique that did not use a set length for foot trimming. Further, Tsuka *et al.* (2014) pictorially demonstrated the consequences of leaving a claw that was too long: claw angle can decrease and there is potential for more weight to be transferred through the plantar-distal aspect of the distal phalanx and the region of the flexor tuberosity. The pathology surrounding the flexor tuberosity demonstrated throughout Chapter 2 suggests that there could be dangers to the anatomy associated with inappropriate force transfer around this region, and functional claw trimming appears to be a skilled task in order to best preserve the functional anatomy of the claw.

The guidelines of Toussaint-Raven (1985) state to cut the medial claw to 75 mm and to trim the lateral claw to it. The results presented in this study show only a 1 mm difference between lateral and medial claws and have assumed that the 75 mm guideline would be applied to both claws. This difference of 1 mm between claws is in line with the differences reported Tsuka *et al.* (2014), although Nuss and Paulus (2006) found no discernible difference between lateral and medial claws. A difference in 1 mm is small and probably within operator error, and it does not seem practicable to suggest that the medial and lateral claw should be trimmed to a different length if set lengths were to be applied.

3.4.3 Landmarks for the proximal limit of dorsal wall length

Definition of the proximal dorsal wall limit has been inconsistent between previous studies and clarification was necessary for this work. The junction between perioplic horn and common integument perhaps seems to be a sensible landmark for future studies (Manske *et al.*, 2002a), as it appears to be the most consistently definable external anatomical structure. In the current study, the proximal limit of wall horn was identified on computed tomography images. Use of this landmark increased measurement repeatability and corresponded well with the proximal limit of perioplic horn (Figure 3.1), varying by up to ±2 mm.

In agreement with increasing recommended claw length, Tsuka *et al.* (2014) suggested 79 mm was more appropriate than 75 mm, and Nuss and Paulus (2006) reported a cut length of 80 mm left 29% of claws with inadequate sole depth. The current study reports longer lengths may be required, but assumed the toe would be trimmed to a point. It is unknown whether leaving a step or a point at the toe has a discernable effect on the functional capacity of the hoof capsule, indicating an area where further research is needed.

3.4.4 Study limitations

A limitation with regard to the management and production history of cows in the current study was time that cows spent on a second unit (Acrehead) prior to culling. This may have contributed to the failure to identify further associations, and as a result 71 % of the observed variation in external dorsal hoof wall length remained unexplained. Alternatively, the unexplained variation could be attributable to unrecorded variables, such as measures of cow stature; carcass weight might have been a poor proxy for this. At present any variation in claw length applied by foot trimming operators must be based solely on their experience and judgement. For practical use, further studies should consider associations with claw length and cow-side measures of stature and conformation that could be assessed by the operator immediately prior to trimming the hoof dorsal wall. The thickness of the sole can be visualized using ultrasonography and

adjustments in claw length could be based on this, and perhaps a hand-held ultrasonographic device could be developed for foot trimming operators to gauge sole thickness. Predictors of dorsal wall length would be of more use at the cow level than at the claw level, as this is where most variation resided (Table 3.2). The difference of 1 mm between lateral and medial claw was likely well within the operating margin for error used by operators.

Data on claw lesions at slaughter were not collected in this study. As lameness occurs most frequently in older, higher yielding cows (Green *et al.*, 2002; Archer *et al.*, 2010b), age would likely be correlated with the presence of claw horn lesions and dorsal wall length. Any curvature of the external hoof wall was not accounted for, and external dorsal wall length was assumed to be a straight line (Figure 3.2). This was considered to be an appropriate simplification for practical application. Further, to simplify the second adjustment, the internal and external aspects of the dorsal wall were considered to be parallel, whilst in fact they diverged from the proximal landmark. Once more, this was considered to be an appropriate simplification.

Computed tomography scanning was a useful imaging modality in this study, enabling non-destructive high resolution assessment of hoof anatomy. However, a limitation was the difficulty in defining the proximal limit of the dorsal wall due to the homogenous radiopacity of the surrounding soft tissues. This was addressed by comparison to dissected claws, but in hindsight the results could have been made easier to interpret had a radiopaque marker been applied to the outside of the claw at the proximal external limit of the perioplic horn, prior to CT scanning.

Further, *minimum* sole thickness of 5 mm was taken from previous recommendations (Laven *et al.*, 2012). Neither the suitability of this measurement nor the *minimum safe* measurement has been tested; operators wishing to leave a greater sole thickness will can increase these recommendations to allow for an increase in sole depth following trimming. Similar adjustments can be made if a different toe angle is desired, although the difference with reported toe angles would be small: the adjustment would need to be 1 mm greater or 1 mm smaller for toe angles of 40 and 55 degrees respectively (which were the limits of toe angles recommended in the literature).

3.5 Conclusions

Based on the results of this study, the null hypothesis can be rejected. The data are strongly in favour of the alternate hypothesis, that 75 mm is an inadequate dorsal wall length to be applied to all cows when foot trimming. An important finding of this work was the large unexplained variation in dorsal wall length, even in this small sample size

from a single farm. There is still little evidence demonstrating that cow-side measures can be used to adjust dorsal wall length when trimming.

Iatrogenic lameness caused by over-trimming could be an important cause of lameness, yet the aim of foot trimming is to maintain appropriate claw structure and function. From the data presented in this work, it appears that the *minimum* recommended claw length to be applied to any Holstein adult dairy cow should be increased to 93 mm, measured from the proximal limit of wall horn, when trimming the toe to a point. Small reductions can be made for younger animals, for example 86 mm would have been appropriate for all first and second lactation cows. If a step is left at the toe leaving a 5 mm sole thickness, 7 mm can be taken from these measurements.

Beyond the effect of age, it is likely that a smaller dorsal wall length is safe for smaller cows, although this study was unable to assess this in a manner that hoof trimming operators could apply when cow-side (carcass weight was the only available measure of cow size). Experienced operators report varying dorsal wall length by size of cow. However, the guidelines are the same for experienced and new operators being trained in foot trimming. It therefore seems imperative that "standard" measures of dorsal wall length used by less experienced operators be increased in future texts and training material, because trimming each claw to 75 mm will over-trim many claws. Finally, future authors should be clear about where the dorsal wall is measured from and whether they advocate the toe being trimmed to a point or left with a step, detailing the size of that step. These measures will reduce the risk of over trimming and allow better comparisons between recommendations from different sources in the future.

4 A Prospective Cohort Study of the Digital Cushion; General Methods and Validation

4.1 Introduction

A long-standing discussion has surrounded whether lame cows go thin or thin cows go lame. Lame cows are likely to be pushed down the social hierarchy (Hoffman *et al.*, 2013), making them less able to compete for feed and subsequently lose body condition (Wells *et al.*, 1993; Espejo *et al.*, 2006). Additionally, several recent studies have identified that low body condition and loss of body condition precede lameness and claw horn lesions, as reviewed in 1.4 (Hoedemaker *et al.*, 2009; Machado *et al.*, 2011; Green *et al.*, 2014; Lim *et al.*, 2015; Randall *et al.*, 2015). It appears that low body condition is a risk factor for lameness, and once lame, cows are at a greater risk of losing further weight. However, why low body condition predisposes cows to lameness is unclear.

It has been suggested that fat is mobilized from the digital cushion during negative energy balance, decreasing its capacity to dissipate shock and predisposing the germinal epithelium of the sole to contusions (Räber *et al.*, 2004). Bicalho *et al.* (2009) reported that the digital cushion was thinner in thin cows. This work was a cross-sectional study and measured "digital cushion thickness" using ultrasonography (their measure included digital cushion and corium) at "Zone 4" of the foot map, on all claws of 501 cows at any stage of lactation, up to and including the 10^{th} month after calving). They compared digital cushion thickness with body condition score (BCS) and reported that the digital cushion was thinner in thin cows, in parity 1 cows and if a lesion (sole ulcer or white line disease) were present. The nadir of BCS and digital cushion thickness were both at around 120 DIM. Cows with a thin digital cushion were more likely to become lame during the lactation, but BCS did not influence lameness occurrence in this study. An important limitation of the study was that a temporal association between BCS, digital cushion thickness and lameness could not be analysed, due to the cross-sectional study design.

In a second study, the same research group used digital cushion thickness, BCS and claw horn disruption lesion presence, all collected at drying off, to predict lameness during the subsequent lactation (Machado *et al.*, 2011); low BCS, thin digital cushion and presence of a claw horn disruption lesion all increased the likelihood of developing a lesion. Digital cushion thickness reportedly correlated with BCS and digital cushion thickness was thinner in cows with claw horn disruption lesions, but a limitation of this study was that cows could have had low BCS or a thin digital cushion thickness as a result of prior lameness. The digital cushion appears diminished in cows that had claw

horn disruption lesions (Lischer *et al.*, 2002; Munk and Capion, 2013), therefore the work of Machado *et al.* (2011) cannot decipher which variable caused the subsequent lameness. In a different *in vivo* study, Toholj *et al.* (2013) measured digital cushion thickness at 30 DIM and used this data to predict lameness at 70 and 180 DIM, and lesions at 180 DIM. Cows were more likely to go lame and develop a sole ulcer if they had a thin digital cushion recorded at 30 DIM. No measures of body fat were included in the study.

From *in vivo* studies of digital cushion thickness, it appears that having a thin digital cushion increases the risk of subsequent claw horn disruption lesions and lameness. A thin digital cushion may also occur after a claw horn disruption lesion (see 1.4.1.1), and Räber *et al.* (2006) suggested that this could be because fatty acids in the digital cushion are used during the inflammatory process, and the cushion becomes depleted and is replaced with scar tissue. It appears that body condition score and digital cushion thickness are correlated, although a previous lameness event could result in both a low body condition and a thin digital cushion.

No work has demonstrated how the digital cushion changes throughout lactation, no work has demonstrated whether the digital cushion changes as body fat changes and no work has demonstrated whether digital cushion *change* leads to lameness; these are all key to understanding whether depletion of the digital cushion during negative energy balance is the mechanism by which being thin or losing body condition predisposes lameness.

4.2 Methods

4.2.1 Aims and objectives

The first aim of the study was to determine how the digital cushion changes throughout lactation and with changes in measures of body fat. The second aim was to discern whether absolute or changes in digital cushion thickness influence future lameness and lesions. Objectives of the study were to (i) enroll a cohort of cows that could be studied throughout a lactation from before calving, (ii) measure the digital cushion thickness at several time points, (iii) assess measures of body fat throughout the study, (iv) identify when cows go lame and (v) assess lesion incidence.

4.2.2 Null Hypotheses

1. Digital cushion thickness does not alter with measures of body fat.

2. Change in digital cushion thickness does not increase the likelihood of impaired mobility or the development of claw horn disruption lesions.

4.2.3 Study design

A prospective cohort study was designed to assess change in the digital cushion throughout lactation, from before calving. Cows were studied from approximately 8 weeks pre-calving to 35 weeks post-calving, during which the hind claws were evaluated at 5 assessment points. Animals were enrolled at the first assessment point, which was at approximately 8 weeks prior to their predicted calving date, and termed "AP-8". The second assessment point occurred between 4 and 10 days post-calving and was termed AP+1 (approximately 1 week post calving). The third assessment point was either 6, 8 or 10 weeks after AP+1 (the period was assigned sequentially within each lactation group), in order to correspond approximately with peak milk production, based on production records on both farms; this assessment point was on average 9 weeks post-calving and was termed AP+9. Assessment Points 4 and 5 were 8 and 20 weeks after AP+9, and were termed AP+17 and AP+29 respectively. Assessment points were automatically prompted by a calendar created in Microsoft Excel.

4.2.4 Study farms

Two high producing herds were selected and were visited weekly from 13th November 2013 until 19th May 2014. The farms were selected for convenience, to ensure ease of access to cows, good handling facilities and willingness of the farm manager to accommodate the study. High producing herds were selected since cows on these units were more likely to undergo body condition loss. Cow data and factors of the management systems that might influence lameness are outlined in Table 4.1. All animals on both farms were trimmed by a professional foot trimmer every 4-6 months; the claws on all feet were trimmed if considered to be over-grown. Additionally, lame cows were treated when identified as lame by the stockperson; this method of lameness management continued as normal throughout the study period.

Both farms fed partial mixed ration; mixed ration was provided *ad lib* at the feed face and concentrates were offered to production in the milking robot for parity >1 and parity 1 animals producing >26 and >22 Litres/day, respectively. Mixed ration constituents and composition are shown in Table 4.2 for each farm, analysed by Biotal Forage Analysis (Worcester, UK). The concentrates provided consisted of sugar beet, wheat, soya, maize, barley, distillers grains, aminomax (amino acid formulation), molasses and oils (megalac); 17.5% crude protein, 9.9% crude fibre, 5.0 % crude oil and fat, 5.6% ash.

Table 4.1: Farm systems and animal data for two study farms used in a prospective cohort study of the digital cushion, hoof lesions and lameness.

Variable	Farm 1	Farm 2
Housing		
Milking system	4 × Lely A3 automatic milking systems	4 × Lely A4 automatic milking systems
Management groups (randomly assigned)	4 groups, 1 robot per group	2 groups, 2 robots per group
Number of cubicles	241	240
Total floor area, excluding cubicles	1,196 m^2	1,016 m^2
Floor type	Rubber matting	Concrete slats
Shed roof type	Pitched, open ridge	Pitched, open ridge
Shed ventilation	Mechanical, via end-walls	Natural, via side-walls
Heifer housing	Cubicle sheds from 6 months old, with rubber mats in cubicles and concrete passageways	At pasture during spring, summer and autumn months from 6 months old, or indoors on deep straw bedding
Cubicle dimensions		
Width	1.1 m	1.06 m
Neck rail height	1.2 m	1.3 m
Depth to brisket board	1.75 m	1.85 m
Kerb height (from passageway)	0.2 m	0.16 m
Feeding		
Water points	2m × 0.6m water troughs (n = 18)	2m × 0.6m water troughs (n = 16)
Feed space length	145 m	126 m
Feed space partitioning	107 m with 154 locking yolks and 38 m with 30 single-animal feed bins (RIC feeders, Hokofarm, NL)	192 locking head yolks
Feed passageway width	4.25m	3.8m
Management		
Feed frequency	1 per day	1 per day
Feed push-up frequency	6 per day (every 2 hours from 8am)	11 per day (hourly 7am to 4pm, once at 9pm)
Foot bathing protocol	3 times per week: 2 × 4% formalin solution, 1 × 5% $CuSO_4$ solution	Fortnightly, alternating between 4% formalin solution and 5% $CuSO_4$
Scraper frequency	Every hour	Every hour
Animal data		
No. of cows milking	Average: 175 (Max: 190)	Average: 201 (Max: 210)
Breed	100% Holstein	All study cows were ≥ 75% Holstein genetics. Historically, Brown Swiss and Ayreshire breeds had been crossed into the herd.
Age at 1st calving, median (mean)	25.6 months (25.8)	26.7 months (26.8)
Milk frequency, per day	2.9	3.5
Mean farm 305 day milk yield	11,380 kg	12,350 kg
Calving interval, median (mean)	366 days (394)	401 days (411)
Lactation length, median (mean)	305 days (310)	311 days (308)

Table 4.2: Average diet composition from each farm throughout the study period, offered as part of partial mixed ration. Up to 26 litres/day of milk production (or 22 litres/day for heifers), this was the only feed available. Above these, concentrates were offered to production in parlour.

Variable	Farm 1	Farm 2
Dry matter (%)	38	41
ME (MJ/kg of DM)	12.1	12.4
CP (g/kg of DM)	160	181
Sugar (g/kg of DM)	32	15
Starch (g/kg of DM)	270	205
NDF (g/kg of DM)	415	480
Oil (g/kg of DM)	55	60

4.2.5 Cow inclusion and exclusion criteria

Cows were studied throughout their 1st, 2nd, 3rd or 4th lactation. All eligible animals approaching 8 weeks prior to calving were enrolled until the necessary sample size was reached in July 2014, which was estimated prior to the study using a sample size calculation (4.2.7).

Cows were ineligible for study if they were entering their 5th lactation or greater or were due to be culled before the end of the subsequent lactation. An assessment point was missed if a cow became unduly stressed by the procedure, if her temperament posed a risk to handlers or herself or for health reasons such as mastitis; data on these cows collected from other assessment points remained in the analysis. If a cow missed ≥3 consecutive assessment points, the cow was excluded from the study. When cows were excluded from the study, reason for exclusion was recorded and data collected from previous assessment points were included in the analysis up until the point of exclusion.

4.2.6 Brief description of data collection

At each assessment point throughout the study, cows were individually loaded into the examination crush and the assessment was performed. Provided here is a brief description of the techniques used during the assessments. The next section describes the techniques in full and explores validation of these techniques.

The routine at each of the 5 assessment points was as follows:

1. Body condition score was recorded on a 1-5 scale with quarter point intervals (Wildman *et al.*, 1982; Edmonson *et al.*, 1989), summarized by AHDB (2014).
2. Withers height was recorded (at AP-8 only) whilst the cow was standing square, facing forward with head level, using a spirit level that measured from the point of Withers to a ruler fixed to an upright of the crush.
3. An ultrasonographic measurement of back fat thickness was taken (4.3.1.2).

4. Both hind feet were lifted in turn and the surface of the horn was prepared to visualize lesions (4.3.1.3).
5. A digital photograph of the base of the foot was taken and stored for lesion analysis (4.3.1.3).
6. Ultrasonographic measurement of sole soft tissue thickness was taken at three sites beneath the distal phalanx (4.3.1.4).

A single observer (R.F.N.) performed all measurements.

Additionally, data were collected continuously throughout the study period by farm management software (e.g. milk yield and body weight), which is described further in 4.3.3, and mobility scoring of all study cows was performed fortnightly throughout the study period (4.3.4).

4.2.7 Sample size calculation

A power calculation was performed to estimate the number of cows required to detect a change in thickness of the digital cushion of 1 mm, which is the difference in digital cushion and corium thickness reported by Bicalho *et al.* (2009) between cows with BCS 2 versus BCS 3. The calculation was based on a 2-sample t-test with alpha = 0.05 and beta = 0.8. The standard deviation in digital cushion and corium thickness on hind claws reported by Bicalho *et al.* (2009) was 2.6 mm. The result of the power calculation was that 108 cows were required in each of two groups to detect a difference in digital cushion and corium thickness associated with a 1-unit difference in BCS. However, the current study was longitudinal in design, with each cow being assessed at 5 assessment points. Cows on both study farms underwent substantial changes in body condition (as demonstrated by farm records) and repeated measures typically increase power (Marshall *et al.*, 1998). Consequently, the power calculation was likely a conservative estimate of the likely power when analysed in a multilevel framework; the sample size target was to have at least 150 cows completing all 5 assessment points

4.2.8 Ethical approval

Local ethical approval was granted by the University of Nottingham School of Veterinary Medicine and Science Ethical Review Committee on 11th September 2013.

4.3 Development and validation of data collection techniques

4.3.1 Ultrasonographic techniques

4.3.1.1 Ultrasound scanner selection

Thickness of back fat and of the soft tissues between the sole horn and the distal phalanx (the digital cushion and corium, to be termed collectively herein as "sole soft tissues") were measured using B-mode ultrasonography. To select the machine, clarity of image of 5 portable ultrasound scanners owned by the School of Veterinary Medicine and Science were compared, when scanning the sole soft tissues through the sole horn of *post mortem* specimens. A variety of linear and curvilinear probes were compared and ultrasound frequencies were varied from 5 to 12 megahertz to gain the clearest image. A MyLab30 scanner (Esaote Europe B V, Cambridge, UK) was selected with a 5 cm linear transducer set at 7.5 megahertz, with a resolution of 0.1 mm. Coupling gel was used at all scanning interfaces.

4.3.1.2 Back fat thickness measurement

To measure back fat thickness, hair over the rump was parted and the transducer was placed 5 to 10 cm cranial to the tuber ischium, perpendicular to the skin on a line between the tuber ischium and the tuber coxae, in order to visualize the fascia profunda (Schroder and Staufenbiel, 2006), as demonstrated in Figure 4.1. Each image was labelled with cow, claw and assessment point, and saved, and the process was repeated to obtain two images on each side of the cow.

Ultrasonograms were exported as JPEG (.jpg) files and after data collection had been completed, were randomized and presented to a blind observer (R.F.N.). The distance from the external border of the skin surface to the fascia profunda was measured manually using electronic calipers in the image analysis software Fiji (Schindelin *et al.*, 2012). The back fat thickness measurement included the following anatomical structures: skin, subcutaneous fat, superficial fascia and interfascial fat (Figure 4.1). Skin thickness in this region is approximately 5 mm and varies little (Schroder and Staufenbiel, 2006), therefore back fat thickness measurements of this magnitude demonstrate little or no subcutaneous fat. Data entry was facilitated by use of a script in Microsoft Excel Visual Basic for Applications (VBA), which converted pixel measurements into linear measurements, identified measurements by cow, claw, assessment point and location, and stored them in a database.

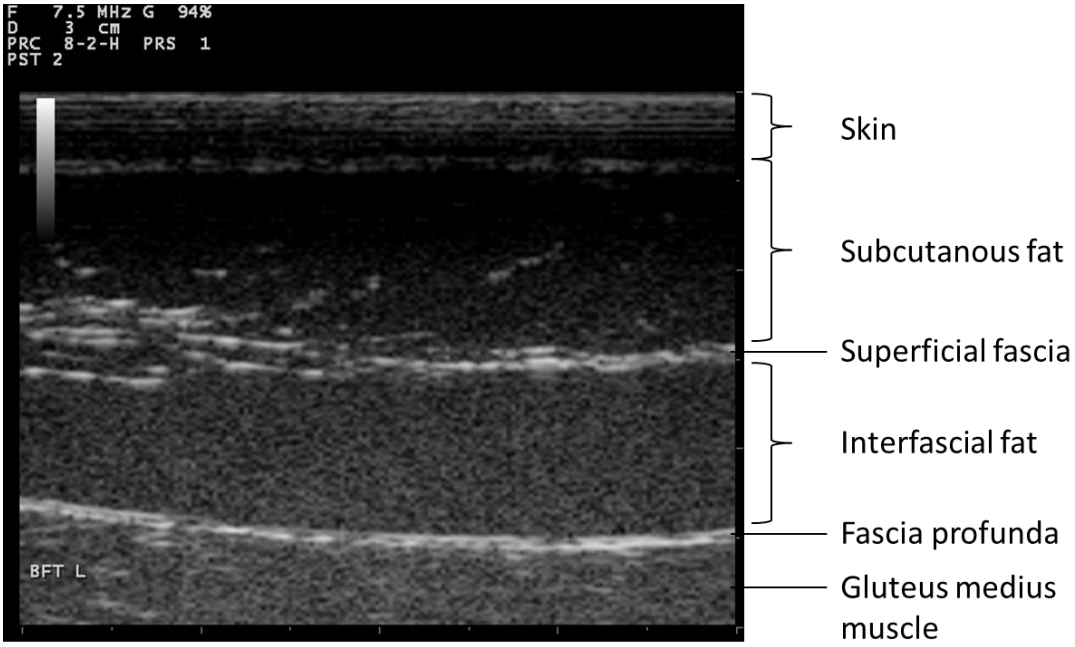

Figure 4.1: Ultrasonogram of back fat thickness. The probe was placed 5 to 10 cm cranial to the tuber ischium, perpendicular to the skin and on a line between the tuber ischium and the tuber coxae, in order to visualize the fascia profunda. Back fat thickness was measured from the external surface of the skin to the fascia profunda, in the middle of the image, as described by Schroder and Staufenbiel (2006).

4.3.1.3 Preparation of hoof for lesion assessment and ultrasonography

During each assessment, each foot was raised in turn and inspected for overgrowth. If the claw was deemed to be over-grown, a functional foot trim was performed according to a modification of the Dutch Method (Toussaint-Raven, 1985); a set claw length was not used, but emphasis was placed on maintaining claw angles (Manske *et al.*, 2002a). When a claw was in shape, a very thin (<1 mm) slice was removed from the surface of the whole sole in order to obtain a smooth surface for ultrasonography and clear visualization of sole and lesions (Leach *et al.*, 1998). A 10 mm circular scale marker was placed on the sole surface at the toe and the heel of each claw. A photograph was taken of the sole using a 12 megapixel digital camera (Cyber-shot DCS-W510, Sony Europe Ltd, Surrey, UK) held square to the claw, 30 cm away. Photographs were stored for lesion analysis after the on-farm data collection was complete.

4.3.1.4 Sole soft tissue measurement

Ultrasonography of the foot was performed using the same scanner and probe as for back fat thickness; ultrasonography sites and an example of an ultrasonogram are provided in Figure 4.2. The probe was placed in a stand-off and on the sole surface in the midline of the claw, perpendicular to the horn to visualize a sagittal plane through the base of the foot, using coupling gel at all interfaces. Images displaying the distal

border of the distal phalanx (identifiable as a thick hyperechoic line) and the inner surface of the sole horn (a thin hyperechoic line), and therefore the thickness of soft tissue between them, were obtained at three sites, as described by Kofler *et al.* (1999): (1) the toe, (2) the highest point of the arch of the distal phalanx and (3) beneath the flexor tuberosity. Repeat images were taken and stored (2 images in total), and randomized for analysis at the end of the study as for back fat thickness.

The ultrasonographic measures beneath the distal phalanx were termed "sole soft tissue thickness" and at each of the three sites, the following soft tissue layers were measured:

- Site 1: Corium thickness beneath the apex of the distal phalanx (the digital cushion does not extend to the toe).
- Site 2: Digital cushion and corium thickness beneath the highest point of the arch of the distal phalanx.
- Site 3: Digital cushion and corium thickness beneath the flexor tuberosity of the distal phalanx.

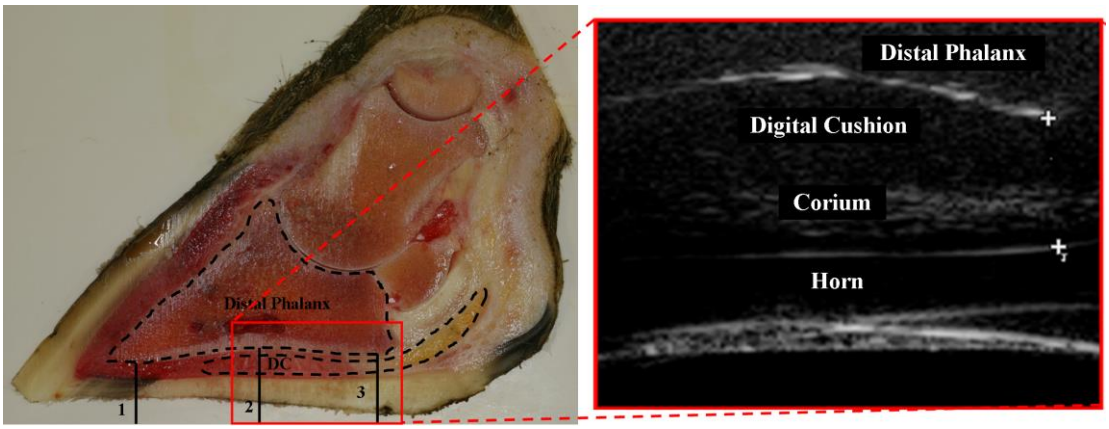

Figure 4.2: (left) Prosection of a bovine digit showing the ultrasound scanning sites for sole soft tissues; (right) Ultrasonogram of the sole soft tissues. On the prosection, the distal phalanx and the digital cushion (**DC**) outlined, and vertical black lines indicate the three measurement sites of sole soft tissue. A red square marks the region in which the sole soft tissues were imaged using ultrasonography. The ultrasonogram demonstrates the tissue layers visible on ultrasonography. The digital cushion and corium could not consistently be distinguished between, but other tissue layers could.

4.3.1.5 Checking data entry

A total of 23,598 ultrasonographic measurements were taken from 827 cow assessment points throughout the study period. Data were plotted against DIM and were inspected visually for outliers. Outliers were re-measured from their ultrasonograms and the vast majority had been accurate measurements. Next, twenty cow assessment points were randomly selected and measurements were taken again from the ultrasonograms, giving 552 duplicate measurements where 'original' and 'checked' values could be compared

against each other. The R-squared value between checked and original was 0.992 and error was normally distributed; agreement was very good.

"Within-assessment point" intra-observer repeatability was assessed by comparing the duplicate measurements taken with assessment points throughout the entire study; at every assessment two measurements of each variable had been taken. A total of 11,338 repeated measurements were recorded and when compared with each other, $R^2 = 0.988$ (Figure 4.3) and residuals were normally distributed. This is expected to be lower than the value for checking data entry, as it includes error in ultrasonographic examination technique as well as error in measurement using Fiji. Still, agreement was very good and variation within assessment point was low (also demonstrated in 5.4, where repeated measure within-assessment point became the bottom level of a mixed effects linear regression model).

In conclusion, intra-observer repeatability of ultrasonographic measurement was very good.

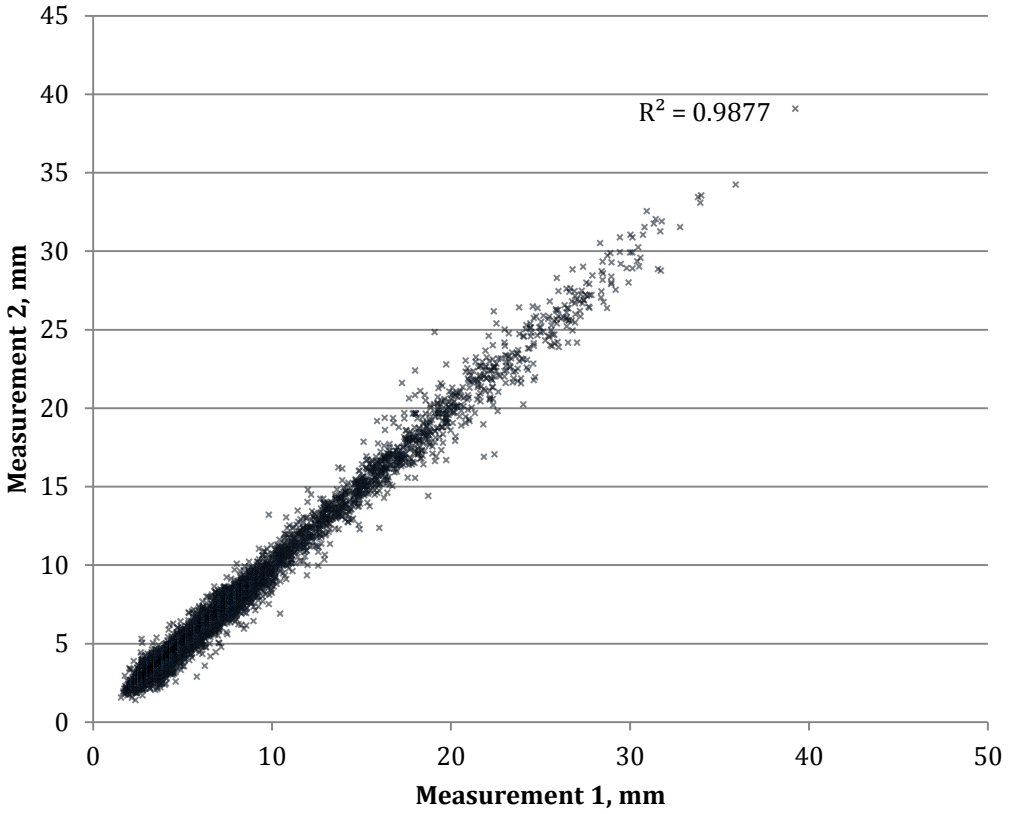

Figure 4.3: Scatter plot of 11,338 repeated within-assessment point ultrasonographic measures of sole soft tissue and back fat thickness.

4.3.2 Lesion scoring

4.3.2.1 Introduction

The literature was reviewed prior to designing the lesion scoring system. Published methodologies vary greatly and the level of detail that they include often depends upon the size and requirements of a study. Many methods for scoring lesions and severity have been reported and there is not yet a globally accepted method. However, some methods have been used frequently and developed and some aspects of scoring systems that other authors have found valuable are presented here. Greenough and Vermunt (1991) scored sole and white line lesions, each being on a 0-5 scale of severity. Leach *et al.* (1997) developed this method and additionally identified deep haemorrhage, yellowing and under-running of the sole. In a subsequent publication, Leach *et al.* (1998) introduced a quadratic weighting to lesions, with greater weightings for higher scoring lesions in order to recognise their increasing clinical severity. This method has been adopted by other authors who value the transformation of the arithmetic scores (Chaplin *et al.*, 2000; Webster, 2001; Webster, 2002; Flower *et al.*, 2005; O'Driscoll *et al.*, 2009). Le Fevre *et al.* (2001) found a very poor correlation between lesion severity and area affected, suggesting that a measure of severity was needed in lesion assessment.

Studies with large numbers of cows tend to adopt less detailed lesion scoring systems, typically including sole haemorrhage, sole ulceration, white line lesions, digital dermatitis, with a range of variations thereon (Manske *et al.*, 2002b; Tadich *et al.*, 2010). Some authors aggregated multiple lesions back to a broader category (Manske *et al.*, 2002b; Machado *et al.*, 2011), or ignored mild lesions entirely (Thomsen *et al.*, 2012) since they have been considered to have little clinical significance (Whay *et al.*, 1997). Authors who have published work analysing lesions consistently value the ability to aggregate detailed scoring systems back into broader classifications for analysis. A method of accounting for the increased clinical significance of more severe lesions is also considered important (Capion *et al.*, 2009).

For use in the current study, a lesion scoring method was required, and the aim was incorporate lesion count, severity, size and location of lesions of interest into a detailed, repeatable scoring system. Further, it was requirement that it could be aggregated back into broader lesion classes.

4.3.2.2 The lesion scoring system

The lesion scoring system was designed based on descriptors in the peer-reviewed literature and lesion descriptors are summarized in Table 4.3. Briefly, sole, heel and toe ulcers were recorded within zone of the foot map (Figure 1.1). Sole heamorrhage (SH), white line haemorrhage (WLH) and white line separation (WLS) were each assigned one

of three severities: mild, moderate or severe; zone was recorded for each lesion. Digital dermatitis and interdigital growths were recorded at the foot level, with digital dermatitis classified according to the M-scale (Dopfer *et al.*, 1997) including the M4.1 category for a chronically re-occurring lesion showing both M1 and M4 stages. Heel horn erosion and under-running were noted.

Sole lesions were circumscribed, and the data entry method allowed one lesion to have multiple areas of severity. Thus, the area of a claw affected by lesions of each type, and the most severe lesion on a claw could be deduced. The same was possible for white line lesions, although these were linear measurements. Every lesion was recorded and lesion count data were available for each claw.

Table 4.3: Lesion names, severities and descriptions used for the lesion scoring system, designed to score lesions on the claws of cows during a longitudinal study of digital cushion and corium thickness and lesion and lameness incidence.

Lesion type (abbreviation)[1]	Zone[1]	Severities	Description	Source
Sole ulcer (SU)	4	N/A	Full-depth penetration of the sole horn in zone 4	(Leach *et al.*, 1998)
Toe ulcer (TU)	5	N/A	Full-depth penetration of the sole horn in zone 5	
Heel ulcer (HU)	6	N/A	Full-depth penetration of the sole horn in zone 6	
Sole haemorrhage (SH)	4/5/6	Mild	Light pink	(Leach *et al.*, 1998)
		Moderate	Dark pink	
		Severe	Very dark pink, or dark red or purple	
White line haemorrhage (WLH)	1/2/3	Mild	Slight haemorrhagic discoloration	(Sogstad *et al.*, 2007)
		Moderate	Moderate haemorrhagic discoloration, up to dark pink	
		Severe	Profound haemorrhage, dark	
White line separation/ fissure (WLS)	1/2/3	Mild	Marks along white line	(Sogstad *et al.*, 2007)
		Moderate	Deep fissures, impacted	
		Severe	Very deep, profound fissure, corium involved, purulent exudate, necrosis, granulation tissue and separation of wall or sole	
Digital dermatitis (DD)	10	M0 to M4.1	M-scale of classification, with the addition of M4.1 for a chronically affected foot that displays the M4 stage in addition to the M1 stage	(Dopfer *et al.*, 1997)

[1]Zones were identified according to the foot map, Figure 1.1.
Other lesions recorded include under-running of the sole, interdigital growth, and "other" (a free text box).

4.3.2.3 Lesion scoring, observer training and assessment of intra-observer repeatability

A single independent observer was trained on a subset of the dataset. Briefly, when scoring lesions, lesions were identified by type, severity scored and circumscribed using the image analysis software Fiji (Schindelin *et al.*, 2012), an open-source platform for the software "ImageJ" (Schneider *et al.*, 2012). The software automatically assigned Cow ID, Claw ID and Assessment Point number to each measurement. The observer was blinded to cow, farm and assessment point.

A workflow diagram for the training and the lesion scoring processes is shown in Figure 4.4 and is summarized here. Briefly, the observer was trained with the scoring system using a subset of the dataset. The observer then undertook intra-observer repeatability tests before beginning the scoring (Cohen's kappa coefficients were calculated in IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The observer was assessed for consistency in identifying lesions, severity classifications and claw regions. After this initial test and repeatability was deemed to be adequate (the results are displayed in "Intra-observer repeatability 1" in Figure 4.4), a subset of images were selected from the main dataset and analysed. Next, the *entire* main dataset was analysed in random order and a blinded manner; the main dataset included the subset scored at the beginning of analysis, which were randomly distributed throughout the order in which images were presented. This enabled intra-observer repeatability checks to be performed between the subset assessed at the beginning of analysis and when the same images appeared at random in the main dataset (for results, see "Intra-observer repeatability 2" in Figure 4.4). The observer was blind to which images were repeated.

4.3.2.4 Intra-observer repeatability results

Agreement between lesion scoring immediately after training was "almost perfect" (Landis and Koch, 1977), with kappa values from 0.83 to 0.96 (Figure 4.4). Intra-observer repeatability checks between the practice dataset and the main dataset was not as high, but still represent substantial agreement (Landis and Koch, 1977): kappa values ranged from 0.66 to 0.76. Since photograph order was randomized, any intra-observer drift throughout the study period ought to be randomly distributed across the dataset. Further, blinding the observer to cow, farm and assessment point reduced the possible influences of bias.

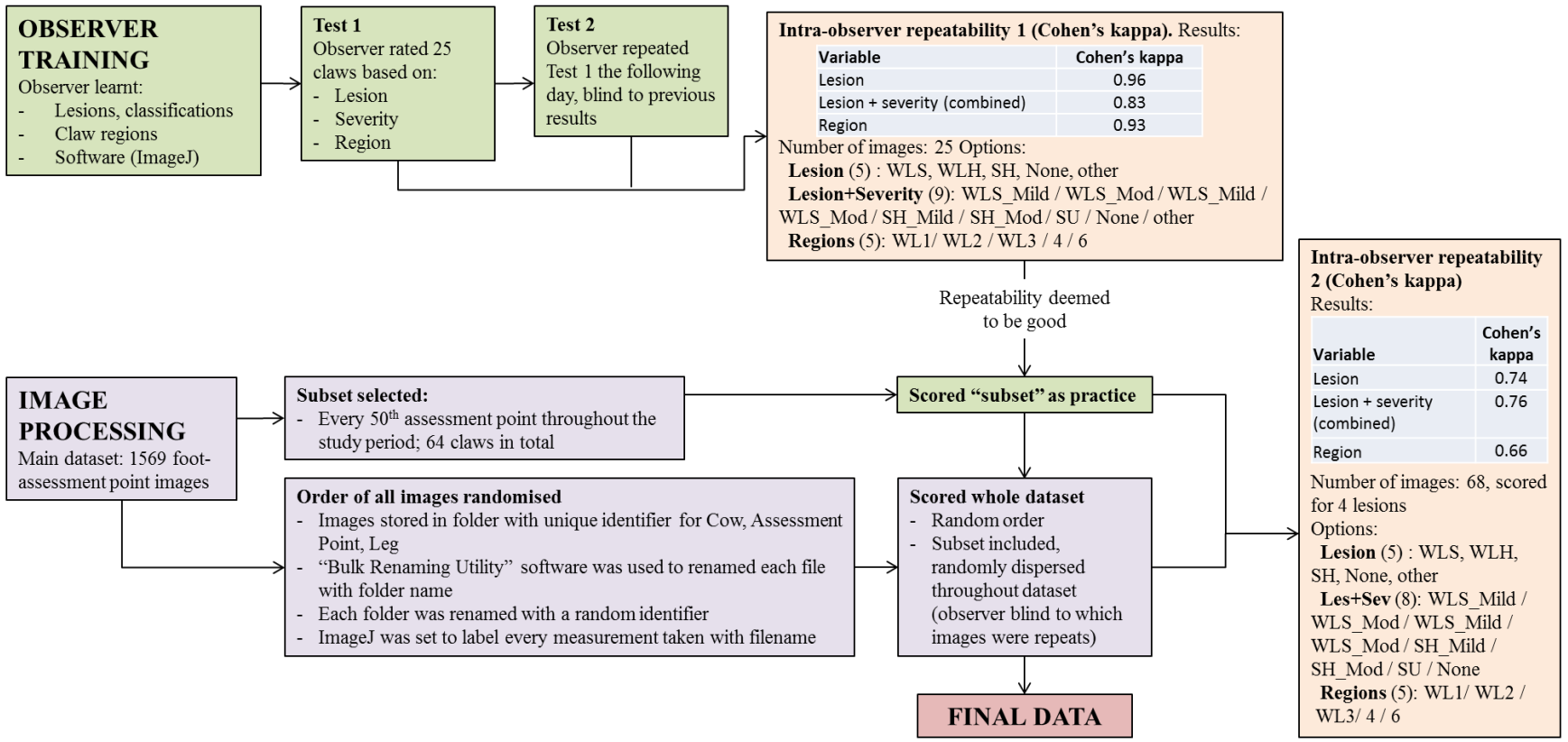

Figure 4.4: Workflow diagram of the lesion scoring process. A single independent observer was trained in the lesion scoring system and then intra-observer repeatability was checked ("Intra-observer repeatability 1"). When repeatability was deemed to be good, the observer scored a subset of the main dataset for practice, then the main dataset, which included the subset randomly dispersed throughout it. Scoring of the main dataset gave the "FINAL DATA". The main dataset had been randomised and the observer was blind to cow, assessment point and farm. Intra-observer checks ("Intra-observer repeatability 2") were also performed at the end of lesion scoring to assess consistency throughout study period.

4.3.3 Data collected using farm management software

Milk yield was recorded automatically by the farm management software at each milking and the milking robots automatically calibrated the milk measurements each week. Body weight was recorded at each milking by farm management software on Farm 1 and weigh scale calibration was built into the milking robots and prompted weekly by farm staff.

Farm 2 did not have weigh scales installed within the milking robots, therefore weigh scales (HD1010 Load Bars, Tru-Test Ltd, Auckland, New Zealand) were installed beneath the assessment point crush on Farm 2. The scales were bolted into the concrete floor and the crush (SA35 Cattle Crush, Wopa, Norfolk, UK) was fixed on top of them, where it remained for the duration of the study period. Bodyweight was recorded at each assessment point using an EziWeigh5 Indicator (Tru-Test Ltd).

The scales on Farm 2 were checked on three days. These check days were normal assessment days at the beginning, middle and end of the study period. On each day, checks were performed both in the morning and at the end of the day, and throughout these days cows were loaded on and off the crush during normal assessments. With the crush empty, the scales were tared. Drums of known weights were loaded onto the crush, and both the weight added and the weight read by the indicator were recorded after each additional load, up to a load of almost 500 kg. The same was recorded as weight was removed from the scales. The correlation between the added weight and the scales reading for two tests are demonstrated in Figure 4.5. The accuracy of the scales was deemed to be good at each test, and no adjustment to cow weight was made for equipment measurement error.

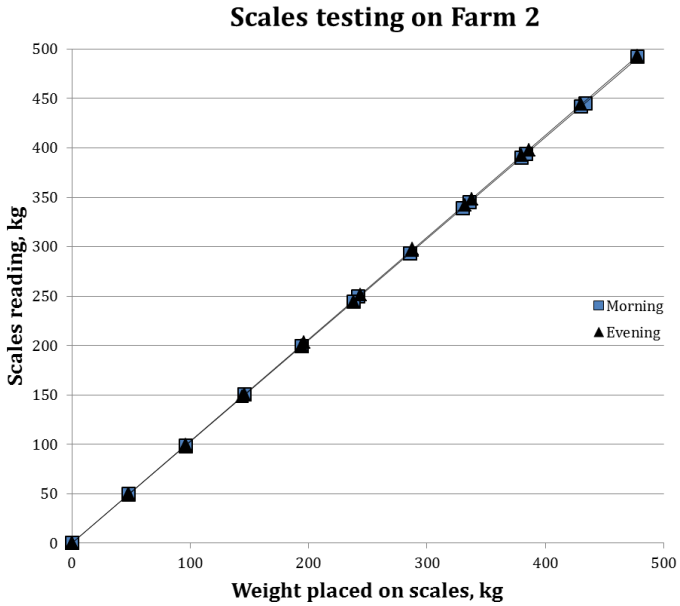

Figure 4.5: Scales reading versus known weight loaded onto the Tru-Test scales that were beneath the assessment crush on Farm 2. Data from two tests, before and after a day of work during which 12 cows were loaded to and from it.

4.3.4 Mobility scoring

4.3.4.1 Methods

Study cows were mobility scored fortnightly from calving to 35 weeks in milk (6 weeks after AP+29), by one independent observer on each of Farm 1 and 2 (Observer 1 and Observer 2, respectively). The observers were blind from the ultrasonographic measurements. Both scorers had been trained to use the mobility scoring system by the same person and had previously used the scoring system in research projects.

The AHDB Dairy mobility scoring system was used, which is the UK industry standard. Briefly, 0 = sound, with even weight-bearing on all limbs, 1 = imperfect mobility but not identifiably lame, 2 = identifiably lame on a leg and 3 = severely lame. Scores 2 and 3 were divided into 2a, 2b, 3a and 3b to allow for greater discretion and aggregation back to four categories if required, full descriptors for the scoring system are presented by Thomas *et al.* (2015a) and shown in Table 4.4. A cow could be given a lame score on each leg (e.g. 2a left hind and 2b right hind) and mobility score was recorded at the leg level. One observer scored on each farm throughout the study period.

Visual assessment of gait is subjective and the literature contains various recommendations to improve consistency of assessment (Whay *et al.*, 1997; Channon *et al.*, 2009). Points that improve consistency of scoring are provided below; these were respected during the study:

- The same observer scores the same cows.
- Ensure the cow is walking freely before a score is taken.
- Observe cows on an even surface.
- Require a “lame” score to be recorded at >1 assessment to be considered lame (this increases specificity of detecting a lame cow, at the cost of sensitivity).

Table 4.4: Mobility scoring descriptors.

Mobility score[1]	Descriptor
0	Walks with even weight bearing and rhythm on all 4 feet, with a flat back. Long fluid strides possible.
1	Steps uneven (rhythm or weight bearing or strides shortened, affected limb or limbs not immediately identifiable).
2a	Mild asymmetry in hind-limb movement. Decreased stride length on affected limb and slightly decreased stance duration with a corresponding increase in limb flight velocity on the nonaffected side. Walking velocity remains normal. Back may be raised.
2b	Moderate asymmetry in hind-limb movement. Decreased stride length on affected limb and a distinct decrease in stance duration. Limb flight on the nonaffected limb is correspondingly faster and the overall walking velocity is reduced. Back usually raised.
3a	Severe asymmetry in hind-limb movement. Marked decrease in stride length on affected limb and very short stance duration. Limb flight on nonaffected limb rapid and walking velocity reduced such that cow cannot keep up with healthy herd. Back raised.
3b	Minimal or non-weight bearing on affected limb. Back raised. Reluctant to walk without encouragement.

[1]Adapted, with permission, from the DairyCo Mobility Score system, the Great Britain industry standard. Scores 2a and 2b and 3a and 3b can be amalgamated back to scores 2 and 3 in this system, respectively.

The two observers had been trained in mobility scoring by the same person and had previously used the scoring system. Inter-observer tests were performed to assess agreement between observers. This was undertaken on one afternoon at Farm 1 and the session progressed as follows:

1. The scoring system was reviewed.
2. Cows were scored in an identical manner to that which KH and NB did on farm:
    a. Walked amongst the communal areas.
    b. Encouraged an individual cow to walk until 6-8 continuous strides had been taken (unless severe lameness inhibited this).
3. Each observer noted a score for the same passage of walk in each cow, always viewed from behind.
4. Observers were blind to each other's scores and did not discuss any scores.
5. Results were blindly entered into the database.

Scores were aggregated into either two, three or four categories. Inter-observer agreement was assessed by calculating Cohen's kappa coefficient in SPSS. Additionally, the numbers of mobility scores assigned to each category were compared to see if either scorer tended to assign higher scores.

4.3.4.2 Results of inter-observer repeatability tests

On one afternoon, 176 cows were mobility scored. Kappa coefficients for the agreement between Observer 1 and Observer 2 for various thresholds are presented in Table 4.5. Figure 4.6 demonstrates the number of mobility scores that each scorer assigned to each category. A chi-square test revealed a difference in scoring; Observer 2 tended to assign higher scores than Observer 1.

Table 4.5: Inter-observer agreement between Observer 1 and Observer 2 using different categories within the mobility scoring system.

Possible outcomes	Kappa
"0-1" / "2-3"	0.46
"≤2a" / "≥2b"	0.66
"0-1" / "≥2a on RH" / "≥2a on LH"	0.43
"≤2a" / "≥2b on RH" / "≥2b on LH"	0.57
"0" / "1" / "2" / "3"	0.40[1]

[1]Weighted (linear) kappa = 0.44
[1]Weighted (quadratic) kappa = 0.50

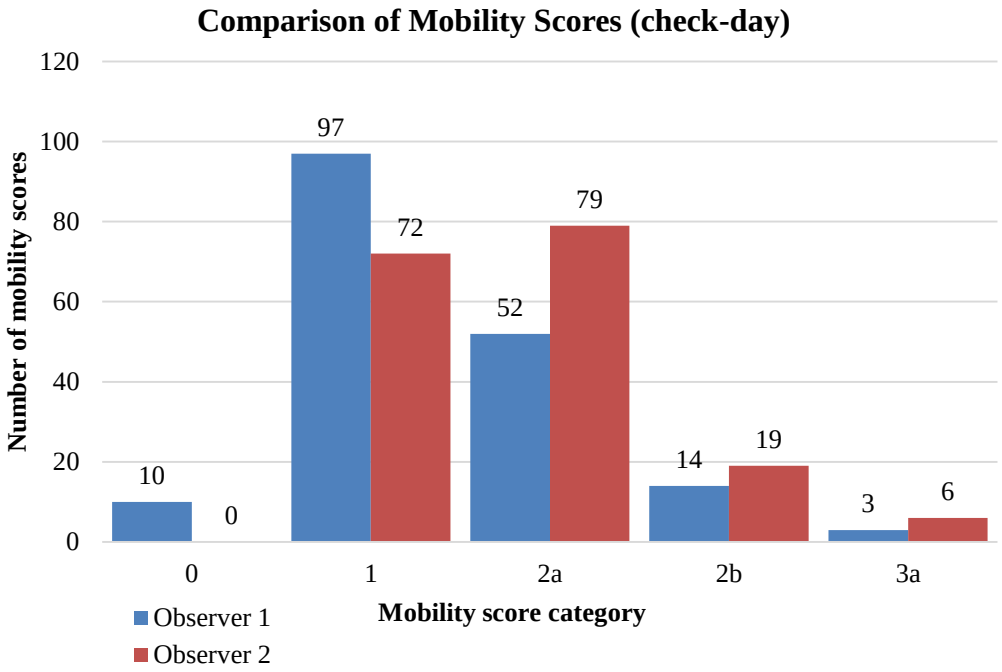

Figure 4.6: Comparison of mobility scores assigned by two observers on a single check-day. The same cows were scored at the same time and the observers were blind to each other's scores.

4.3.4.3 Discussion

Kappa values from inter-observer repeatability checks for mobility scoring ranged from 0.40 to 0.66, which are considered to represent "moderate" and "substantial" agreement (Landis and Koch, 1977). KH and NB agreed particularly well when deciphering between "severely lame" (≥2b) and "not severely lame" (kappa = 0.66).

Locomotion or mobility scoring frequently has poor inter-observer repeatability (Channon *et al.*, 2009) and these current results were compared to those reported in the literature. Out of 16 peer-reviewed studies (published since 2007) that reported the use of multiple observers to visually assess the gait of dairy cattle, 5 stated that they had tested inter-observer repeatability. Four studies reported results for tests, of which one was a percentage agreement test; this can be considered an inappropriate test as it does not account for chance agreement (Birkimer and Brown, 1979). Of the remaining three studies, two reported kappa values, which ranged from 0.15 on a 5-point scale to 0.56 on a 2-point scale (Thomsen *et al.*, 2008; Channon *et al.*, 2009); the other study reported R^2 value of the correlation between scores from different observers (Bicalho *et al.*, 2007a). The inter-observer repeatability scores presented in this work appear to be in line with the published literature. Intra-observer variation was not tested, which could be a limitation of the study; there could have been intra-observer drift over the duration of the study.

4.4 Discussion

Throughout the entire data collection and data processing period, the degree to which errors were made was reduced where possible, by using consistent protocol when taking measurements, observer training and automating some aspects of lesion scoring and ultrasonogram measuring (i.e. identification of images, cow and feet). Error is particularly important if it is systematic, but if random it may not influence the results. The measurement period was long (about 18 months) and observer drift could have occurred. This was avoided for ultrasonographic measurement and lesion analysis by scoring all images at the end and randomizing order of measurement. For mobility scoring this was not possible, but both observers were experienced in mobility scoring at the beginning of the study, which could have reduced observer drift. This work demonstrates that subjective categorizations of lesions and mobility scores were not perfect, but were good when compared with literature published in the area.

4.5 Summary of dataset

The study began on farm in November 2013 and ran until May 2015; enrolment finished in July 2014 when 179 cows had been enrolled. To summarize the dataset compiled throughout the study, it consisted of:

- Assessment point data:
    - Body condition score.
    - Back fat thickness.
    - Ultrasonographic measures of sole soft tissue thickness, measured at three sites beneath the distal phalanx (all three measurements incorporated corium thickness and two incorporated the digital cushion).
    - Lesions (type, region and severity).
- Fortnightly mobility scores.
- Daily milk yield and body weight (except body weight on Farm 2, which was at each assessment point).
- Cow data:
    - Age.
    - Lactation number.
    - Stature.
- Farm system data.

Analyses of the dataset are presented in the subsequent two chapters. Chapter 5 presents analysis of associations between body fat measures and sole soft tissue thickness, and Chapter 6 presents analyses of how back fat thickness, sole soft tissue thickness and how changes in each variable were associated with future lesion and lameness incidence.

5 Mixed-Effects Linear Regression Analysis of Sole Soft Tissue Thickness throughout Lactation

5.1 Introduction

Epidemiological work has demonstrated that body condition loss preceded lameness events, whether lameness was defined by visual detection of impaired mobility (Lim *et al.*, 2015; Randall *et al.*, 2015) or treatment incidence of lesions (Green *et al.*, 2014). Bicalho *et al.* (2009) reported that body condition score was positively associated with digital cushion thickness, an association that could be biologically plausible because the digital cushion contains adipose tissue (Räber *et al.*, 2004; Räber *et al.*, 2006); lipid could be deposited to and mobilized from the digital cushion during periods of positive and negative energy balance.

Further, having a thin digital cushion appears to predispose subsequent lameness from claw horn disruption lesions (Machado *et al.*, 2011; Toholj *et al.*, 2013). A possible mechanism for the temporal association between body condition loss and lameness is that fat is mobilized from the digital cushion during negative energy balance, which leads to depletion of the digital cushion, poorer cushioning of forces during foot strike, greater peak forces on the germinal epithelium and contusions within it, and subsequent lameness. However, previous work assessing the digital cushion has been cross sectional, with the digital cushion on a claw only being assessed once (Bicalho *et al.*, 2009; Machado *et al.*, 2011; Toholj *et al.*, 2013). Whether the digital cushion becomes thinner as body fat is mobilized is yet to be demonstrated. This is a key step in demonstrating whether digital cushion depletion with body condition loss is a mechanism by which cows go lame.

A longitudinal study was undertaken to assess sole soft tissue thickness at five assessment points, between eight weeks before calving and 29 weeks post-calving. Materials, methods and validation for the study were described in Chapter 4. In this chapter, associations between measures of body fat and sole soft tissue thickness (ultrasonographic measurements of the digital cushion and corium beneath the distal phalanx: 4.3.1.3 and Figure 4.2) are explored.

5.1.1 Aims and Objectives

The primary aim of this work was to determine how sole soft tissue thickness changed with changes in body fat measures. A secondary aim was to identify other variables that might influence sole soft tissue thickness, and to explain variability in sole soft tissue thickness that was not associated with back fat thickness. This enabled a correlation between back fat thickness and sole soft tissue thickness to be appropriately assessed.

5.2 Materials and Methods

5.2.1 Null hypothesis

Sole soft tissue thickness does not alter with measures of body fat.

5.2.2 Study design

Chapter 4 describes the methods and validation of a prospective cohort study that repeatedly measured the sole soft tissue thickness throughout lactation. A cow had been eligible for enrolment if approaching its 1st, 2nd, 3rd of 4th calving and if there had been no intention to cull the cow before the 35th week of the lactation throughout which it would be studied. From sample size estimations (4.2.7) on data presented by Bicalho *et al.* (2009), the aim had been to assess a minimum of 150 cows at each of the 5 assessment points, and 179 cows had been enrolled into the study.

5.2.2.1 Description of study herds

Cows on two high yielding robotic milk dairy herds were studied and average cow production was >11,500 litres per 305-day lactation. Cows in the milking herds were housed year-round in cubicle sheds; the production systems on both farms were described in 4.2.4 and summarized in Table 4.1.

Cows were assessed at five assessment points, at approximately 8 weeks prior to calving ("AP–8") and during the 1st, 9th, 17th and 29th week after calving ("AP+1", "AP+9", "AP+17" and "AP+29", respectively), with some variation (4.2.3). At each assessment point, ultrasonographic measurements were taken of the soft tissues beneath the sole at three sites (4.3.1.4 and Figure 4.2, reviewed in Figure 5.1) and were termed "sole soft tissue thickness". At each site, the following soft tissue layers were measured:

- Site 1: Corium thickness beneath the apex of the distal phalanx (the digital cushion does not extend to the toe).
- Site 2: Digital cushion and corium thickness beneath the highest point of the arch of the distal phalanx.
- Site 3: Digital cushion and corium thickness beneath the flexor tuberosity of the distal phalanx.

An ultrasonographic measure of back fat thickness was taken (4.3.1.2, Figure 4.1), as well as body condition score (4.2.6) and body weight (4.3.3). Digital photographs were taken for lesion analysis, which was performed by a blinded observer in a randomized order after on-farm data collection had finished; Table 4.3 presents lesion classifications and section 4.3.2.2 explains the lesion scoring process.

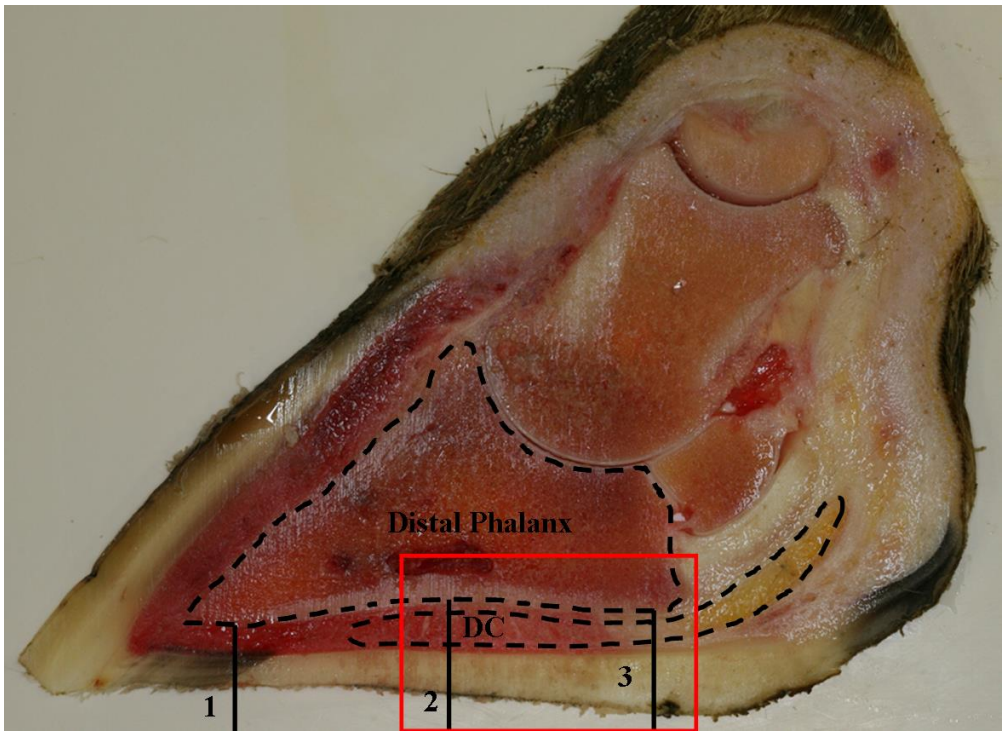

Figure 5.1: Prosection of a bovine digit, with the distal phalanx and the digital cushion (DC) outlined. Vertical black lines indicate the three sites at which sole soft tissue thickness was measured using ultrasonography. The digital cushion is present at sites 2 and 3, and corium is present at all sites.

5.2.3 Data visualization

Data were initially visualised using charts constructed in Microsoft Excel (2010) and in GraphPad Prism (version 6 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Pearson correlation coefficients were calculated in Minitab 17.

5.2.4 Linear regression modelling of sole soft tissue thickness

Mixed-effects linear regression models were constructed to assess variation in the thickness of the sole soft tissues. Two models were constructed with separate outcomes: sole soft tissue thickness at both sites 2 and 3 (Figure 5.1). Models were constructed in MLwiN (Rasbash *et al.*, 2012) using iterative generalized least squares algorithms and a forward stepwise procedure. The final model took the format:

$$Y_{ijkl} = \alpha + \beta 1X_l + \beta 2X_{kl} + \beta 3X_{jkl} + \beta 4X_{ijkl} + f_l + v_{kl} + u_{jkl} + e_{ijkl}$$
$$f_l \sim N(0, \sigma^2_f)$$
$$v_{kl} \sim N(0, \sigma^2_v)$$
$$u_{jkl} \sim N(0, \sigma^2_u)$$
$$e_{ijkl} \sim N(0, \sigma^2_e)$$

where Y was the outcome of the four level linear regression model, which was an ultrasonographic measurement of sole soft tissue thickness at *either* Site 2 or Site 3 in separate models, subscripts i, j, k and l denote the ith repeated measure within the jth assessment of the kth claw of the lth cow respectively, α was the intercept, β1, β2, β3 and β4 represent vectors of coefficients for the fixed effects, X_l, X_{lk}, X_{jkl} and X_{ijkl} represent fixed effect variables at cow, claw, claw-assessment point and repeat measure levels respectively and f_l, v_{kl}, u_{jkl} and e_{ijkl} denote the residual error at each level (assumed to be normally distributed with mean 0 and variance σ^2). The hierarchical structure of the model is demonstrated in Figure 5.2.

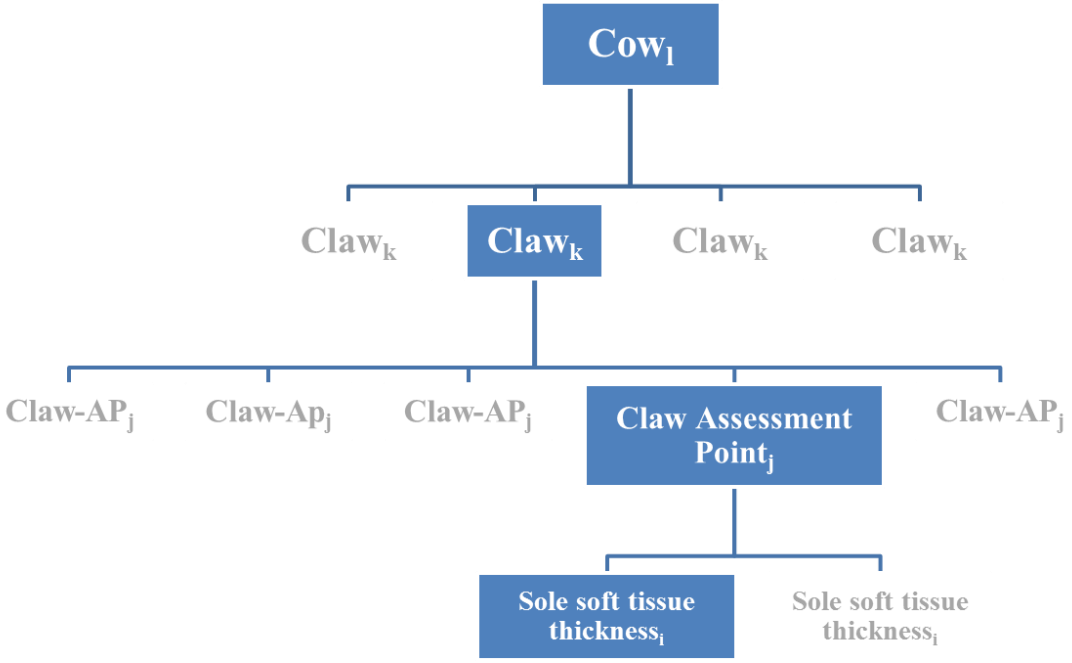

Figure 5.2: Overview of the hierarchical structure of a mixed effects linear regression model of sole soft tissue thickness. Three random effects were included (cow, claw and claw-assessment point) and the outcome was the ith repeat measure (n = 2) of sole soft tissue thickness at the jth assessment point (n = 5: approximately either -8, +1, +9, +17 or +29 weeks relative to calving) of the kth claw (n = 4) of the hind legs of the lth cow (n = 179).

Cow level explanatory variables included lactation number, farm, withers' height and the occurrence of a claw horn disruption lesion throughout the study period. Claw level variables identified if the claw was lateral or medial, as well as lesion occurrence on a claw throughout the study period. Assessment point level variables were time (days into study, with the study start date = 1), assessment point number, days in milk (DIM), back fat thickness, body condition score, body weight at time of assessment, lesion occurrence and corium thickness at Site 1. No explanatory variables existed at the repeated measure level (within assessment point), but this level was retained in the model to assess the variance between ultrasonographic measures at the same site at the same assessment point. Polynomials of all linear

variables and biologically plausible interactions were tested. Dummy variables were used to partition subsets of data that poorly fitted the model.

5.2.5 Linear regression model checking

The Wald test was applied to determine whether fixed effects should remain in a model; essentially, a variable was significant when the coefficient was $\geq 1.96 \times$ SE, as the 95% confidence interval does not include 0 (i.e. $P \leq 0.05$). Residuals were inspected visually at each level. Data points with high influence were removed from the model and the model refitted to evaluate changes in model coefficients; a technique described in 3.3.3.1. The likelihood ratio test was used to compare subsets of models, assessing whether the additional complexity of using additional terms and higher model levels improved model fit (Dohoo *et al.*, 2009).

Biologically plausible interactions were tested. Interactions were plotted using the customized predictions function in MLwiN. Fixed effects of interest were given a range of values (typically including the mean and plus and minus one standard deviation of the mean), whilst other fixed effects took the value of their respective means. This enabled the outcome to be plotted as a prediction from model parameters.

5.3 Descriptive results

5.3.1 Overview of the dataset

The study ran from November 13th 2013, the last assessment point was on 6th April 2014 and the last mobility scores taken on 19th May 2014. A total of 827 assessment points were performed, with data collected from 179, 176, 167, 163 and 158 cows at each of the five assessment points. The median number of days from AP–8 to calving was 56 (IQR: 35 to 64) and from calving to AP+1, AP+9, AP+17 and AP+29 was 7 (IQR: 5 to 10), 62 (52 to 74), 118 (107 to 130) and 202 (192 to 215) respectively. One hundred and five cows were enrolled on Farm 1 and 74 on Farm 2. By lactation number (1, 2, 3 and 4), 70, 45, 42 and 28 cows were enrolled and 66, 40, 29 and 23 completed the study.

5.3.2 Cows that left the study

Twenty one animals left the study, for the following reasons: two were barren when enrolled and did not calve, two were excluded for afflictions that severely compromised mobility that were not related to outcome measures of lameness or claw horn disruption lesions (one developed obturator paralysis at calving and one developed severe foul in the foot), seven became sick and were not assessed for welfare reasons (four had severe mastitis and three had undiagnosed illness), eight

were culled (four for not getting back in calf, three for poor production, one cow for recumbency) and two died (one was diagnosed as an abomasal ulcer and one was not investigated *post mortem*).

5.3.3 Missing data

Some ultrasonographic measurement and lesion data were missing, for the following reasons. If a cow became distressed during an assessment and some data had already been collected, the assessment was finished early and data already recorded was used in the analysis. If a clear ultrasonographic image could not be attained, a measurement was missed. If a block was present, the non-blocked claw was still measured but no ultrasonographic measurement could be taken from the blocked claw; this occurred at <10 claw assessment points.

5.3.4 Description of back fat thickness and sole soft tissue thickness

Table 5.1 displays the means and standard deviations of back fat thickness and sole soft tissue thickness at all three assessment sites. Median BCS was 3.5 and the range was from 1.5 to 4.5. Back fat thickness, corium thickness at Site 1 and sole soft tissue thickness at sites 2 and 3 are plotted against BCS in Figure 5.3; there was a distinct association between back fat thickness and BCS. Between body condition scores of 2.5 and 4.5, a 1 unit change in body condition score corresponded with a 10 mm change in back fat thickness. The magnitude of the effect was smaller below BCS 2.5. Figure 5.3 illustrates sole soft tissue thickness plotted against back fat thickness. Average body weight was 647 kg (SD: 72.2).

The nadir of sole soft tissue thickness both for sites 2 and 3 occurred at AP+1 (Table 5.1). The corium at the toe was thinnest at AP+9. Minimum mean back fat thickness was at AP+9 and AP+17.

Pearson correlation coefficients between corium thickness at Site 1 and each of sole soft tissue thickness at sites 2 and 3 were 0.29 and 0.15 respectively, and between sole soft tissue thickness at sites 2 and 3 was 0.66 ($R^2 = 0.43$).

Table 5.1: Ultrasonographic measurement data collected at 5 assessment points (AP) during a prospective cohort study of sole soft tissue thickness in dairy cows. Back fat thickness was measured over the gluteal muscles. The sole soft tissue were measured at three sites, in the midline of the claw when non-weight bearing: (1) at the apex of the distal phalanx, (2) at the highest point of the arch beneath the distal phalanx and (3) beneath the flexor tuberosity of the distal phalanx; the digital cushion is present at sites 2 and 3. Standard deviations and number at each assessment point are shown.

	Back fat thickness (mm)	Corium thickness only (mm)	Sole soft tissue thickness (mm)	
AP[1]	(SD, n[2])	Site 1 (SD, n[3])	Site 2 (SD, n[3])	Site 3 (SD, n[3])
–8	18.9 (5.7, 170)	3.71 (0.67, 674)	7.43 (1.04, 671)	5.22 (0.91, 670)
+1	16.6 (5.9, 175)	3.57 (0.69, 696)	7.24 (0.98, 695)	4.68 (0.87, 696)
+9	11.1 (5.0, 167)	3.21 (0.60, 661)	7.36 (1.08, 661)	4.89 (0.90, 660)
+17	10.9 (5.3, 163)	3.35 (0.57, 641)	7.47 (1.03, 639)	5.02 (0.96, 639)
+29	13.3 (5.8, 152)	3.49 (0.60, 603)	7.68 (1.02, 599)	5.20 (0.97, 597)
All data	14.3 (6.4, 827)	3.47 (0.67, 3,275)	7.43 (1.06, 3,265)	4.99 (0.95, 3,262)

[1]Assessment point, weeks relative to calving
[2]Number of cows measured; two repeat measures taken on each side of the cow (left and ride) at each assessment point
[3]Number of claws measured; two repeat measures taken at each site at each assessment point

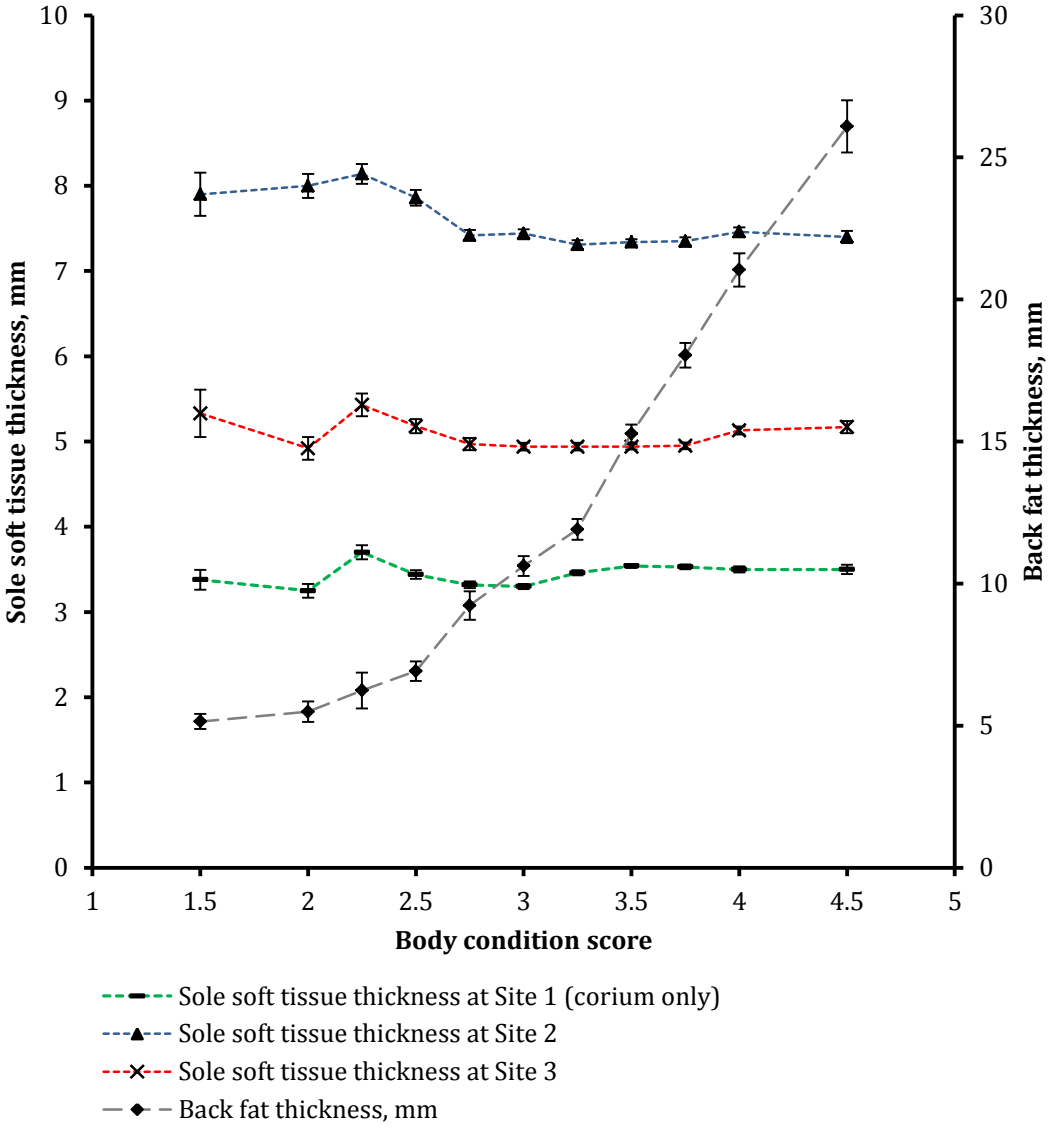

Figure 5.3: Sole soft tissue thickness measured at three sites and back fat thickness plotted against BCS , for all data collected during a prospective cohort study of sole soft tissue thickness and measures of body fat. Measurements were taken at five assessment points; all data are at the claw-assessment point level. Mean and standard error are shown. The numbers of sole soft tissue measurements within each BCS category were 4, 13, 20, 41, 55, 117, 123, 207, 139, 74 and 29 respectively. The back fat thickness measurement includes skin thickness, which is approximately 5-6 mm thick; therefore back fat thickness measures of this magnitude represent virtually no subcutaneous fat being present at the site.

5.3.5 Lesion incidence

Figures 5.4 and 5.5 display lesions (sole and white line lesions respectively) present on claws at an assessment point, with a different severity of lesion displayed on each chart. A lesion appears on a chart if it is the most severe lesion of that type on a claw (types: sole haemorrhage or ulcer, white line haemorrhage and white line separation). Colours within columns indicate the most severe lesion on the same claw at the next assessment point. There was little difference in prevalence of mild sole and mild white line lesions between assessment points. Severe sole haemorrhage and white line lesions peaked at AP+17 and sole ulcers peaked at AP+29.

Figure 5.4 illustrates sole ulcer occurrence, and the most severe lesion present on the same claw at the next assessment point. It shows that 1 claw has a sole ulcer present at AP–8 and AP+1, at AP+1 and AP+9, and at AP+17 and AP+29, whilst three claws were affected by a sole ulcer at both AP+9 and AP+17. Of the 14 claws with a sole ulcer at AP+17, at the previous assessment point, 3 had had a sole ulcer as the most severe sole lesion, 4 a severe sole haemorrhage, 3 a moderate sole haemorrhage and 3 a mild sole haemorrhage, therefore 1 claw had developed a sole ulcer without having displayed a lesion at the previous assessment point. Of the 17 claws that had a sole ulcer at AP+29, 13 had displayed either a moderate sole haemorrhage, a severe sole haemorrhage or a sole ulcer at the previous assessment point, 4 had displayed a mild sole haemorrhage. No claws with a sole ulcer had displayed no lesion at the previous assessment point.

Of the 26 claws displaying severe sole haemorrhage lesions at AP+9, 1, 6 and 13 had displayed severe sole haemorrhage, moderate sole haemorrhage or mild sole haemorrhage lesions at AP+1; 6 had displayed no lesion. Lesions present at AP+17 appear to cure more frequently than lesions present at AP+9.

White line separation lesions appear to occur at a similar stage of lactation to sole haemorrhage lesions, and possibly severe white line haemorrhage, but there was a higher rate of moderate white line haemorrhage lesions earlier, at AP+9 (Figure 5.5).

5.3.6 Visualising sole soft tissue thickness at Site 3 plotted against back fat thickness

Back fat thickness is plotted against sole soft tissue thickness in Figure 5.6 and sole soft tissue measurements taken at the time of a sole ulcer are distinguished between. This suggests that the digital cushions of claws where a sole ulcer was present was thicker than the overall trend of cows where no sole ulcer was present.

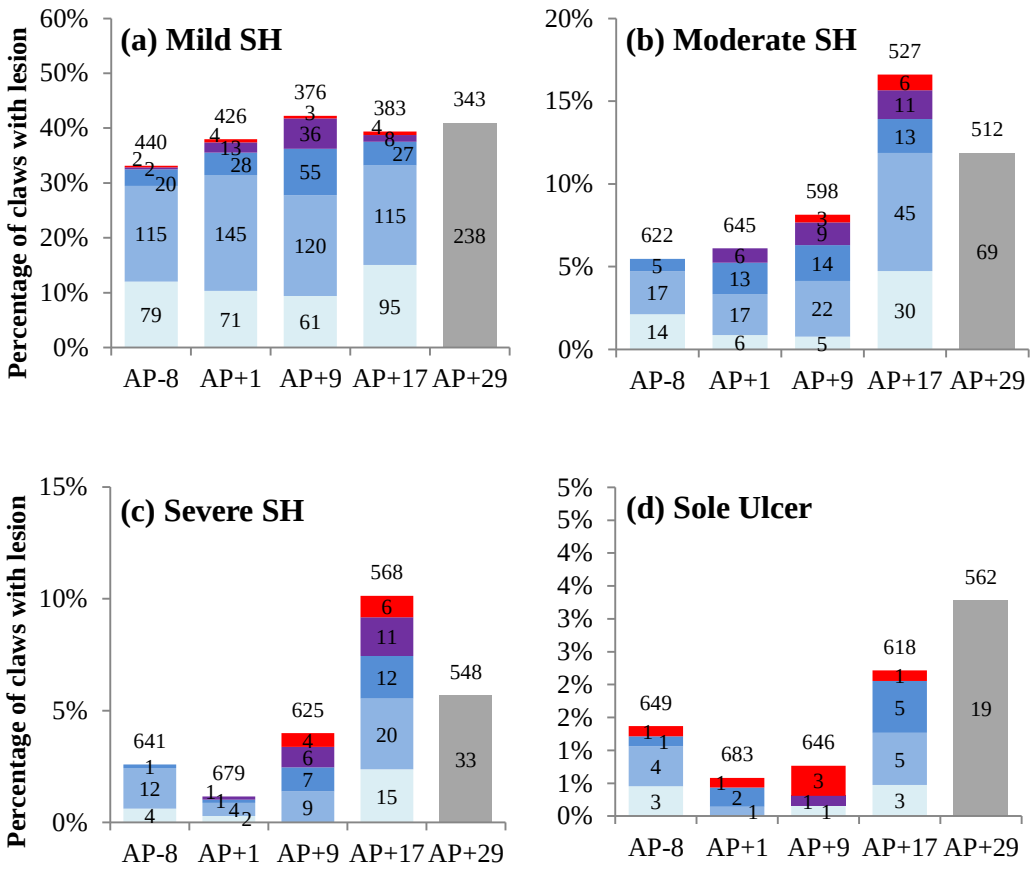

Figure 5.4: Sole lesion data from a longitudinal study of sole soft tissue thickness, by assessment point, severity and progression by next assessment point. Total column height shows the percentage of claws on which the most severe lesion was a mild (a), moderate (b) or severe (c) sole haemorrhage, or a sole ulcer (d), as a percentage of all claws at each assessment point. (Lesion severity was assumed to increase in this order.) Colours within columns show the percentage of claws with that particular lesion (data label = absolute number of claws) that display a lesion of a particular severity at the next assessment point. Key: Sole Ulcer, Severe SH, Moderate SH, Mild SH, No Lesion, Not followed up. Data labels above columns show the number of claws without the lesion to which the chart is dedicated (e.g. (a) the most severe sole lesion was not a Mild SH on 440 claws at AP–8). There were 658, 687, 651, 632 and 581 claws at each assessment point. All data describe a single most severe sole lesion on each claw at each assessment point.

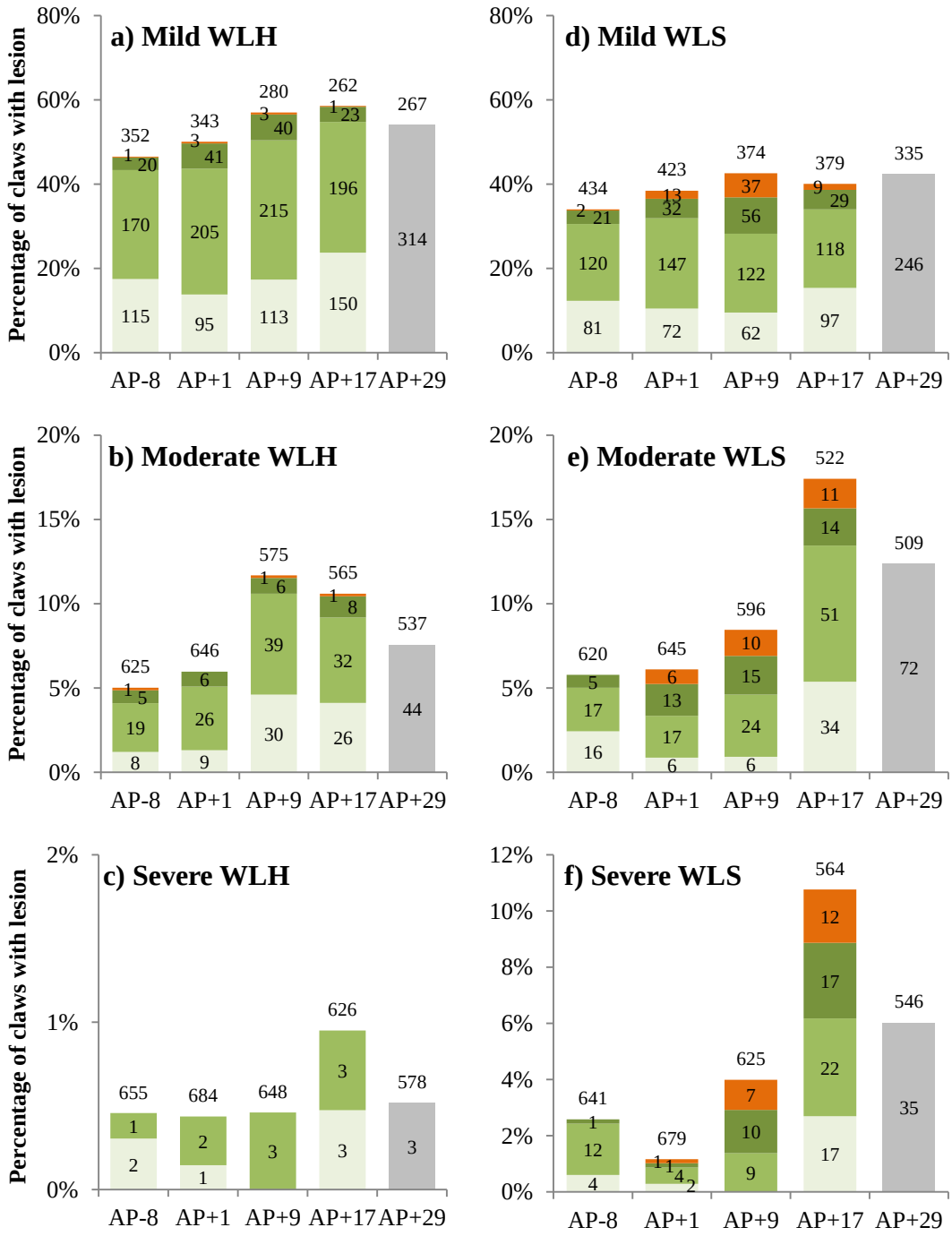

Figure 5.5: White line lesion data by assessment point, severity and progression by next assessment point. Charts (a), (b) and (c) refer to white line haemorrhage and charts (d), (e) and (f) refer to white line separation. Total column height shows the percentage of claws on which the most severe lesion Mild, Moderate or Severe, as a percentage of all claws at each assessment point. Colours within columns show the percentage of claws with that particular lesion (data label = absolute number of claws) that display a lesion of a particular severity at the next assessment point. Key: Severe, Moderate, Mild, No lesion, Not followed up. All data describe a single most severe white line lesion of either time (haemorrhage or separation) on each claw at each assessment point.

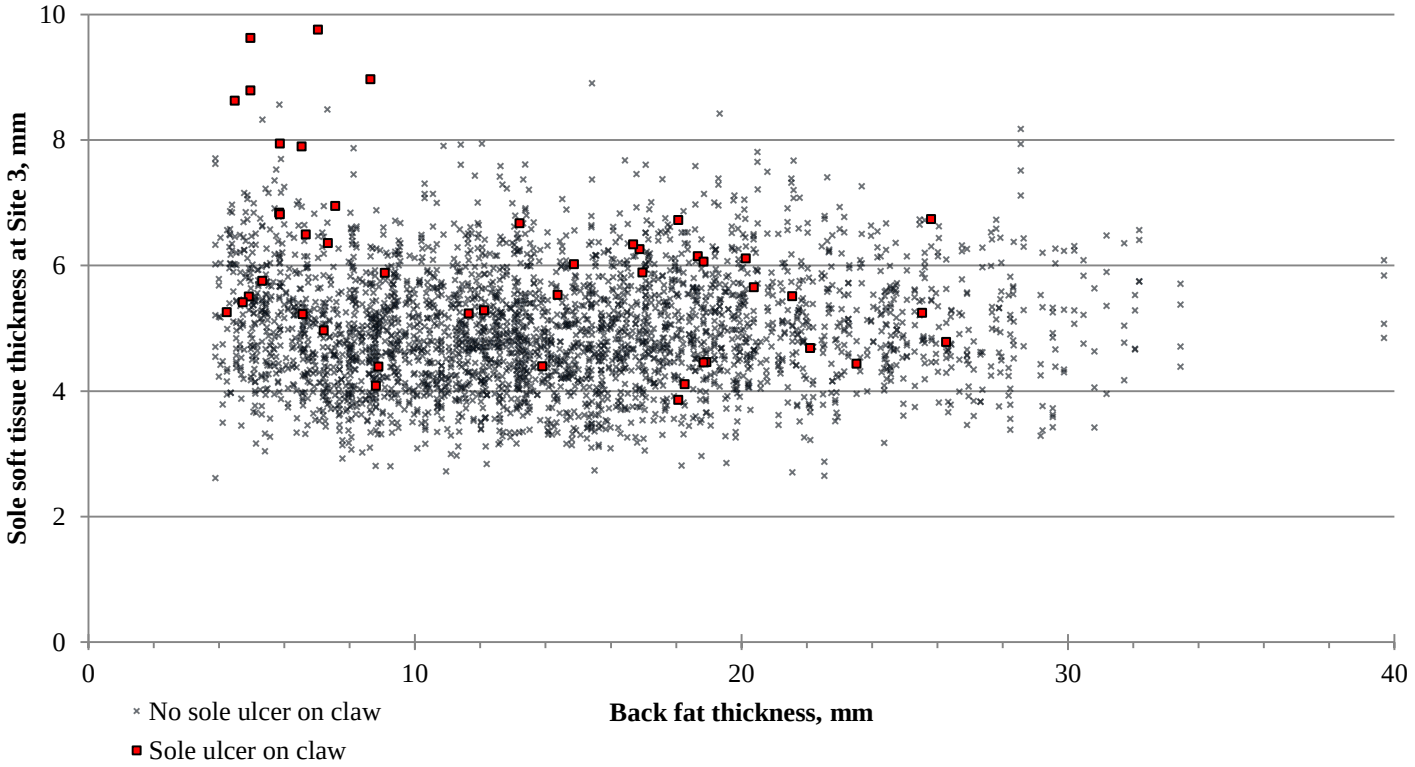

Figure 5.6: Sole soft tissue thickness at the claw-assessment point level plotted against back fat thickness for all cows in the modelled dataset. Sole soft tissue measurements when a sole ulcer was present on a claw are distinguished from measurements when a sole ulcer was not present. Data were collected during a prospective cohort study of the sole soft tissue thickness on the hind claws, from 179 cows in either lactation 1, 2, 3 or 4.

5.4 Modelling results: a 4-level mixed effects model of sole soft tissue thickness

5.4.1 The model dataset

The dataset for the final models consisted of 6,550 measures of sole soft tissue thickness at Site 3 (i.e. a combined measurement of digital cushion and corium thickness beneath the flexor tuberosity of the distal phalanx) from 3,275 assessments of 716 hind claws of 179 cows. The final models were 4-level mixed effects models with the outcome sole soft tissue thickness. The model hierarchy is presented in Figure 5.2, with random effects to denote "cow", "claw" and "claw-assessment point". As stated in 5.3.4, sole soft tissue thickness at sites 2 and 3 were correlated (R^2 = 0.43); models with either term as the outcome presented very similar results and differences in these models are described later in 5.4.5.

5.4.2 Overview of model

The final model is presented in Table 5.2 and had the outcome sole soft tissue thickness at Site 3 (directly beneath the flexor tuberosity of the distal phalanx). This model left 61 % of the null model variance unexplained. Of this unexplained variance, 48 % was at the claw-assessment point level.

Sole soft tissue thickness on the lateral claw was 0.89 mm greater (CI: 0.84-0.95) than on the medial claw. Cows on Farm 1 had a sole soft tissue thickness 0.27 mm greater (CI: 0.14 to 0.40) than those on Farm 2. Sole soft tissue thickness at AP+1 fitted the model poorly, being 0.33 mm thinner (CI: 0.28 to 0.39) than at other assessment points; this difference was not explained by other variables tested. (This unexplained difference is demonstrated and expanded upon later in Figure 5.10, where both back fat thickness and sole soft tissue thickness are plotted against DIM, grouped by assessment point). Withers' height and polynomial terms of time (which had a small effect size) were significant and retained in the model. Other significant variables were involved in interactions and are described below.

Table 5.2: A linear regression model of sole soft tissue thickness beneath the flexor tuberosity of the distal phalanx, measured during a prospective cohort study of 179 dairy cows. Cows were assessed at 5 assessment points (**AP**) between 8 weeks prior to and 29 weeks post calving. Explanatory variables included ultrasonographic measures of back fat thickness (**BFT**), lateral or medial claw, time throughout study, lesion data throughout the study period, claw, withers height, farm and corium thickness at the apex of the distal phalanx (Site 1). Interactions are shown.

Response:			Sole soft tissue thickness at Site 3 (mm)		
	Mean (SD)[1]	No. of units[2]	Coefficient	Lower 95% CI	Upper 95% CI
Fixed Part					
Intercept			4.69		
Assessment Point (AP)$_i$					
AP-8, +9, +17 or +29		2,579	Baseline		
AP+1		696	-0.335	-0.389	-0.282
Claw$_k$					
Medial		358	Baseline		
Lateral		358	0.892	0.836	0.948
Farm$_l$					
1		105	Baseline		
2		74	-0.269	-0.403	-0.135
Time$_j$, days	259 (109)				
Time^1			-9.40×10^{-5}	-5.34×10^{-4}	3.46×10^{-4}
Time^2			1.93×10^{-5}	1.48×10^{-5}	2.38×10^{-5}
Time^3			2.32×10^{-9}	-9.92×10^{-9}	1.46×10^{-8}
Time^4			-2.01×10^{-10}	-2.97×10^{-10}	-1.05×10^{-10}
BFT$_i$ (categories)					
≥6 mm		2,991	Baseline		
<6 mm		284	0.221	0.123	0.319
Corium at apex$_i$, mm	3.47 (0.65)		0.101	0.0603	0.141
Withers height$_l$, cm	144 (4.08)		0.0360	0.0197	0.0522
Cow SU/SevSH incidence$_l$					
Never occurred		147	Baseline		
Occurred		32	-0.237	-0.370	-0.105
Sole ulcer$_i$					
Absent		3228	Baseline		
Present		47	0.531	0.349	0.714
DD M2 lesion$_i$					
Absent		3233	Baseline		
Present		42	-0.223	-0.411	-0.0351
BFT$_i$ (continuous), mm[3]	1.43 (0.64)		0.132	0.0687	0.195
Interactions with BFT$_i$[4]					
BFT$_i$ × AP+1$_i$			-0.184	-0.268	-0.100
BFT$_i$ × Corium at apex$_i$			-0.137	-0.192	-0.0819
BFT$_i$ × sole ulcer present$_i$			-0.761	-1.02	-0.502
BFT$_i$ × DD M2 lesion present$_i$			-0.605	-0.974	-0.235
Random Part			σ2 (SE)	% remaining at each level	
Level:					
l: Cow		179	0.161 (0.021)	28.6%	
k: Claw		716	0.075 (0.009)	13.4%	
j: AP		3,275	0.270 (0.008)	47.9%	
i: Repeated measure		6,550	0.057 (0.001)	10.1%	
Total variance:		Remaining: 0.566		Explained: 38.7%	

Terms are significant when the 95% confidence interval does not include 0 (Wald Test, $\alpha = 0.05$).
Subscripts i, j, k and l denote the lowest level of the model at which a term varied (5.2.4).
Linear terms are centred around the grand mean.
[1]Mean and standard deviation for continuous variables.
[2]Number of units in each category, for categorical variables. Depends on the lowest level at which the term varies.
[3]Coefficients for continuous back fat thickness measurements relate to a 10 mm difference.
[4]Baseline of each interaction term is the baseline for the coefficient not in the interaction, when back fat thickness = 0.

5.4.2.1 Effects of body fat measures

No association was found between body condition score and sole soft tissue thickness at Site 3. However, sole soft tissue thickness was positively correlated with back fat thickness. Back fat thickness also interacted with other terms: assessment point (sole soft tissue was thinner and not correlated with back fat thickness at AP+1), corium thickness at Site 1 (the association became smaller with increasing corium thickness at Site 1) and lesion presence (back fat thickness was negatively correlated with sole soft tissue thickness when either a sole ulcer or an M2 digital dermatitis lesion was present). The mean effect of a 10 mm increase in back fat thickness on sole soft tissue thickness was a 0.13 mm increase, for measures of sole soft tissue thickness at AP-8, +9, +17 and +29, based on the mean corium thickness at Site 1 and when no sole ulcer or M2 digital dermatitis lesion was present. The effects of interactions between back fat thickness and other variables on sole soft tissue thickness are described below and displayed in Figures 5.7 to 5.9.

As stated above, back fat thickness was positively correlated with sole soft tissue thickness. However, a categorical variable demonstrated that when back fat thickness was <6 mm, sole soft tissue thickness was 0.22 mm thicker (CI: 0.13 to 0.32) than when back fat thickness was ≥ 6 mm (Table 5.2: "BFT_j (categories)"); approximately 10% of back fat thickness measurements were < 6 mm. Back fat thickness < 6 mm corresponded with BCS ≤2 and virtually no subcutaneous fat. Figure 5.7 shows a prediction of sole soft tissue thickness at Site 3 from the model parameters, and this effect is visible at the 10^{th} percentile of back fat thickness, where sole soft tissue was thicker than predicted by the rest of the regression line, in cows not displaying a lesion.

Further, when a sole ulcer was present on a claw at an assessment point, the digital cushion was thicker than at other assessment points. Additionally, when a sole ulcer was present, there was a negative correlation between back fat thickness and sole soft tissue thickness at Site 3. This interaction is plotted in Figure 5.7. There was also an interaction between back fat thickness and presence of an M2 digital dermatitis lesion, which is shown in Table 5.2 ("BFT_j × DD M2 lesion $present_j$") and had a similar shape to the effect of sole ulcer plotted in Figure 5.7, although for clarity, the effect of an M2 lesion being present is not shown in Figure 5.7.

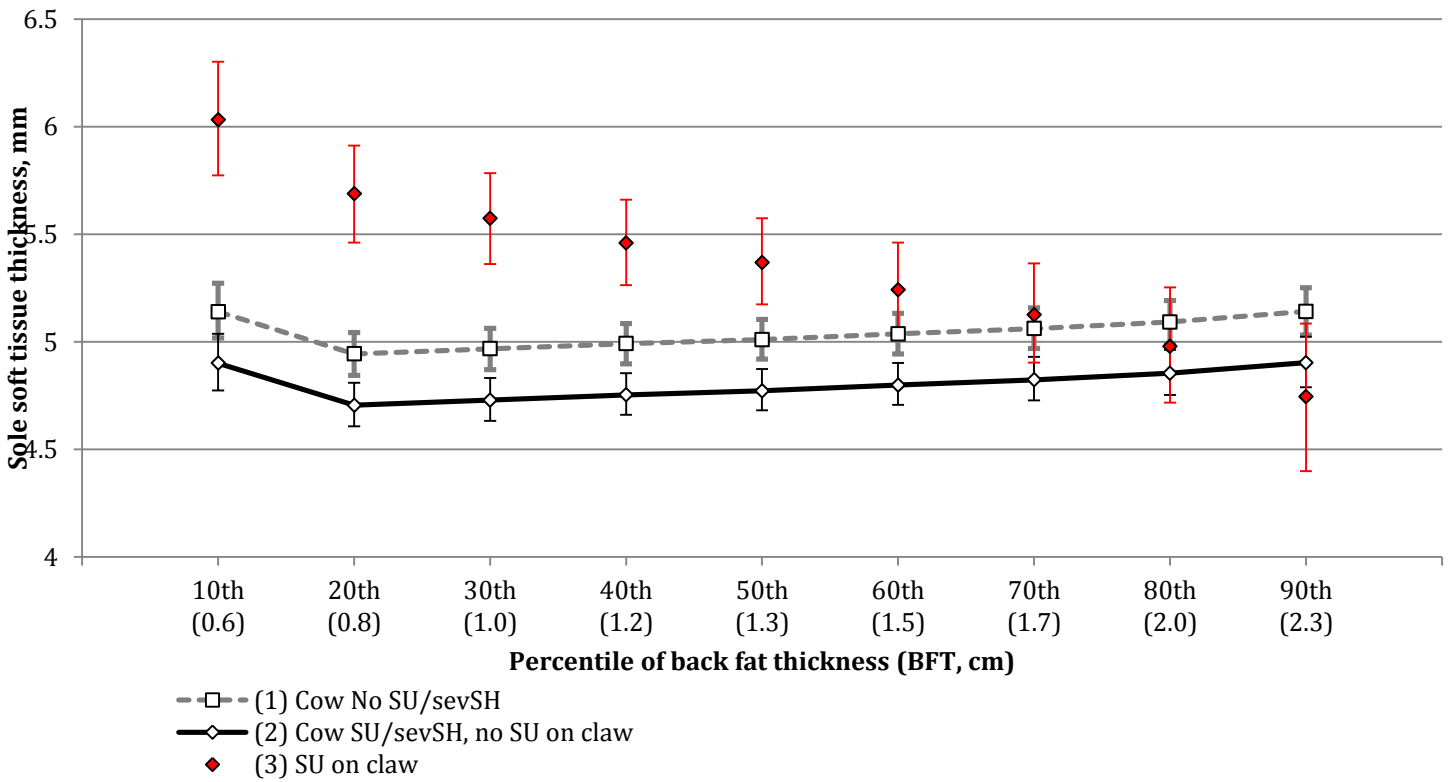

Figure 5.7: Predictions of sole soft tissue thickness at Site 3, plotted against deciles of back fat thickness (with absolute values of back fat thickness at each percentile), with data grouped by lesion occurrence. The model was a linear regression model with the outcome sole soft tissue thickness beneath the flexor tuberosity, collected between 8 weeks prior to and 29 weeks post calving, in 179 cows studied throughout one of the first four lactations. The model is presented in Table 5.2, and the predictions are based on measures of sole soft tissue when no M2 digital dermatitis lesion was present and measures not at AP+1. Data displayed are grouped as follows: (1) cow did not develop a sole ulcer or severe sole haemorrhage during the study, (2) cow did develop a sole ulcer or severe sole haemorrhage during the study and a sole ulcer is not present on the claw at the assessment point, and (3) sole ulcer present on the claw at the assessment point. The numbers of sole ulcers that occurred within each decile were 11, 7, 4, 1, 2, 3, 3, 7, 5 and 4. (The numbers of severe sole haemorrhages within each decile were 22, 28, 26, 18, 19, 8, 12, 5, 9 and 5). Error bars show 95% confidence intervals.

Secondly, the interaction between back fat thickness and sole soft tissue thickness is shown using predictions from the model in Figure 5.8. The coefficient for the model was negative and as corium thickness at Site 1 increased, the effect size for the correlation between back fat thickness and sole soft tissue thickness became smaller (Table 5.2: "back fat thickness × Corium at Site 1"). Corium thickness at Site 1 was thinnest at AP+9 and AP+17 (Table 5.1).

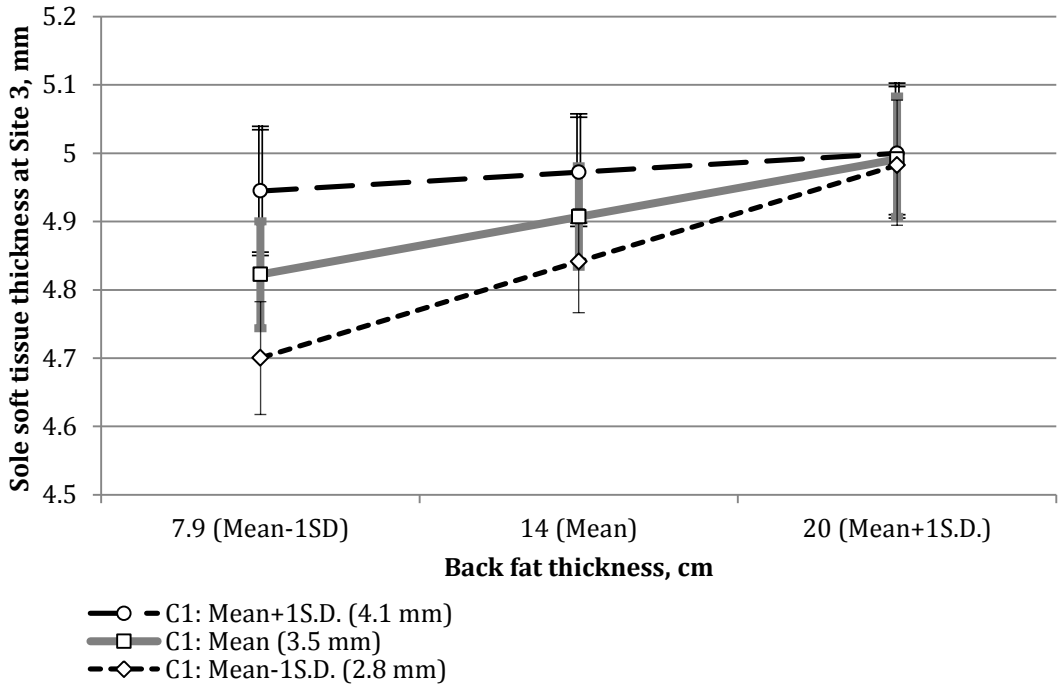

Figure 5.8: Predictions of sole soft tissue thickness at Site 3, for three measures of corium thickness beneath the apex of the distal phalanx, demonstrating an interaction between back fat thickness and corium thickness. The three lines represent the association between back fat thickness and sole soft tissue thickness beneath the flexor tuberosity for three different values of corium thickness at Site 1: the mean, mean+1 and mean-1 SD. Error bars show the 95% confidence intervals. Predictions are based on measures of sole soft tissue when no sole ulcers and no M2 digital dermatitis lesions were present. Error bars show 95% confidence intervals.

Thirdly, whilst sole soft tissue at Site 3 was thinner at AP+1, additionally back fat thickness was not positively correlated with sole soft tissue thickness at this assessment point as it was at others. This interaction is plotted as a prediction from the model in Figure 5.9.

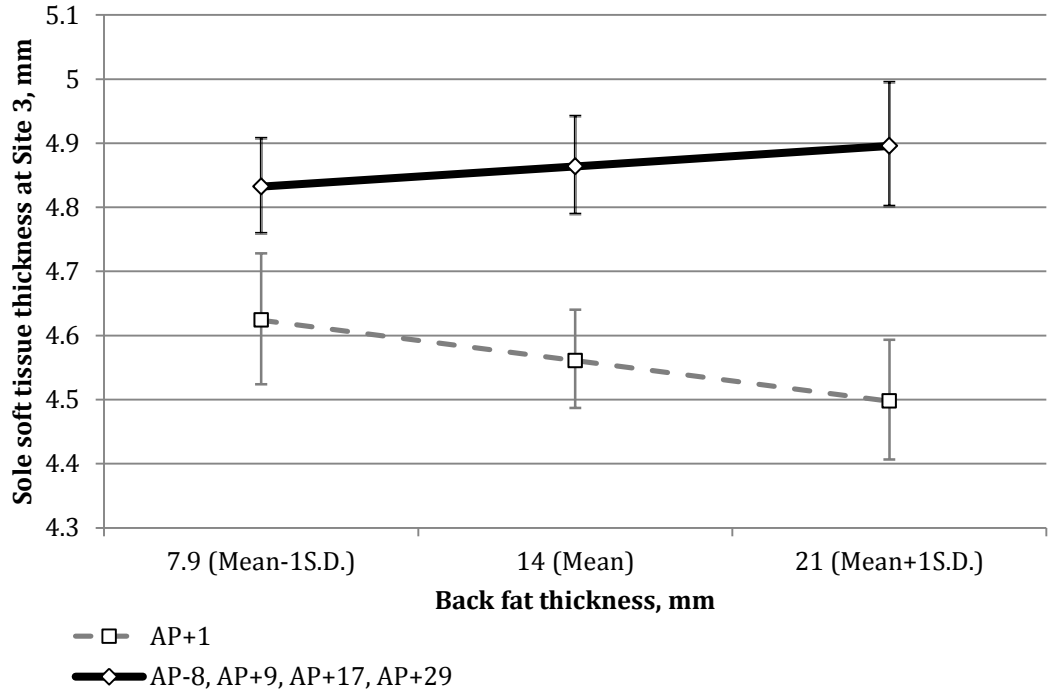

Figure 5.9: Predictions of sole soft tissue thickness at Site 3 demonstrating an interaction between back fat thickness and assessment point; sole soft tissue thickness was positively correlated with back fat thickness, except for at AP+1. The predictions are based on a linear regression model with the outcome sole soft tissue thickness beneath the flexor tuberosity, collected between 8 weeks prior to and 29 weeks post calving, in 179 cows studied throughout one of the first four lactations. The model was based on measures of sole soft tissue when no sole ulcers and no M2 digital dermatitis lesions were present. Error bars show 95% confidence intervals.

5.4.2.2 The effects of lesion incidence

In addition to the interaction between back fat thickness and sole ulcer presence, if a cow experienced a sole ulcer or a severe sole haemorrhage during the study, the cow's mean sole soft tissue thickness across all claws was 0.24 mm thinner (CI: 0.11 to 0.37, Table 5.2: "Cow SU/SevSH incidence"), *except* on a claw when a sole ulcer was present. Both this cow-level effect of lesion occurrence and the claw-assessment point level effect of sole ulcer presence are plotted in Figure 5.7, compared to the sole soft tissue thickness of cows that do not develop a sole ulcer or a severe sole haemorrhage during the study period. The sole soft tissue thickness at Site 3 was not thickened at the assessment point when a sole haemorrhage was visible (compared to the baseline of sole soft tissue thickness of cows that develop a lesion during the study period; data not shown).

Further to this interaction, the dataset shows that sole ulcers were over-represented in thin cows ($P < 0.001$; chi-squared test with one degree of freedom): of the 827

assessment points, 73 had back fat thickness measurements <6 mm, and of the 47 sole ulcers that were recorded, 11 occurred when back fat thickness was <6 mm. Additionally, the highest sole ulcer incidence coincided with when cows were thin: of the 73 back fat thickness measurements that were <6 mm, 68 occurred at AP+9 or later.

5.4.2.3 Other associations, non-significant variables and remaining variance

Stature, lactation number and body weight were all correlated with each other. Stature and body weight were significant in the final model, with positive effects. Stature was retained in the final model; when included, there was no additional effect of body weight.

A categorical variable distinguished between the sole soft tissue thickness in cows before and after a lesion had occurred. There was no difference in thickness of the digital cushion after a sole ulcer compared with prior to the sole ulcer. No significant correlations were found between sole soft tissue thickness and white line lesion incidence, whether variables described white line haemorrhage lesions, white line separation lesions, either, or different severities of either lesion.

In the final model, 48% of the remaining variance was between assessment points of the same claw. Table 5.2 additionally shows that 14% of the remaining variance occurred between claws of the same cow, 28% between claws of different cows, and 10% between measures of the same claw at the same assessment point.

5.4.3 Summary of the final model

The model shows that sole soft tissue thickness was thicker (1) on the lateral claw compared with the medial, (2) on Farm 1 compared with Farm 2, (3) in the thinnest cows, (4) when a sole ulcer was present, (5) in taller cows, (6) with increasing corium thickness at Site 1 and (7) with greater back fat thickness. The digital cushion was thinner in cows that experienced a sole ulcer or severe sole haemorrhage during the study period, in claws at the time of an M2 digital dermatitis lesion and at AP+1 (immediately after calving); no variable was found to explain the thinness of the sole soft tissue at AP+1. Polynomial terms of time were significant, describing changes in sole soft tissue thickness throughout the study from Day 1 of the study period (13th November 2013), although it is unclear why these changes occurred with time. Back fat interacted with other variables, and the association between back fat thickness and sole soft tissue thickness in different circumstances are plotted as predictions from model parameters in Figures 5.7 to 5.9.

5.4.4 Comparison of the final model with an alternative model

A squared term of DIM was correlated with back fat thickness and was significant in an alternative to the final model, in addition to significant effects of back fat

thickness, although the effect size of back fat thickness reduced. Figure 5.10 demonstrates that a squared term of DIM could describe both back fat thickness and sole soft tissue thickness. An alternative model that included DIM was considered as an alternative to the final model presented. Model parameters were very similar in the alternative model and since DIM itself could not have had an effect on sole soft tissue thickness, it is unclear what the polynomial term of DIM indicated. DIM could be an indicator of variables that were associated with sole soft tissue thickness, such as hormonal changes throughout lactation or milk production, or it could simply be another descriptor of back fat thickness (the two were correlated). By design, this prospective cohort study could not distinguish an effect of DIM from an effect of back fat thickness, so the final model excludes DIM to allow better assessment of the effects of interest.

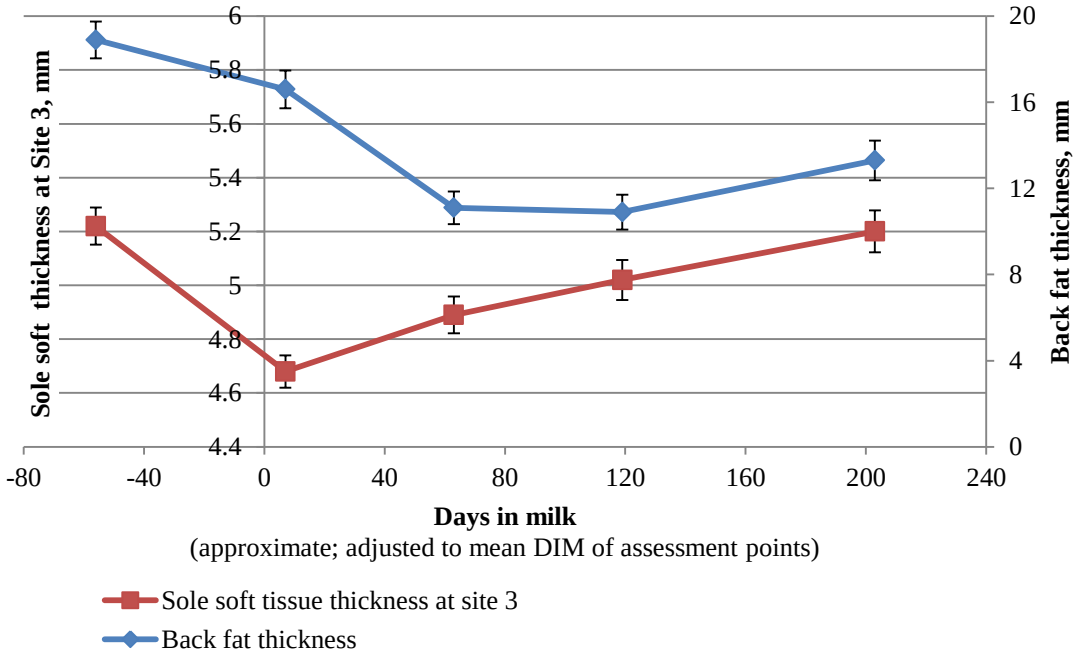

Figure 5.10: Back fat thickness and sole soft tissue thickness at Site 3 from 5 assessment points plotted against days in milk, from a longitudinal study of 179 cows. Error bars show the 95 % confidence interval around the mean; number of measures and standard deviations are shown in Table 5.1.

5.4.5 Differences in the models when sole soft tissue thickness at Site 2 was the outcome

As mentioned in 5.3.4, the sole soft tissue thickness at sites 2 and 3 were correlated (R^2 = 0.43). The model differed very little when sole soft tissue thickness at Site 2 was used as the outcome. Where it differed substantively from the model that had the outcome sole soft tissue thickness at Site 3 was as follows. An interaction "back fat thickness <6 mm × SU on a claw at an AP" was not significant, and there was a significant effect of lactation number that explained a large degree of the cow-level variance (lactation 2 and 4 had a thicker digital cushion at Site 2, compared with

lactation 1 animals, data not displayed). The model explained 41% of the null variance of sole soft tissue thickness at Site 2, with lactation number explaining much of the cow-level variance.

5.5 Discussion

5.5.1 Summary of results

This study measured the thickness of the sole soft tissue beneath the distal phalanx at five assessment points, once before calving and at four assessment points throughout lactation. The null hypothesis stated that sole soft tissue thickness does not alter with measures of body fat. Back fat thickness was positively correlated with sole soft tissue thickness between claw-assessments and the results support the alternate hypothesis. This effect was small and depended upon many other variables, most notably being that sole soft tissue thickness of cows that developed a sole ulcer or a sole haemorrhage during the study was thinner than other cows, except on a claw when a sole ulcer was present, when it was thickened.

As with all statistical models of this type, this does not confirm that changes in back fat thickness and changes in sole soft tissue thickness have a similar cause; it cannot be concluded that sole soft tissues became thin as a result of fat mobilization that was associated with negative energy balance and loss of back fat. Neither can the model determine whether sole soft tissues became thin as a result of other effects that are described by a polynomial term of DIM. It can however demonstrate that both were correlated. The current work demonstrates that sole soft tissue thickness does alter with back fat thickness between assessment points throughout lactation, whilst highlighting that back fat thickness is only one of many variables associated with sole soft tissue thickness.

5.5.2 Comparison of sole soft tissue thickness measures with prior work

There are similarities between the results presented in this work and those of previous works that have assessed the soft tissues of the sole. In concurrence, this work found associations between cushion thickness and body fat measures (Bicalho *et al.*, 2009) and digital cushion was thicker on the lateral claw (Kofler, 1999; Lischer *et al.*, 2002; Bicalho *et al.*, 2009). However, there were also differences with previous work. This study found that the digital cushion was thinnest at AP+1 (4-10 days post-calving); Bicalho *et al.* (2009) had reported the nadir of sole soft tissue thickness coincided with the nadir of BCS, at approximately 120 DIM. The association between back fat and sole soft tissue thickness found in this work was smaller than Bicalho *et al.* (2009) reported (discussed later: 5.5.2.1). Further, the sole soft tissue thickness reported in this work was approximately 50 % thinner than that reported by Bicalho *et al.* (2009), but was very similar to measurements reported in other work (Kofler *et al.*, 1999; Toholj *et al.*, 2013).

5.5.2.1 A possible difference in scanning site of the sole soft tissue, compared with Bicalho et al. (2009)

The sole soft tissue thickness reported by Bicalho *et al.* (2009) was thicker than that reported in the current and in other work. This difference could be due to a difference in ultrasonography site, or there could be genetic differences between the cows studied in this work and by others. In this work, the ultrasonography transducer was held perpendicular to the plantar aspect of the claw in the midline, a technique particularly precisely described by Kofler *et al.* (1999). Conversely, the scanning site described by Bicalho *et al.* (2009) was "at the typical sole ulcer site", and was described as being over the "medial" (infer "axial") aspect of zone 4 of the foot map (Figure 1.1). Bicalho *et al.* (2009) describe that their scanning site was in line with the middle pad and reference Räber *et al.* (2004), but this presents an incongruity: previous authors (e.g. Lischer *et al.*, 2002; Räber *et al.*, 2006) and the current thesis find the middle pad to be in the midline of the sole when using external landmarks. If the Bicalho *et al.* (2009) work used a scanning site more axially and over the typical sole ulcer site as described, it seems likely that they scanned the axial pad rather than the middle pad. Figure 5.11 shows a possible difference between the scanning sites.

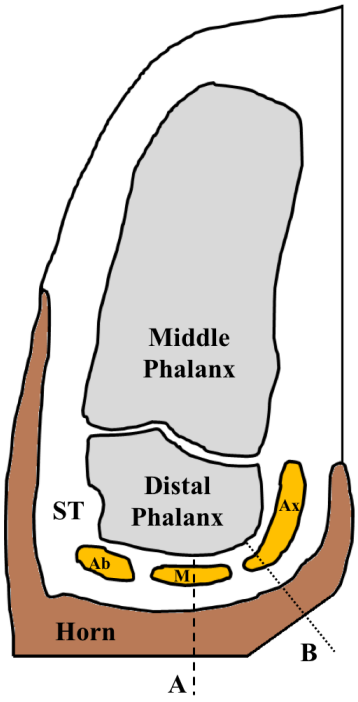

Figure 5.11: Diagram of a transverse section through a claw to demonstrate different possible reported scanning sites of the sole soft tissues. The middle and distal phalanges are annotated. The axial, middle and abaxial fat pads of the digital cushion are shown ("**Ax**", "**M**" and "**Ab**"), all other soft tissue structures are marked "**ST**" and the hoof capsule is marked "Horn". Two lines demonstrate the intended ultrasound scanning sites to image the digital cushion, beneath the flexor tuberosity of the distal phalanx, (A) as performed in this study and (B) as inferred from the Bicalho *et al.* (2009) work. Figure constructed by tracing over a photograph of a dissected claw to select anatomical points of interest, for clarity.

The current work scanned at site A in Figure 5.11. Site B is a possible scanning location used by Bicalho *et al.* (2009), inferred from their description of the scanning site. It appears that this would scan the axial pad, which has a larger volume of fat (Räber *et al.*, 2004). This might explain two key differences seen between the results of Bicalho *et al.* (2009) and those of this work. Firstly, the difference in anatomical location could explain differences in the absolute values of sole soft tissue thickness. Secondly, the middle fat pad is reported as being the smallest pad (Räber *et al.*, 2004); perhaps, scanning at site B would target a larger fat depot that constituted a greater proportion of the tissue thickness, and altered more as body fat was mobilized or stored. This might have limited the ability of the current work to detect changes in sole soft tissue thickness with back fat thickness of the magnitude described by Bicalho *et al.* (2009). From these results, however, the digital cushion seems to be about more than just the fat content. This is explored throughout the remainder of this chapter and the results provide some compelling information.

5.5.3 Nadir of sole soft tissue thickness

A profound finding of the current study was that the nadir of sole soft tissue thickness occurred at AP+1, 4-10 days post-calving. This could be a result of periparturient effects of the hormone relaxin: Tarlton *et al.* (2002) found the suspensory apparatus of the distal phalanx to have greater laxity around calving, in a study that using timed slaughter to make comparisons between maiden and in-calf heifers. The suspensory apparatus of heifers slaughtered around calving distended more than in maiden heifers when the same tension was applied. The authors suggested that relaxin, which mediates distension of the reproductive tract for parturition by activating metalloproteinases that degrade collagen, has the same effect on structures throughout the body such as the suspensory apparatus of the hoof. This global effect of relaxin on other collagen structures is recognised in other mammals too (Samuel *et al.*, 1998). This could cause the distal phalanx to sit lower in the hoof capsule around calving and be observed as thinner at AP+1.

Intriguingly, this mechanism would not explain why the corium thickness at the toe (Site 1) was thinnest at AP+9 (at 63 DIM). Aspects of the suspensory apparatus are located directly above the toe (Mulling and Budras, 2003), and if relaxin had caused laxity in the suspensory apparatus, the corium at Site 1 ought to be thinner immediately after calving, too. One possible explanation for this discrepancy is presented by Dietz and Heyden (1990), who describe a crossover point at the cranial aspect of the digital cushion in front of which the distal phalanx does not press down towards the sole. Still, with laxity of the suspensory apparatus, the distal tip of the distal phalanx would still be expected to sit lower in the hoof and the corium at this site ought to be thinner; this remains an interesting paradox.

5.5.4 Days in milk and sole soft tissue thickness

DIM was significant in the final model but excluded as it was correlated with back fat thickness. This effect of DIM could simply be another descriptor of back fat thickness or it could represent a "stage of lactation" effect that has not been directly measured, such as hormonal changes throughout lactation; the previous section already suggests that there could be hormonal influences on the sole soft tissue thickness at calving, and there could additionally be an effect of hormones that vary through lactation. In their cross-sectional study, the data of sole soft tissue thickness presented by Bicalho et al. (2009) had a similar shape when plotted against DIM (except they did not find the nadir to be near calving, but at 90-120 DIM). These authors did not include a term of stage of lactation in their statistical models and found sole soft tissue thickness to be different in cows with different body conditions. Inspecting the data of Bicalho et al. (2009) that demonstrates how sole soft tissue thickness changed throughout lactation, a polynomial term of DIM might have also correlated with body condition score and made the association between body condition score and sole soft tissue thickness difficult to decipher.

5.5.5 Back fat thickness and sole soft tissue thickness

The differences in sole soft tissue thickness with back fat thickness were significant in this work, although were not of the magnitude reported in previous work (Bicalho *et al.*, 2009). Previous studies have used body condition score as a descriptor of fatness, but in the current study body condition score did not appear to be correlated with sole soft tissue thickness (Figure 5.3). However, body condition score and back fat thickness were correlated and back fat thickness was correlated with sole soft tissue thickness; back fat thickness appears to have been a more sensitive measure of fatness. To aid comparison between this and other work, a 1 unit change in body condition score corresponded with approximately 10 mm difference in back fat thickness between BCS values of 2.5 and 4.5 (Figure 5.3), in accordance with previous works (Schroder and Staufenbiel, 2006). Bicalho *et al.* (2009) reported that a 10 mm difference in back fat thickness equated to a 1 mm difference in sole soft tissue thickness. These differences were far greater than differences observed in the current study, which observed a 0.13 mm difference in sole soft tissue thickness with a 10 mm difference in back fat thickness.

Epidemiological studies found that body condition scores of ≤ 2.25 were associated with the greatest risk of lameness (Green *et al.*, 2014; Lim *et al.*, 2015; Randall *et al.*, 2015). This equates to ≤ 7 mm of back fat thickness. Since the back fat measurement incorporates approximately 5 to 6 mm of skin in the gluteal region (Schroder and Staufenbiel, 2006), cows have virtually no subcutaneous fat at these body condition scores. Regional adiposity is an increasingly studied topic in human biology; it is predominantly of interest for its implications in metabolic dysfunction with obesity and cardiovascular risk. However, the literature describes some important concepts that might extrapolate into bovine physiology too. Different fat-storing tissues within

a body have very different metabolic roles and abilities to mobilise fats (Tchkonia *et al.*, 2013). Even within a tissue type, for example subcutaneous adipose tissue, fat can be mobilized preferentially from different regions, sometimes to conserve fats with structural roles. Examples of the mechanisms that regulate fat storage and mobilsation at the tissue level are concentrations of hormone-sensitive lipase on the membrane of cells (Sztalryd and Kraemer, 1994) and rate of basal lipolysis (Lass *et al.*, 2011). Regional adiposity and rates of lipolysis vary between genetic sub-groups within the human population (Kohli and Lear, 2013), and possibly do with genetic differences in cows, too. In the cow, this could mean that the fat of the digital cushion is preferentially reserved during negative energy balance, and whilst sole soft tissue thickness might incorporate subcutaneous fat, it might not be mobilized at the same rate and under the same conditions (e.g. body fat levels) as subcutaneous fat.

Differences in regional adiposity, demonstrated in Man between genetic subgroups, could also be present in different genetic groups of cows. In the current study, a second order interaction between lesion occurrence, back fat thickness and lactation number approached significance ($P = 0.08$, data not shown), when sole soft tissue data at the time of a sole ulcer was excluded as it was abnormally thickened. This suggested that the association between back fat thickness and sole soft tissue thickness was different between cows that developed lesions and those that did not, and in cows that developed lesions, the association differed between lactations. Perhaps some cows can mobilise fat from the digital cushion at a greater rate than other cows, which is controlled at the genome level and occurs at the detriment of their own health. After all, cows have been bred for high production and the highest producing cows have a great capacity to mobilise body fat in early lactation. In these cows, mobilization of fat from the digital cushion could predispose lesion formation. Clearly, this points to complex physiological interactions that the current study neither confirms nor explains, but suggests that there is much to be explored surrounding differences in the physiologic capacity of cows to mobilise fats from different tissues, and the possible consequences of this to their own health.

The digital cushion appears to have a functional component with a different fatty acid composition to subcutaneous fat elsewhere in the body (Räber *et al.*, 2006) and might be a region of fat that is preferentially retained during negative energy balance. Perhaps, fat is conserved in the digital cushion during negative energy balance until the cow drops below a particular body condition or reaches a particular physiological state, beyond which fat is mobilized from the digital cushion. These thresholds could vary between cows, but could be once most subcutaneous fat has been mobilized (approximately 7 mm of back fat thickness or BCS 2.25). Further, when fat is lost from the digital cushion, a lesion may develop which makes the digital cushion appear thickened, which could have obscured associations between back fat thickness and sole soft tissue thickness in this study.

It is important to note that thickness of the sole soft tissue was assumed to be an indicator of white adipose tissue in the digital cushion, an assumption that was based on previous work by other researchers (Bicalho *et al.*, 2009). To the author's knowledge, this ultrasonographical method as an indicator of fat content has not been validated, although it has been validated as a method for assessing thickness of the sole soft tissue beneath the sole of the foot (Kofler *et al.*, 1999; Bicalho *et al.*, 2009; Cecen *et al.*, 2015). Further, it has not been shown whether a cow does mobilise fat from the digital cushion during negative energy balance. A first step to further explore whether cows can mobilise fat from the digital cushion is presented in Appendix 1, where stereological techniques were used to assess the morphology of adipose tissue in cows culled at a variety of body condition scores (study subjects were 16 of the SRUC cows studied in Chapters 2 and 3). Provisionally, fatter cows appeared to have fatter cells (Appendix Figure 1), which from a cross-sectional perspective might suggest that cows mobilise fat from the digital cushion during negative energy balance. In theory, this could lead to thinning of the sole soft tissue during negative energy balance, although the work by no means presents proof.

5.5.6 Corium thickness and sole soft tissue thickness

There was a strong association between back fat thickness and corium thickness at Site 1, beneath the toe. However, the digital cushion does not extend to the toe (Räber *et al.*, 2004), and it is unclear why there is such an association. This could be an effect of wider hormonal influences rather than an effect of back fat thickness, in a manner similar to the correlation between DIM and back fat thickness.

Sole soft tissue thickness appeared to correlate with back fat thickness in both final models when corium thickness at Site 1 was thin. The corium at Site 1 was thinnest at AP+9 and AP+17, it increased slightly with increasing lactation number and was thinner in cows that experienced lesions. The model did not fully assess all possible interactions as power was limited. It could be that back fat thickness and sole soft tissue thickness was only associated with back fat thickness at AP+9 and AP+17, or the differences in associations could be a genetic difference that was not captured in this study. Further, the thickened corium thickness at the toe could indicate inflammation or venous congestion; with a lesion, blood flow to the foot could increase, and inflammation could compromise drainage from the whole foot, causing tissues to become thickened. This idea is explored further in the next section (5.5.7.1).

5.5.7 Lesion incidence and sole soft tissue thickness

5.5.7.1 Sole ulcers and severe sole haemorrhage lesions

A novel finding of this work was that whilst sole soft tissue thickness was thinner before either a sole ulcer or a severe sole haemorrhage occurred, it appeared to be thicker when a sole ulcer was present on a claw. This thickening when a sole ulcer

was present might have been due to inflammation in the underlying tissues. Sole soft tissues, however, were not thicker when a sole haemorrhage was present. This could suggest that sole haemorrhage lesions were not associated with inflammation that the ultrasonographic method could detect. Or, it could be because sole haemorrhage lesions represent historic disruption within the soft tissues of the sole and a lesion on the surface of the claw horn has taken time to grow out; by the time the lesion was observed, pathology in the underlying tissues had resolved. Further, sole lesions occurred more in thinner cows, and sole soft tissue thickness in cows with back fat thickness < 6 mm was thicker than predicted by the regression line, even when a lesion was not present on the surface of a claw. This unexplained thickness could have been due to a haemorrhage lesion that was present within the claw, but not present on the surface. This possible underlying pathology could be explored further, perhaps using infrared thermography (Oikonomou *et al.*, 2014b).

The interaction between back fat thickness and sole ulcer presence (Figure 5.7) demonstrate that the sole soft tissues were particularly thickened when a sole ulcer occurred and the cow was thin. This also coincided with AP+9 and AP+17, although the digital cushion was still thickened when a sole ulcer occurred at other assessment points.

5.5.7.2 Repeated lameness

The finding that the sole soft tissues are thickened during an active sole ulcer could tie in with work discussed in Chapter 2. Once a cow has become lame she is more likely to go lame in the future (Hirst *et al.*, 2002; Green *et al.*, 2014) and it is plausible that prolonged inflammation around the sole ulcer site elicits changes in and bone modelling on the flexor tuberosity of the distal phalanx (2.6). This abnormal bone modelling could then predispose future lameness by causing greater peak forces on the germinal epithelium of the sole. Thomas *et al.* (2015a) demonstrated that NSAIDs aided recovery as part of a combination of treatments for claw horn disruption lesions, and perhaps resolving inflammation at the sole ulcer site earlier could reduce the degeneration of structures which further predisposes lameness.

5.5.7.3 White line lesions

No significant associations were found between white line lesion incidence and sole soft tissue thickness. Whilst the lesion scoring system used recorded even small lesions, the number of severe white line lesions observed was small and for this reason the study could not suitably assess variables associated with white line lesions. The peak incidence of both sole lesions and white line lesions was at the same assessment point (Figures 5.4 and 5.5), yet they were not associated with thin (or thickened) digital cushion. A measure of sole soft tissue thickness near the white line, rather than in the midline of the sole, might provide more appropriate data on the thickness of the sole soft tissue as related to white line lesion incidence (if there

is an association). After all, this study found links between sole soft tissue thickness and sole lesions, when assessing the sole soft tissue in the region at which sole lesions were observed.

5.5.7.4 Recovery of the digital cushion

This study found that there was no difference in sole soft tissue thickness after a sole ulcer compared with before the ulcer. Previous work has suggested that changes within the digital cushion during an active lesion are important for its future function, for example Räber *et al.* (2006) found reduced arachidonic acid levels in older cows and suggested that the fatty acid could have been used in inflammatory processes during previous lesions; it is a precursor to inflammatory mediators. This process could contribute to depletion of the digital cushion, which could then be replaced with scar tissue and have poorer shock dissipating capacity. The current work cannot adequately assess how the digital cushion recovered from lesions, for the following reasons. Assuming that lesions in first lactation animals were first lifetime lesions, any thin digital cushion prior to a lesion in parity ≥2 animals could be due to lesions in previous lactations. Only 5 heifers developed sole ulcers and this analysis lacked the power to assess whether the digital cushion in heifers recovered after ulceration. Work assessing the recovery of the digital cushion would need to study the animal before and throughout first lifetime lesions, to ensure a "normal" digital cushion was being studied before the lesion.

Contradictory to these results, post mortem studies have found that the digital cushion was thinner in cows culled with ulcers (Lischer *et al.*, 2002; Tsuka *et al.*, 2012). In such studies, exudation post-mortem could have led to the digital cushions being observed as thinner in cows with ulcers present, and the thinness was an artefact. Or, perhaps the horn remained ulcerated while the underlying tissue healed and scarred, and left a thinner digital cushion. Again, this suggests that the process by which sole ulcers heal, and whether the surrounding soft tissues can regain their original structure and function, is not fully understood.

5.5.7.5 Digital dermatitis

When an M2 digital dermatitis lesion was present, the digital cushion was thinner, and back fat thickness was negatively correlated with sole soft tissue thickness. This is difficult to explain. Perhaps, cows that develop digital dermatitis resemble a sub-population that has not been captured in this analysis, for example a group of heifers, or animals that spend longer standing; by some means this could have been associated with having a thinner digital cushion at an assessment point. It could resemble genetic differences in cows that are susceptible to DD. The negative association between back fat thickness and sole soft tissue thickness when a lesion was present could be that a lesion occurred and the cow lost condition as a result of being able to cope poorly in the herd. This theory would assume that sole soft tissue

thickness remained thickened as the cow lost condition, which could be a result of increased vascular supply to the foot. This association remains difficult to explain.

5.5.8 Techniques

5.5.8.1 Back fat thickness versus body condition score

The assessment of body fat primarily reported is back fat thickness; analysis using body condition score categories was unyielding. The digital cushion is the subcutis layer of integument, and fat in it is subcutaneous fat. Ultrasonographic measurements of back fat thickness correlate well with body condition in this and previous reports (Schroder and Staufenbiel, 2006). Other authors report that differences in muscle mass at different stages of lactation can lead to inconsistent body condition score categorization, particularly by over-estimating condition during early lactation and under-estimating condition during the dry period (Schroder and Staufenbiel, 2006). These stages of lactation were of key interest to the current study and back fat thickness was a more appropriate choice than body condition score. Further, back fat thickness is an objective measurement and does not have the poor repeatability associated with body condition scoring (Kristensen *et al.*, 2006).

5.5.8.2 Ultrasonography of sole soft tissue thickness

Ultrasonography of the sole soft tissue has been validated for measuring the thickness of the soft tissues of the sole of the foot (Kofler *et al.*, 1999; Bicalho *et al.*, 2009; Cecen *et al.*, 2015). In the current study, it was not used as a measure of fat content of the digital cushion in the way that ultrasonography was used as a measure of subcutaneous fat in the gluteal region. Previous work had suggested that the ultrasonographic measurement of the digital cushion might be an indicator of fat content (Bicalho *et al.*, 2009), but the current study in part highlights the limitations of the technique as an indicator of fat content; many other variables affected sole soft tissue thickness. Future validation work could look at how to use ultrasonography to measure fat content of the digital cushion, either by testing different ultrasonography sites, or by using other methods to quantify fat content of the digital cushion, such as standing magnetic resonance imaging or x-ray scanning (e.g. DEXA or computed tomography scanning).

5.5.8.3 The use of blinded observers

Throughout this study, observers were blinded to other results wherever possible, as described in several sections of Chapter 4. As such, the observer measuring the digital cushion (from images) was blind to which cow the images were taken from, at which assessment point they were taken, the body condition of the cow and lesions present on the claw. The observer assessing lesions was blind in a similar manner, and the mobility scorer was blind to sole soft tissue thickness (although could not be

blinded from body condition score). This reduced the effects of bias and enabled the assumption that any error in measurement of sole soft tissue thickness or lesion incidence was random.

5.5.8.4 Statistical analysis

The statistical techniques enabled the assessment of correlations between back fat thickness and sole soft tissue thickness of a claw at different assessment points. This was accounting for differences in sole soft tissue thickness that occurred with other variables such as lesion presence, and a random effect allowed cows to have a different intercepts. Mixed effects statistical models were designed specifically for datasets that contain repeated measures (Rasbash *et al.,* 2012). A key question that remains from this analysis is how back fat thickness and sole soft tissue thickness predispose a cow to lesions and lameness, and these questions are the focus of Chapter 6.

5.5.9 Remaining variance

The model left approximately 61 % of the variability in sole soft tissue thickness unexplained (Table 5.2: "Random part"). Variance at the bottom level (repeated measure of sole soft tissue thickness within claw-assessment point) accounted for 10 % of the total remaining variance; there was on average a 0.24 mm difference in measurement thickness between repeated scans of the same site at the same assessment point. This incorporates intra-observer variability both when ultrasound scanning and when measuring sole soft tissue thickness from the ultrasonograms.

Of the unexplained variance, 28 % remained at the cow level. This could, for example, resemble genetic differences or differences with parity that were not significant (lactation number was significant when models had the outcome sole soft tissue thickness at Site 2 and explained much of the cow-level variance). Little variance remained at the claw (within cow) level once the fixed effect of lateral (versus medial) was included in the model. 48% of the remaining variance was at the claw-assessment point level; this indicates that the sole soft tissue varied greatly between assessment points and variables were not found to explain this variation. A factor that could randomly affect sole soft tissue thickness at any assessment point could be local oedema, interstitial fluid or peripheral vasodilation, which could accumulate during lying or with a higher environmental temperature. If a cow had recently been lying or if it was a warmer day, the sole soft tissue might be thickened due to increased interstitial fluid, and such possible explanatory variables were not recorded. Other hormonal influences that have not been captured could also be playing a role. Remaining variance in the final models was normally distributed and model fit was good.

5.5.10 Limitations and future work

This was a prospective cohort study, and no intervention was made. The study can therefore not inform us of the benefits of managing a variable, such as back fat thickness, in controlling the outcome or lameness. Additionally, it was not possible to determine whether changes in sole soft tissue thickness were a result of changes in back fat thickness, by DIM or other variables that were not assessed. Intervention studies could better assess this, and the current work does point usefully to variables that would need to be considered whilst undertaking such a study. Key areas that this work highlights are associations between lesions, sole soft tissue thickness and local inflammation, as well as that sole soft tissues appear to be thinner shortly after calving.

The digital cushion was measured non-weight bearing, and this might not resemble its functional capacity. Further, it seems likely that this study measured sole soft tissue thickness at a different location to that described by Bicalho *et al.* (2009) and this work found associations between back fat thickness and sole soft tissue thickness to be of very different magnitude to those reported by Bicalho *et al.* (2009).

5.6 Conclusion

This work suggests that sole soft tissue thickness does vary with back fat thickness during lactation, although the association depends on interactions with many other variables. The digital cushion was thickened when sole ulcers were present, which predominantly occurred in thin cows, and could have been an indication of inflammation in the tissue. This work highlights the complexity of the associations between measures of body fat, sole soft tissue thickness and lesions.

6 Logistic Regression Modelling of Lameness and Lesion Incidence

6.1 Introduction

Several reports have found that thin sole soft tissues increased the likelihood of a cow becoming lame from claw horn disruption lesions, either during the current lactation if the sole soft tissue were measured during the current lactation (Bicalho *et al.*, 2009; Toholj *et al.*, 2013), or during the subsequent lactation if measured at drying off (Machado *et al.*, 2011). Results in Chapter 5 found that cows that developed sole ulcers or severe sole haemorrhages had thinner sole soft tissue thickness, except when a sole ulcer was present on a claw; at the assessment point at which a sole ulcer was present, the sole soft tissues were thicker.

Loss of body fat has been shown to precede the onset of lameness, measured by both visual detection (Lim *et al.*, 2015; Randall *et al.*, 2015) and lesion treatment (Green *et al.*, 2014). A key hypothesis surrounding this association is that fat is lost from the digital cushion during negative energy balance, causing the digital cushion to become thinner. As a result, forces during foot strike are inadequately dissipated, causing contusions within the germinal epithelium of the sole, haemorrhage, disruption of cell proliferation and eventually ulceration. The analysis in Chapter 5 demonstrated that back fat thickness was associated with sole soft tissue thickness with a small effect size (Table 5.2); cows underwent considerable body condition loss and whilst sole soft tissue thickness was correlated with body condition, the associated changes in sole soft tissue thickness were small. Many other variables had an impact on back fat thickness, and the nadir of sole soft tissue thickness was immediately after calving, before cows had lost considerable body condition. It appears that whilst low body fat might contribute to having a thin sole soft tissue, it is only one of many variables that are associated with sole soft tissue thickness, and it is clear whether primarily back fat thickness or sole soft tissue thickness are important in the onset of lesions or lameness. Further, no work has demonstrated whether *change* in thickness of the sole soft tissues leads to lameness; only minimum thickness at a single assessment point has been shown to predispose lameness or lesion incidence (Bicalho *et al.*, 2009; Machado *et al.*, 2011; Toholj *et al.*, 2013).

Another factor that appears to contribute to the thickness of the sole soft tissues is prior lesion incidence: the digital cushion appears to be thinner in claws that have displayed claw horn disruption lesions (Lischer *et al.*, 2002; Munk and Capion, 2013), and authors have suggested that inflammation in the region of the digital cushion utilizes fats from it and causes the formation of scar tissue, leaving a thinner digital cushion with poorer force dissipating capacity (Lischer *et al.*, 2002; Räber *et al.*, 2006). Lameness has been shown to precipitate further lameness, especially if

lameness was associated with claw horn disruption lesions (Hirst *et al.*, 2002); this association could be a result of scarring of the digital cushion with previous lameness. As such, thin sole soft tissue that reportedly predisposed lameness in previous work (e.g. Bicalho *et al.*, 2009; Machado *et al.*, 2011; Toholj *et al.*, 2013) might have been a result of prior lameness rather than low body condition. Further, low body condition that was associated with sole soft tissue thickness and increased risk of lameness (Machado *et al.*, 2011) could also have been a result of prior lameness. To address this, Randall *et al.* (2015) assumed that first lameness after first calving was first lifetime lameness and demonstrated that in cows that went lame for the first time in second lactation, body condition loss preceded lameness (although the association was not true in heifers). Whilst it is difficult to assess whether the assumption is safe, it could be a useful assumption in investigating first lifetime lameness.

The dataset constructed in Chapter 4 (and used for the analysis in Chapter 5) contains repeated measures of sole soft tissue thickness (i.e. a combined measure of digital cushion and corium thickness beneath the flexor tuberosity of the distal phalanx), measures of body fat, lesion incidence data and fortnightly mobility scores from calving until 35 weeks in milk. The dataset could be used to assess the effects of sole soft tissue thickness and body fat measures on future lesion and lameness incidence.

6.1.1 Aims and Objectives

The aim of the analysis in this chapter was to determine how sole soft tissue thickness affected the likelihood of lesions and lameness later in the study period. Specifically, it sought to determine whether *change* in sole soft tissue thickness, *minimum previous* sole soft tissue thickness, or sole soft tissue thickness *at the previous assessment point* predisposed lesions or lameness. Using the dataset compiled in Chapter 4, the objectives were to define "lameness" and "lesions" as functions of data available, and to test each as the outcome in statistical models that used prior data as predictors. Such predictors would include measures of body fat, sole soft tissue thickness, and cow-level explanatory variables.

6.1.2 Null Hypothesis

Sole soft tissue thickness does not influence the incidence of lameness or lesions later in lactation.

6.2 Materials and Methods

6.2.1 Study design

Chapter 4 describes the materials, methods and validation of a prospective cohort study where 179 cows were enrolled and the sole soft tissue thickness on each hind claw was repeatedly assessed throughout lactation. There were 5 assessment points, at 8 weeks prior to calving (AP–8) and at 1, 9, 17 and 29 weeks post-calving (AP+1, AP+9, AP+17 and AP+29), for 70, 45, 42 and 28 cows in lactations 1, 2, 3 and 4 respectively.

Back fat thickness and sole soft tissue thickness were measured at each assessment point. Back fat thickness was measured ultrasonographically over the gluteal muscles (4.3.1.2), and sole soft tissue thickness at three sites beneath the distal phalanx. In Chapter 5, the sole soft tissue at Site 3 (beneath the flexor tuberosity, Figure 4.2) had been the primary outcome used for analysis and had the greatest associations with lesions present on the claw at the time of assessment. Sole soft tissue thickness at Site 3 was therefore used in the analysis during this chapter.

Digital photographs were taken of each claw at each assessment point. Image order was then randomised and presented to a single blinded observer to assess lesions (4.3.2.2). Different severities of lesions were recorded, as described in Table 4.3.

In addition to data collected at each assessment point, all cows had been mobility scored fortnightly by a blind observer between calving and 35 weeks of lactation. The mobility scoring system was based on a 0 to 3 scale, outlined in Table 4.4 and lame cows were identified using this mobility scoring system. Briefly, scores 0 or 1 described cows with normal or imperfect gait, where no obvious lameness could be detected. Scores 2 and 3 were assigned when a cow appeared lame on a leg, and were sub-classified into “a” and “b” to denote increasing severities within either category, as described by Thomas *et al.* (2015a). When a score 2 or 3 was assigned, the lame leg was identified, and both hind legs could be scored as lame at a single mobility score (e.g. 2A on the left hind *and* 2B on the right hind). As such, mobility scoring data were specific to each hind leg studied. In the dataset, there were 48 recordings of mobility score ≥2A on the forelimbs, and all mobility scores from that cow at that scoring were excluded from the analysis.

6.2.2 Introduction to statistical methods

Statistical models were constructed with binary outcomes being either (1) lesion versus no lesion on a claw at an assessment point or (2) lame versus not lame on a leg at a mobility score. Models were constructed testing different definitions of “lesion” or “lame”; “lesion” described a lesion type and severity on a claw at an assessment point, whilst “lame” referred to the severity of leg mobility scores at one or multiple consecutive fortnightly mobility assessments. Models were constructed

as multilevel logistic regression models in MLwiN (Rasbash *et al.*, 2012), which equate to Cox proportional hazards models, and when including a time element can be termed survival analyses or frailty models (Cox and Oakes, 1984; Goldstein, 2003; Yang and Goldstein, 2003). The outcome consisted of repeated measurements of each claw or limb: lesions were assessed on a claw at repeated assessment points, and lameness was assessed at repeated mobility scores.

6.2.3 Introduction to survival analysis

Survival analysis is an area of statistics that concerns the amount of time until an event, such as death, or in the context of this thesis, lameness or a lesion occurring. Some types of survival analyses calculate the duration to an event. Other types test time of an event as an explanatory variable such that the model reports whether the outcome is more or less likely to occur at a particular time point. Other explanatory variables can then be tested in addition to the effect of time. A typical model that can be used for survival analysis is the Cox proportional hazards model (Cox and Oakes, 1984), denoted as:

$$h_1(t) = h_0(t)\exp(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots)$$

where $h_1(t)$ is the hazard function (i.e. the probability of an outcome occurring, such as being lame, compared with the survival function of being sound), $h_0(t)$ is the baseline hazard (i.e. the probability of an outcome occurring when all other constants are 0; in essence this is the intercept), X_1 and X_2 are fixed effects variables and β_1 and β_2 are coefficients of the fixed effects variables.

In the Cox proportional hazards model, "time" of the outcome can be tested as an explanatory variable. Time can be tested as categorical variables (such as 0-1 or 2-3 months post calving), therefore the coefficients associated with each time category show how likely a positive outcome is to occur within the time period. The Cox proportional hazards model hence becomes a survival analysis, as each time period has a corresponding probability of the outcome occurring. Other explanatory variables can be tested in the same model, and demonstrate the hazard of the outcome occurring *in addition to* the effect of time. Therefore, positive coefficients show that the outcome is more likely to occur than predicted by the time category alone, and negative coefficients show it is less likely to occur.

Alternative to testing time with categorical variables in the Cox proportional hazards model, time can also be modelled as a continuous variable with polynomial functions. This would allow, for example, the hazard associated with time to take the shape of a curve, if appropriate. Again, the effect size of other explanatory variables (such as changes in body condition prior to an assessment) would relate to the hazard of the outcome occurring *in addition to* the effect of time.

Cox proportional hazards models can be constructed in MLwiN in a multilevel format, with unit (e.g. claw) having repeated measures. A claw remains in the model until the outcome occurs, or if the outcome does not occur, it is censored. "Time" is associated with each assessment, therefore testing time of the assessment as a covariate gives the probability of an outcome occurring at a time point. Once the outcome occurs, the claw can be excluded from the model (or retained in, with a dummy variable to denote repeated outcome; as explained in 6.2.4). Other variables can then be introduced to determine whether they also have an effect on the probability of the outcome occurring. These survival models equate exactly to Cox proportional hazards models (Cox and Oakes, 1984; Goldstein, 2003), the full proof of which is presented by Yang and Goldstein (2003). These models could also be termed frailty models, and when tested in multilevel frameworks and can be termed multilevel survival analyses.

6.2.4 Survival analyses of the incidence of claw horn disruption lesions

Lesion incidence, assessed at each of five assessment points, was tested as the outcome in two subsets of multilevel survival analyses, as follows: Model 1 had the outcome presence or absence of a lesion at AP+9, AP+17 or AP+29, which enabled data collected at the two previous assessment points to be tested as explanatory variables as all claws at these assessment points had data from two previous assessment points; Model 2 had the outcome presence or absence of a lesion at assessment points after calving (AP+1 to AP+29) and the purpose of this model was to test the effect of data collected at the previous assessment point on lesion incidence. Further, each model was constructed twice, (a) with data from all animals and (b) with data from heifers only.

The lesion models were constructed around separate definitions of the outcome "Lesion". In separate models, the outcome was sole ulcer, severe sole haemorrhage, severe white line haemorrhage or severe white line separation, or combinations of the lesions as follows:

- Sole ulcer or severe sole haemorrhage.
- Severe white line separation or severe white line haemorrhage.
- Sole ulcer or severe sole haemorrhage or severe white line separation or severe white line haemorrhage.

Models were constructed in MLwiN (Rasbash *et al.*, 2012) using iterative generalized least squares algorithms and a forward stepwise procedure. The models had 3 levels and took the format:

$$\text{Lesion}_{ijk} \sim \text{Bernoulli (probability} = \pi_{ijk})$$
$$\text{logit}(\pi_{1ijk}/\pi_{0ijk}) = \alpha + \beta 1X_j + \beta 2X_{ij} + \beta 3X_{ijk} + v_k + u_{jk}$$
$$v_k \sim N(0, \sigma^2_v)$$
$$u_{jk} \sim N(0, \sigma^2_u)$$

where the outcome "$Lesion_{ijk}$" described whether a lesion of a particular type (specified above) was present at the ith assessment point of the jth claw of the kth cow, π_{1ijk} was the probability of a lesion being present at that assessment point, α was the intercept, $\beta1$, $\beta2$ and $\beta3$ represent vectors of coefficients, $X_j + X_{ij} + X_{ijk}$ represent fixed effects variables at the cow, claw and claw-assessment point levels respectively, and v_k and u_{jk} represent the residual error at the cow and the claw level. Cow level data tested as explanatory variables included lactation, farm, withers' height and milk yield (cumulative milk production during the first 60, 100 and 160 DIM). A claw level categorical variable distinguished between lateral and medial claw and a claw-assessment point level variable denoted whether a claw had previously displayed a lesion (specific to the outcome, i.e. this variable identified repeat lesions). Days in milk (DIM) was tested as the time variable up to the fourth power. Corium thickness at Site 1 at the previous assessment points was tested. Additionally, the following measures of back fat thickness and sole soft tissue thickness at Site 3 were tested separately and together in the models:

- Back fat thickness and sole soft tissue thickness at the previous assessment point.
- Back fat thickness and sole soft tissue thickness at two assessment points previously.
- Minimum previous sole soft tissue thickness at any assessment point.
- Change in back fat thickness and change in sole soft tissue thickness between previous consecutive assessment points.
- Maximum change in back fat thickness and maximum change in sole soft tissue thickness between any previous assessment points, i.e. the difference between the thickest previous and the subsequent thinnest previous measurement.

Initially, once a claw had displayed the outcome lesion in a particular model, the claw remained at risk in the model and the previous occurrence of a lesion was indicated using a dummy variable. However, previous occurrence of a lesion strongly predicted the occurrence of a lesion and the influence of other fixed effects became non-significant. Therefore, models were re-constructed with the outcome "first incidence of a lesion on a claw during the study period" and subsequent records were excluded from the analyses; a claw only remained in the model until the first outcome lesion occurred.

All final models are survival analyses to *first* lesion on a claw, after which the claw was excluded from the analysis. If the outcome lesion had occurred on a claw at assessment points prior to those included in the model (i.e. AP-8 or AP+1 for Model 1, AP-8 for Model 1), the claw was not included in the analysis.

In some final models, a polynomial term of "time" correlated with back fat thickness, and obscured significant associations between back fat thickness and the outcome (in a similar way to that which occurred in Chapter 5). Therefore, in some models, the

fixed effect of time was excluded. Without the effect of time, these models would strictly no longer be survival analyses, but are still proportional hazards models in a multi-level framework. Where final models are survival analyses, they will be called as such. Where final models have the time element excluded, they will simply be called logistic regression models.

6.2.5 Survival analyses of lameness incidence

Multilevel survival models were constructed with the outcome "lameness", as detected by mobility score and recorded at the leg level. Each leg had fortnightly mobility scores between calving and 245 DIM. Two separate models were constructed; Model 3 had the outcome "lame versus not lame at any mobility score between calving and 245 DIM" and Model 4 had the outcome "lame versus not lame for mobility scores within set periods after a mobility score, of 30-90 days, 90-150 days or 30-150 days".

Separate models were constructed, where "Lame" was defined as one of the following descriptions of one or multiple consecutive mobility scores:

- A single mobility score of 2A or greater on a leg.
- A single mobility score of 2B or greater on a leg.
- Two consecutive mobility scores that included two scores of 2A or greater on the same leg.
- Two consecutive mobility scores that included two scores of 2A or greater on the same leg, where one had to be 2B or greater.
- Three consecutive mobility scores that included at least two scores of 2A or greater on the same leg.
- Three consecutive mobility scores that included two scores of 2A or greater on the same leg, where one score was 2B or greater.

Where multiple mobility scores were required to define the outcome as lame, the first mobility score at which a cow was scored mobility 2A or greater was taken as lame. For example, for the last outcome listed above, if 5 consecutive mobility scores were as follows: 1, 1, 2A, 1, 2B, the third mobility score was considered lame.

The models had 3 levels and took the format:

$$Lame_{ijk} \sim \text{Bernoulli (probability} = \pi_{ijk})$$
$$\text{logit}(\pi_{1ijk}/\pi_{0ijk}) = \alpha + \beta 1X_j + \beta 2X_{ij} + \beta 3X_{ijk} + v_k + u_{jk}$$
$$v_k \sim N(0, \sigma^2_v)$$
$$u_{jk} \sim N(0, \sigma^2_u)$$

where the outcome "$Lame_{ijk}$" denoted either a lame or non-lame state (as defined above) at the ith mobility score of the jth leg of the kth cow, π_{1ijk} was the probability a leg being lame at that mobility score, α was the intercept and v_k and u_{jk} were random effects at the cow and the leg levels, respectively. DIM was included up to

the fourth power. Cow level data were tested as explanatory variables, as in the previous logistic regression models that had "Lesion" as the outcome; these included lactation number, farm, Withers' height and claw horn disruption lesion occurrence. Back fat thickness and sole soft tissue thickness from previous assessment points were tested as in the "Lesion" model, although for sole soft tissue measurements, separate explanatory variables were constructed to describe the thickness on the medial and on the lateral claw of the leg, as well as an average measure across both claws.

As with the lesion models, repeat lameness events were initially identified using a dummy variable, then models were reconstructed omitting a leg after the first case of lameness. Models were also repeated excluding cows that had experienced an M2 digital dermatitis lesion at any point during the study. All reported models are survival analyses to first lameness event.

6.2.6 Logistic regression model checking

Terms were considered significant if the 95 % confidence interval around the mean effect did not include the value 1 ($P \leq 0.05$). Data were clustered within cow and within either claw (for the lesion models) or within leg (for the lameness models), therefore two random effects were included in each model that permitted this hierarchical structure (as shown in the model equations). Goodness of fit was assessed at the bottom level using posterior predictions described by Hosmer and Lemeshow (1989), with and without random effects, as follows. The probability of the outcome occurring was predicted from the model. Predicted probabilities were then ranked and summed within each 20^{th} percentile and compared with the observed data; this assessed the ability of the model to predict the observed data across a spectrum of probabilities. Fit was considered to be good if a Pearson's chi-square test found no difference between the actual frequency of the outcome on which the model was based and the predicted frequency of the outcome (i.e. if $P > 0.05$). Predicted probabilities were also cumulated within each lactation group and compared to the observed data in the same manner, in order to assess model fit within each lactation group. Residuals at higher levels were inspected to assess normality. Models were simplified by excluding random effects if model fit remained good.

6.3 Results: Description of the dataset

Descriptive data on sole soft tissue thickness, back fat thickness and lesion incidence were presented in the previous chapter (5.3). As for mobility score data, there were 5,142 mobility scores of the hind legs. Figure 6.1 summarises mobility score data, showing the number of scores of each category in 30-day periods from calving, and the proportion of all scores that each mobility score category constituted. There was a peak in 2B scores at 120-150 DIM and prevalence of 2B scores remained high at subsequent mobility scores. Figure 6.2 presents the incidence of *first* leg lameness for each mobility score 2A, 2B and 3A. Again, a peak of 2B scores occurred at 120-150 DIM, which coincided with the highest incidence of 3A scores.

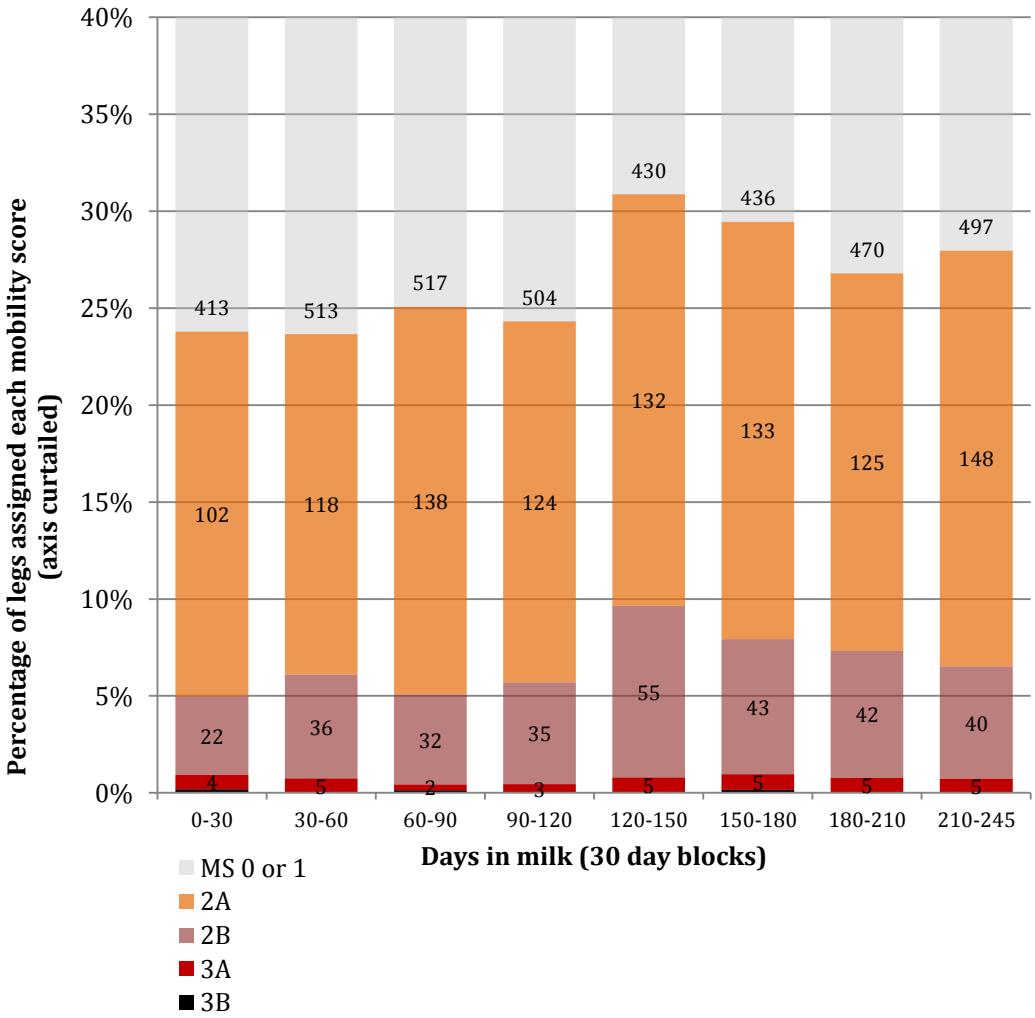

Figure 6.1: Fortnightly hind-leg mobility score data collected between calving and 35 weeks in milk during a longitudinal study of sole soft tissue thickness and lameness. Scores are grouped by 30-day periods between calving and 35 weeks in milk. Mobility scores are at the leg level. Numbers of scores for each category are shown, as well as proportion of all scores that were in each category (scores 0 and 1 are combined).

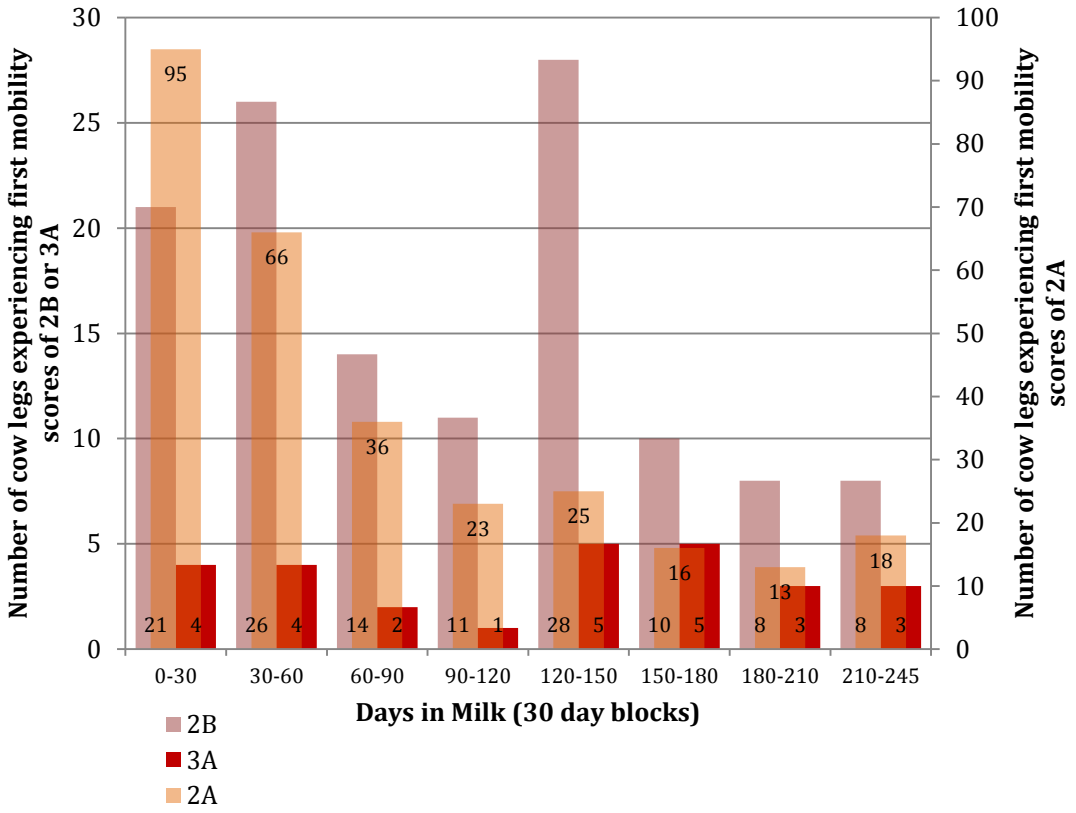

Figure 6.2: Incidence of *first* lameness after calving, in cows on a longitudinal study of sole soft tissue thickness and lameness. Data are at the leg-level and are of hind leg mobility scores only. Mobility scores are grouped into 30 day periods throughout lactation. For each descriptor of mobility score, a leg can only appear once. First mobility scores of 2A, 2B and of 3A or greater on each leg are shown.

6.4 Results: Logistic regression analyses of lesion incidence

6.4.1 Model 1a: logistic regression analysis of lesion incidence at and after AP+9

6.4.1.1 Description of dataset

The outcome for Model 1 was the first presence of either a sole ulcer or a severe sole haemorrhage on a claw at AP+9, AP+17 or AP+29, compared with no lesion being present. Animals of all lactations were included and 1,616 claw assessments of 624 claws of 167 animals met the requirements of the model; 100 claws displayed a sole ulcer or severe sole haemorrhage at an assessment point that was included in the analysis.

6.4.1.2 Results

Model 1 (Table 6.1) demonstrates that either sole ulcers or severe sole haemorrhages were more likely to occur on the lateral claw compared with the medial (OR: 11.4, CI: 6.2-21.0), in lactation 3 or 4 cows (OR: 3.5, CI: 1.8-6.7 and 2.5, 1.2-5.3 respectively), with a thinner sole soft tissue on the claw at the previous assessment point and if back fat had been thinner at the previous assessment point. Further, had a cow had thicker back fat thickness two assessment points previously followed by a thinner back fat thickness at the previous assessment point (i.e. had lost body condition) the odds of a lesion being present were significantly increased.

Table 6.1 shows the odds ratios associated with a 1 mm thicker sole soft tissue thickness at the previous assessment point. Correspondingly, the odds of a lesion being present at an assessment point were higher if sole soft tissue thickness was 1 mm thinner at the previous assessment point (OR: 1.7, 1.3-2.3). Likewise, thin sole soft tissue thickness two assessment points previously increased the odds of a lesion developing (OR: 1.6, 1.2-2.2, model not shown.) Sole soft tissue thickness at the same assessment point as lesion observation did not influence the odds of lesion presence, except when the outcome described only the presence of a sole ulcer; in these models that are not shown, a 1 mm *thicker* sole soft tissue increased the odds of a sole ulcer being present (OR: 2.0, 1.4-2.9); i.e. the sole soft tissues were thicker when a sole ulcer was present. Neither in the current model nor in any models tested did change in thickness of the sole soft tissue between previous assessment points influence the odds of lesion presence at an assessment point; only sole soft tissue thickness prior to an assessment point influenced the odds of a lesion being present.

Table 6.1 shows the odds ratios associated with a 10 mm thicker back fat thickness at either of the two previous assessment points. Correspondingly, thinner back fat thickness at the previous assessment point was associated with higher odds of a lesion being present (OR: 3.4, CI: 1.8-6.7 for a 10 mm thinner back fat thickness). Further, having thicker back fat two assessment points previously increased the odds

of lesion presence, given thin back fat at the previous assessment point; i.e. a reduction in back fat increased the odds of subsequent lesion presence. When back fat thickness two assessment points previously was not included in the model, a thinner back fat thickness at the previous assessment point increased the odds of a lesion being present (OR: 2.2, 1.5-3.1 for a 10 mm difference, model not shown); this demonstrates that back fat thickness at previous assessment points were correlated, but a reduction between assessment points increased the odds of lesion incidence.

Table 6.1: Model 1a: logistic regression analysis of lesion incidence on the hind claws at repeated assessment points, at and after AP+9, from a longitudinal study of sole soft tissue thickness and lesion incidence. Claws remained in the analysis until first lesion was recorded; the outcome was either sole ulcer or severe sole haemorrhage, compared with the lesions being absent. Explanatory variables were back fat thickness (**BFT**) at the two previous assessment points ("Previous AP" and "2 previous AP"), and sole soft tissue thickness (**SST**) of the claw at the previous assessment point. Cow and claw level random effects were tested, but model fit was best when random effects were excluded.

	Model 1a: Lesion versus no lesion at AP+9, AP+17 or AP+29 ("Lesion" = first sole ulcer or severe sole haemorrhage)				
	Coefficient: -1.71				
Variables	Mean (SD)[1]	No. of assessments (lesions)[2]	Odds Ratio	Lower 95% CI	Upper 95% CI
Claw					
Medial		873 (17)	Baseline		
Lateral		743 (83)	11.36	6.15	20.97
Lactation number					
1		697 (37)	1.36	0.72	2.55
2		425 (15)	Baseline		
3		290 (31)	3.48	1.80	6.72
4		204 (17)	2.51	1.20	5.28
BFT, mm[3]					
Previous AP	13.1 (6.0)		0.29	0.15	0.57
2 previous AP	15.8 (6.3)		1.79	1.02	3.14
SST at previous AP, mm[4]	4.8 (0.9)		0.59	0.44	0.80

DIM was tested to the fourth polynomial. DIM was correlated with back fat thickness and when included, the effects of back fat thickness were not significant and DIM was excluded from the final model.

[1]Means and standard deviations of continuous variables.

[2]Number of claw assessments within each category (number of first sole ulcer or severe sole haemorrhage that occurred within each category).

[3]Coefficients shown for a 10 mm difference in back fat thickness.

[4]SST thickness at two assessment points previously was correlated with the term of SST included in the model, and could have been used as an alternative descriptor of SST.

6.4.1.3 Model 1a: other findings and non-significant results

The model found no difference in lesion incidence between farms. No measures of a change in sole soft tissue thickness between previous assessment points had a significant effect on the odds of a lesion; only *absolute* sole soft tissue thickness prior to an assessment point influenced the odds of a lesion being present. Corium thickness at the toe was significant only when neither back fat thickness nor sole soft tissue thickness were in the model (thinner corium thickness at the toe increased the odds of lesions, models not shown); when either were included, corium thickness at the toe was not significant. Stature was not significant in the model. The assessment point at which a prior measurement had been taken (e.g. if the previous sole soft tissue thickness had been taken at AP+1) was tested as a categorical variable and was not significant.

The reported model was a fixed effects model and it fitted the data well. Model fit was poor when random effects were included at the cow or the claw levels, therefore the mixed effects model was not reported. In 6.6, Model 1a is used as an example of how the Hosmer-Lemeshow posterior prediction can be applied to assess fit of logistic regression models.

6.4.1.4 Alternative models to Model 1a

In the presented models, DIM was excluded as an explanatory variable. Including DIM, the effects of back fat shown in Table 6.1 was reduced and became non-significant (P = 0.08). A polynomial term of DIM and back fat thickness were correlated, as demonstrated in the previous chapter (Figure 5.10). Therefore, for the same reason as in the sole soft tissue thickness linear regression model in Chapter 5 (Table 5.2), DIM was excluded from the analysis.

To fully explore the data, a variety of different models were constructed using different descriptors of sole soft tissue thickness. The effect of sole soft tissue thickness collected at specific assessment points was tested, and regardless of the assessment point at which a sole soft tissue thickness was measured, thinner sole soft tissue increased the likelihood of lesions at all future assessment points (models not shown). Further, Model 1a was re-constructed as two separate models, one with first sole ulcer incidence and one with first severe sole haemorrhage incidence; thin back fat and thin sole soft tissue preceding both lesions (models not shown).

The effects of back fat thickness and of sole soft tissue thickness on the medial claw were tested alone (similar to Model 1, model not shown). Both sole soft tissue on the medial claw and back fat were thinner prior to a lesion being present on the medial claw (significant). A decrease in back fat thickness prior to a lesion formation tended to increase the odds of a lesion being present on the medial claw, although not significantly; the number of lesions on the medial claw was small (n = 17). Thus, the associations between each of back fat thickness and sole soft tissue thickness seen in

Model 1a appear to be true on the medial claw, although power was insufficient to fully assess these possible associations.

6.4.2 Model 1b: logistic regression analysis of lesion incidence at and after AP+9, using heifer data only

Model 1b used lesion data from heifers only and is shown in Table 6.2; the outcome was the incidence of first sole ulcer or severe sole haemorrhage in heifers, when either lesion was present at or after AP+9. The dataset consisted of 705 assessments of 258 claws of 66 heifers, and 37 claws developed lesions at either AP+9, AP+17 or AP+29. As with Model 1a, the lateral claw was at higher odds of developing a lesion than the medial and the odds of a lesion were higher at an assessment point if either sole soft tissue or back fat had been thin at the previous assessment point. Farm was significant in this model: heifers were at higher odds of developing a lesion on Farm 2 than on Farm 1. Back fat thickness measured at 2 assessment points previously was not significant, although the coefficient was positive as in Model 1a (not shown).

Table 6.2: Model 1b: logistic regression analysis of first lesion incidence on hind claws of heifers at and after AP+9, from a longitudinal study of sole soft tissue thickness and lesion incidence. The binary outcome was the first presence or absence of either a sole ulcer or severe sole haemorrhage and claws remained in the analysis until the first lesion was recorded on it. Explanatory variables were back fat thickness (**BFT**) at the previous assessment point and sole soft tissue thickness (**SST**) of the claw at the previous assessment point. Cow and claw level random effects were tested, but model fit was best when random effects were excluded.

	Model 1b: Lesion versus no lesion at AP+9, AP+17 or AP+29 ("Lesion" = first sole ulcer or severe sole haemorrhage)				
	Coefficient: 0.014				
Variables	Mean (SD)[1]	No. of assessments (lesions)[2]	Odds Ratio	Lower 95% CI	Upper 95% CI
Claw					
Medial		401 (8)	Baseline		
Lateral		304 (29)	9.94	6.13	26.66
Farm					
1		369 (8)[3]	Baseline		
2		336 (29)	3.60	2.36	8.56
BFT at previous AP, mm[4]	13.6 (5.7)		0.29	0.18	0.73
SST at previous AP, mm[5]	4.7 (0.8)		0.45	0.33	0.86

DIM was tested to the fourth polynomial and made the effect of back fat thickness not significant (p = 0.08). It was excluded from the final model.
On Farms 1 and 2, n = 36 and 30 heifers respectively.
[1]Means and standard deviations of continuous variables.
[2]Number of claw assessments within each category (number of sole ulcer or severe sole haemorrhage that occurred within each category).
[3]All 8 lesions that occurred on the medial claw occurred on Farm 2 (by chance, this is the same number that occurred on the lateral claw on Farm 1; i.e. it is not a number error).
[4]Coefficients shown for a 10 mm difference in back fat thickness.
[5]SST thickness at two assessment points previously was correlated with the term of SST included in the model, and could have been used as an alternative descriptor of SST.

6.4.3 Model 2a: survival analysis of lesion incidence post-calving

6.4.3.1 Description of the dataset

Model 2a had the outcome "presence or absence of first sole ulcer or severe sole haemorrhage, when it occurred after calving" (i.e. AP+1, AP+9, AP+17 and AP+29). Data from 2,255 assessments of 676 claws of 176 cows were available, and 113 claws displayed a lesion at one of the assessment points. The model was similar to Model 1, but included all lesion assessments after calving, where claws had not displayed a lesion at AP-8. Explanatory variables included data from the previous assessment point.

6.4.3.2 Results

The final model is presented in Table 6.3. As in Models 1a and 1b, sole soft tissue thickness at the previous assessment point was significant: a thinner sole soft tissue increased the odds of a lesion being present. Various descriptors of sole soft tissue thickness from previous assessment points were significant, but were correlated and not included in the same models; where this occurred is stated in footnotes in the model tables. In Model 2a, the descriptor of sole soft tissue thickness presented is the *minimum previously recorded* sole soft tissue thickness, categorized as quartiles, within claw (lateral or medial) and within lactation number. This descriptor adjusted for differences in thickness of the sole soft tissue between claws and different lactations. The model estimated that a claw with a minimum previous sole soft tissue thickness within the lowest quartile (within claw and within lactation number) was at much higher odds of having a lesion at an assessment point compared with a claw in the upper quartile (OR: 4.2, CI: 2.0 to 9.0).

A polynomial term of DIM was included in this model (making it a survival analysis) and back fat thickness at previous assessment points had no additional effect on lesion incidence. An additional linear term for sole soft tissue thickness was not significant.

Table 6.3: Model 2a: survival analysis predicting first sole ulcer or severe sole haemorrhage on a claw at any assessment point (AP) post calving, from a longitudinal study of sole soft tissue thickness and lesion incidence. A claw was excluded after a first severe sole haemorrhage was recorded on it. Minimum previous sole soft tissue thickness was categorised as quartiles, within lactation number (1 to 4) and within claw (lateral or medial).

	Model 2a: Lesion versus no lesion after calving ("Lesion" = first sole ulcer or severe sole haemorrhage)			
	Coefficient: -5.9			
Variables	No. of assessments (lesions)[1]	Odds Ratio	Lower 95% CI	Upper 95% CI
Claw				
Medial	1212 (17)	Baseline		
Lateral	1043 (96)	8.14	4.78	13.87
Lactation number				
1	950 (37)	1.11	0.62	1.97
2	570 (20)	Baseline		
3	432 (34)	2.89	1.59	5.25
4	303 (22)	2.50	1.30	4.81
Min. Previous SST[2, 3]				
Quartile 1	562 (50)	4.20	1.97	8.95
Quartile 2	559 (31)	2.67	1.22	5.83
Quartile 3	570 (23)	2.08	0.93	4.64
Quartile 4	564 (9)	Baseline		

DIM was included to the fourth polynomial in the final model. Coefficients not shown.

[1]Number of claw assessments within each category (number of sole ulcer of severe sole haemorrhage that occurred within each category).

[2]Minimum previous sole soft tissue thickness, categorised by quartiles (low to high) within claw and within lactation number.

[3]Sole soft tissue thickness at the previous assessment point was also significant.

6.4.4 Model 2b: logistic regression analysis of lesion incidence post-calving, using heifer data only

6.4.4.1 Description of the dataset and results

Model 2b was based on data from heifers only and is presented in Table 6.4. The dataset contained 946 claw assessments of 262 claws of 66 heifers, and a lesion occurred 37 times. Unlike in Model 2a, Model 2b could not fit the categorized term "minimum previous sole soft tissue thickness" as very few lesions occurred in the upper two quartiles of sole soft tissue thickness. Further, there were large differences between farms and between lateral versus medial claws, with only 37 claws being recorded as displaying a lesion at any assessment point, therefore the model could not compute all categories. Sole soft tissue thickness as a linear term was significant in the model and model fit was good; a 1 mm thinner *minimum previous* sole soft tissue thickness increased the odds of a lesion occurring at any assessment points (OR: 4.2, CI: 2.0 to 5.9). There was an additional effect of back fat thickness: thin back fat at the previous assessment point increased the odds of a lesion being present.

Table 6.4: Model 2b: logistic regression analysis predicting first sole ulcer or severe sole haemorrhage on a claw at any assessment point (AP) post calving in heifers, from a longitudinal study of sole soft tissue thickness and lesion incidence. Claws were excluded after a first severe sole haemorrhage was recorded on that claw.

	Model 2b: Lesion versus no lesion after calving in heifers ("Lesion" = first sole ulcer or severe sole haemorrhage)				
	Coefficient: 2.2				
Variables	Mean (SD)[1]	No. of assessments (lesions)[2]	Odds Ratio	Lower 95% CI	Upper 95% CI
Claw					
Medial		494 (8)	Baseline		
Lateral		452 (29)	16.46	9.89	46.53
Farm					
1		530 (8)	Baseline		
2		416 (29)	3.38	2.24	7.83
BFT at previous AP, mm[3]	15.0 (6.0)		0.24	0.15	0.63
Minimum previous SST, mm	4.6 (0.8)		0.24	0.17	0.51

DIM was tested to the fourth polynomial, which caused back fat thickness to be not significant (p = 0.09) as the two were correlated, and DIM was excluded from the model.

[1]Means and standard deviations of continuous variables.

[2]Number of claw assessments within each category (number of sole ulcer or severe sole haemorrhage that occurred within each category).

[3]Coefficients shown for a 10 mm difference in back fat thickness.

6.4.4.2 An alternative model to Model 2b that included DIM

As in Model 1a and Model 1b, DIM was excluded from Model 2b as it was correlated with measures of back fat thickness. When included, back fat thickness had a coefficient in the same direction as in Model 2b (i.e. negative: thinner back fat thickness increased the odds of a lesion being present) but was not significant (P = 0.09).

6.4.5 Summary of lesion incidence models

Models 1 and 2 demonstrate that having a thin sole soft tissue thickness increased the odds of either a sole ulcer or a severe sole haemorrhage being present on a claw. Different descriptors of sole soft tissue thickness at previous assessment points were correlated and not significant in the same models, therefore different descriptors are shown in Models 1 and 2. Being thin, or even loss of back fat between two previous assessment points, increased the odds of a lesion occurring. When DIM was included in the model, the effects of back fat reduced and became non-significant, as DIM was correlated with the overall shape of back fat thickness. However, there appeared to be an additional effect of back fat thickness. DIM was therefore excluded from Models 1a, 1b and 2b in order to demonstrate the effect of back fat thickness.

Both being thin and loss of back fat predisposed lesion incidence in Model 1a. Being thin or having a thin sole soft tissue thickness preceded lesion formation in heifers. A greater reduction in thickness of the digital cushion did not increase the odds of a lesion occurring in any animals.

The odds of a lesion developing were greater on the lateral claw and in parity 3 and 4 animals. There was no difference in lesion incidence between farms, except in heifers: lesion incidence in heifers on Farm 2 was much higher than on Farm 1. Neither back fat thickness nor sole soft tissue thickness were found to increase the odds of white line lesions occurring and models with white line lesions as the outcome are not displayed.

6.5 Results: Survival analyses of lameness incidence

6.5.1 General description of the dataset for lameness models

The dataset for the models that had outcomes describing lame mobility scores consisted of 5,142 fortnightly leg-mobility scores recorded between calving and 245 DIM from 173 cows, of which 3,780 were non-lame (score 0 or 1), 1,020 were 2A, 305 were 2B, 34 were 3A and 3 were 3B (Figure 6.1). As in the lesion models, repeat cases of the outcome (lameness, in this case) were strongly predicted by previous lame mobility scores. After a leg had been mobility scored lame, subsequent mobility records for that leg were excluded from the dataset and the models presented are survival analyses to the first lameness event. In the reported models, the outcome "lame" was the first leg mobility score >1 within 3 consecutive fortnightly mobility scores that included at least one "2A" and at least one "2B", versus not lame.

Excluding mobility scores that followed a lame event reduced the number of mobility scores available for analysis. Using the stated definition of lameness, 3,946 leg mobility scores of 346 legs of 173 cows were available for analysis (i.e. 1,196 leg-mobility scores had occurred after a lameness case on that leg), and 110 legs were lame at some point between calving and 245 DIM. By lactation, 31, 20, 30 and 29 legs of cows in lactations 1, 2, 3 and 4 became lame respectively.

6.5.2 Model 3: survival analysis of lameness by mobility score

6.5.2.1 Description of the dataset and results

The outcome for Model 3 was lameness at any mobility score between calving and 245 DIM. The dataset consisted of all 3,946 leg mobility scores described above, and 110 legs became lame during the study period. Model 3 contains a random effect at the cow level and is presented in Table 6.5; Model 3 shows that having a 1 mm thinner sole soft tissue *on the lateral claw* at AP-8 (prior to calving) made a leg more likely to be scored lame at any subsequent mobility score (OR: 1.4, 1.1-1.8). The likelihood of lameness also increased with lactation number. The random effect, which improved model fit, suggested that if a cow went lame on one hind leg, the other hind leg was at increased probability of lameness compared to the legs of cows that experience lameness on neither leg.

6.5.2.2 Other results and non-significant findings

Sole soft tissue thickness on the medial claw at AP-8 did not influence the odds of lameness during that lactation. Loss of back fat thickness between AP-8 and AP+1 tended to increase the odds of lameness (P = 0.06, small effect size, model not shown).

Table 6.5: Model 3: survival analysis predicting the occurrence of first lameness on a leg, between calving and 245 DIM (with lameness defined as at least one "2A" and one "2B" in 3 consecutive fortnightly mobility scores), in a longitudinal study of sole soft tissue thickness and lameness incidence. Explanatory variables were lactation number and sole soft tissue thickness on the lateral claw at the first assessment point, approximately 8 weeks prior to calving ("SST-Lat AP-8"). A cow-level random effect was included in the model.

	Model 3: Lame versus not lame at a mobility score				
	Coefficient: -0.65				
Variables	Mean (SD)[1]	No. MS (lame)[2]	Odds Ratio	Lower 95% CI	Upper 95% CI
Fixed part					
SST-Lat AP-8, mm	5.64 (0.84)		0.71	0.55	0.92
Lactation					
1		1779 (31)	Baseline		
2		1051 (20)	1.18	0.62	2.26
3		658 (30)	4.04	2.16	7.57
4		458 (29)	4.36	2.25	8.44
Random part	Variance: 0.79		SE: 0.24		

Days in milk and polynomials up to the power 4 included; coefficients not shown.
[1]Means and standard deviations of continuous variables.
[2]Number of claw assessments within each category (number of sole ulcer or severe sole haemorrhage that occurred within each category)

6.5.3 Model 4: survival analysis of lameness incidence, using data on parity >1 cows only

6.5.3.1 Description of the dataset

The next set of models were constructed around mobility scores that occurred within time periods following an assessment point, to assess the effects of sole soft tissue thickness during lactation on lameness. Model 4 was a fixed effects survival analysis with the outcome "first leg-lameness at a mobility score", where mobility scores were only included when they occurred within the 90-150 days following an assessment point. Data from parity >1 animals only were used as when heifers were included, model fit was poorer. The model consisted of 1,778 lameness assessments of 178 legs of 100 parity >1 animals; 55 legs were scored lame.

6.5.3.2 Results

Model 4 is presented in Table 6.6. Cows in lactations 3 and 4 were more likely to go lame than those in lactation 2. Cows with sole soft tissue thickness *on the lateral claw* of 1 mm thinner during the period 90-150 d prior to a mobility score increased the odds of being lame at that mobility score (OR: 1.7, CI: 1.2 to 2.3). Sole soft tissue thickness on the medial claw did not influence the odds of lameness. Days in milk was retained in the model. For measures of sole soft tissue thickness included in

the model, the assessment point at which the measure was tested and was not significant.

6.5.3.3 Non-significant findings

When the model was reconstructed to include assessments within the 30-90 days after calving, having a thinner sole soft tissue thickness during the 30-90 d prior to a mobility score did not increase the odds of lameness ($p = 0.13$).

There was no additional effect of back fat thickness to the effect of sole soft tissue thickness. However, when sole soft tissue thickness was excluded from the model, a 10 mm thinner back fat thickness at 2 assessment points prior to a mobility score increased the odds of lameness (1.5, 1.1-2.1, model not shown), as did back fat thickness at 3 assessment points prior to a mobility score. Back fat thickness at the previous assessment point did not influence lameness (the corresponding median number of days between the previous assessment point and the mobility score was 35, range: 1 to 119, IQR: 19 to 50).

Table 6.6: Model 4: survival analysis predicting first lameness incidence on a leg of parity >1 cows, between calving and 245 DIM in a longitudinal study of sole soft tissue thickness and lameness incidence. Lameness was defined as at least one "2A" and one "2B" in 3 consecutive fortnightly mobility scores. Legs were included in the model if they had an ultrasonographic measurement of sole soft tissue thickness on the lateral claw during the previous 90-150 days.

	Model 4: Lame vs not lame at a mobility score				
	Coefficient: 4.15				
Variables	Mean (SD)[1]	No. MS (lame)[2]	Odds Ratio	Lower 95% CI	Upper 95% CI
SST-Lat prev90-150d, mm	5.55 (0.86)		0.65	0.47	0.90
Lactation					
2		885 (17)	Baseline		
3		543 (20)	2.17	1.12	4.21
4		350 (18)	2.73	1.38	5.41

DIM to the fourth polynomial was included; coefficients not shown.

[1]Means and standard deviations of continuous variables.

[2]Number of claw assessments within each category (number of sole ulcer or severe sole haemorrhage that occurred within each category)

6.5.4 Survival analysis of lameness incidence in heifers

To assess the effect of prior back fat thickness and sole soft tissue thickness on first lameness in heifers, both Models 3 and 4 were re-constructed using heifer data only. This was assumed to be first-lifetime lameness. The dataset consisted of 1,516 heifer mobility scores, of which 27 were lame. By farm, 878 mobility scores were included from Farm 1, of which 6 were lame, and 638 from Farm 2, of which 21 were lame. Sole soft tissue was thinner in heifers on Farm 2, but the fixed effect of Farm was significant. When farm was not included in the model, thin sole soft tissue during the

30-90 days prior to a mobility score increased the odds of lameness (OR: 0.6, 0.3 to 1.0 for a 1 mm increase in sole soft tissue thickness, P = 0.05, model not shown). There were also differences in back fat thickness between heifers on farms, but back fat thickness had no additional effect on lameness incidence beyond the effect of Farm. Sole soft tissue measurements during the 90-150 or 30-150 days prior to a mobility score did not increase the odds of lameness.

To explore further whether sole soft tissue thickness had an effect on lameness within farm, sole soft tissue thickness were categorized as quartiles within farm, in a similar manner in Model 3 where "minimum previous sole soft tissue thickness" was split into quartiles within lactation group and within claw. The model estimated that within farm, there was no effect of thickness of the sole soft tissue either during the 30-90 days or the 90-150 days prior to a mobility score on lameness in heifers, even between the thickest and the thinnest quartiles of sole soft tissue thickness. Likewise, there was no additional effect of back fat thickness on lameness in heifers, beyond a difference in back fat thickness between farms: mean back fat thickness in heifers was 1.57 cm (SD: 0.68) on Farm 1 and 1.32 (SD: 0.43) on Farm 2.

6.5.5 Lameness models: non-significant findings

Variables that were not significant in the lameness models included stature, incidence of digital dermatitis, sole soft tissue thickness on medial claw and farm. The inclusion of cows that experienced digital dermatitis during the study period did not affect model parameters and these cows were included in the models.

6.5.6 Alternative models

Models were tested where the definition of "lameness" as the outcome was different, for example when a lame event was defined as two consecutive mobility scores >1. Similar associations between sole soft tissue thickness or back fat thickness and lameness were observed; that is, thin sole soft tissue thickness increased the odds of lameness at future mobility scores. This was not true, however, when lameness was defined by a single mobility score, whether that was 2A or any greater score.

6.5.7 Summary of lameness incidence models

In the reported models, the outcome "Lame" was considered to be the first leg mobility score >1 within 3 consecutive fortnightly mobility scores that included at least one "2A" and at least one "2B". Lameness strongly predicted future lameness, therefore the reported models only concern first lameness during the study period. Having a thin sole soft tissue on the lateral claw predicted future lameness in parity >1 animals. There was no *additional* effect of back fat thickness, although when sole soft tissue thickness was not accounted for in Model 3, thin back fat thickness at calving was associated with higher odds of future lameness.

In heifers, lameness incidence was higher on Farm 2 (although this difference was not apparent beyond the heifer group). Sole soft tissue thickness of heifers on Farm 2 was also thinner. The model estimated that the difference in lameness incidence was best explained by a farm effect, and within farm, having a thin sole soft tissue did not appear to predispose lameness in heifers.

6.6 Results: Fixed effects versus mixed effects models

Some of the reported models are fixed effects models. Random effects were tested in all models: cow and claw levels for the lesion models, or cow and leg levels for the lameness models. In some models, Hosmer-Lemeshow posterior predictions showed that the models only predicted the observed data adequately when random effects were not included, therefore when random effects resulted in poorer model fit, they were omitted.

As an example of a model in which a cow-level random effect made model fit poor, posterior predictions based on a fixed effects model (Model 1a) and based on an alternative mixed effects model (Model 1a with the inclusion of a cow-level random effect), are shown in Table 6.7. Both are compared with the observed data. Table 6.7 shows that the model without a cow-level random effect predicted the observed data (predicted versus observed was not different, chi-squared test, P = 0.11) whilst the model with the random effect did not predict the observed data (P <0.001).

Table 6.7: Hosmer-Lemeshow posterior predictions for Model 1a and an alternative model that included a cow-level random effect. The outcome for both models was lesion on a claw at an assessment point and explanatory variables were lactation number and sole soft tissue thickness. The two models were based on the same data and the only difference was the inclusion or exclusion of cow as a random effect.

Model:	Model 1a Fixed effects model		Alternative to Model 1a, with a cow-level random effect	
20th Percentile	Expected outcome	Predicted outcome	Expected outcome	Predicted outcome
1	4	2	0	2
2	7	5	1	5
3	6	11	4	11
4	19	24	11	23
5	64	58	84	70
Total	*100*	*100*	*100*	*111*

Chi-squared tests compared the predicted outcome to the expected outcome (i.e. the data on which the model was based); the null hypothesis was that the distributions were the same, $\alpha = 0.05$.
Model 1a: P = 0.11; model fit was good
Alternative model: P < 0.001; the model prediction did not fit the data on which the model was based, i.e. model fit was poor.

6.7 Discussion

6.7.1 Summary of model results

The analysis presented in this chapter used data from a prospective cohort study to determine the effects of back fat thickness and of sole soft tissue thickness on the incidence of claw horn disruption lesions and lameness. Addressing the null hypothesis (6.1.2), *change* in sole soft tissue thickness did not influence the incidence of lameness or lesions, but thin sole soft tissue at a single assessment point *did* increase the odds of subsequent lesion and lameness incidence.

6.7.2 Lesion incidence

Models 1 and 2 demonstrate that sole soft tissue thickness on a claw was thinner before either a sole ulcer or a severe sole haemorrhage. This applied for various descriptors of sole soft tissue thickness: thickness at the previous assessment point and the minimum previous sole soft tissue thickness. Also, thin sole soft tissue at any particular assessment point increased the odds of lesion incidence at any future assessment point (these models were variations on Models 1 and 2, not shown).

6.7.2.1 The effect of sole soft tissue thickness on lesion incidence

These results concur with previous work that reported thin sole soft tissue thickness precedes incidence of sole ulcers (Machado *et al.*, 2011; Toholj *et al.*, 2013). The outcome used by Machado *et al.* (2011) also included incidence of white line disease; their outcome consisted of 114 incidences of sole ulcer and 26 of white line disease. From the description of their analysis, it is not clear whether thin sole soft tissue thickness predicted incidence of white line disease alone, or whether it only truly predicted sole ulcer incidence, with white line disease included in the same lesion category. In the current analysis, thin sole soft tissue thickness did *not* predict incidence of severe white line lesions as defined in Table 4.3 (i.e. separation or haemorrhage lesions). However, the models only predicted severe sole lesions (sole ulcer and severe sole haemorrhage), and very few white line lesions that were considered to be severe were recorded, therefore the analyses may have lacked power to predict severe white line lesions. It ought to be noted that the lesion severity classifications had not been validated, therefore a "severe" sole haemorrhage lesion may not be "biologically equal" in severity to a "severe" white line lesion.

The finding of the current study that thin sole soft tissue preceded the incidence of severe sole haemorrhage is a novel finding. The definition of severe sole haemorrhage lesions in this analysis was "sole haemorrhage: very dark pink, dark red or purple"; size of the lesion was not considered, only its presence or absence. Severe sole haemorrhage and sole ulcer were both predicted by thin sole soft tissue thickness, likely being due to different stages of the same disease process. Previous work may not have reported associations with sole haemorrhage due to lower

sensitivity of lesion assessment, due to fewer number of lesion assessments or because lesions were only identified when animals were lame with a lesion (Machado *et al.*, 2011; Toholj *et al.*, 2013); the current study recorded precisely which lesions were present at 5 assessment points. The current study did not find associations between prior sole soft tissue thickness and mild and moderate sole lesions, and might have lacked the sensitivity to predict milder lesions. However, severe lesions were well predicted, using sole soft tissue thickness measurements.

Both sole soft tissue thickness and back fat thickness were significant in the same models. Previous work has suggested that thinning of the sole soft tissue is due to mobilization of body fat, yet additionally these results (Models 1a, 1b and 2b) and those presented in Chapter 5 suggest that sole soft tissue thickness and back fat thickness in part explain different aspects of lesion incidence. The measure of sole soft tissue thickness could have been an imprecise indicator of fat content of the digital cushion (discussed in 5.5.8.2), but since sole soft tissue thickness remained significant in the model, it suggests there was an additional effect of sole soft tissue thickness that was not associated with back fat thickness. Perhaps another structural component of the sole soft tissue has a major influence on future lesion formation; this could be the structure and function of the connective tissue.

6.7.2.2 The effect of back fat thickness on lesion incidence

In addition to the effect of sole soft tissue thickness, being thin and loss of back fat both increased the odds of lesion incidence. It could be that sole soft tissue thickness measurements only partly described the amount of fat within the digital cushion, and fat at a different site to that measured was more important in the formation of lesions (such as the axial site, measured by Bicalho *et al.* (2009) and discussed in 5.5.2.1). Therefore, body fat measures could be playing a bigger role in the size of the digital cushion than this study captured. Alternatively, the additional effect of back fat thickness to sole soft tissue thickness on lesion incidence could suggest that the mechanism by which body condition loss predisposes claw horn disruption is more complex than loss of fat from and thinning of the digital cushion. There could be earlier metabolic effects that predispose cows to both body condition loss and lesion development. Systemic inflammation in transition and early lactation is a topic receiving research attention at the moment as being behind many diseases of dairy cattle that have an association with parturition (Bradford *et al.*, 2015), and this systemic inflammation in the peri-parturient period could lead to lameness; Zhang *et al.* (2015) found increased acute phase proteins in the blood of cows that went lame in early lactation, prior to parturition and lameness. This theory is explored further in the General Discussion (7.5), but for now, it can be hypothesized that cows may go lame following body condition loss for reasons beyond fat mobilization from the digital cushion.

6.7.2.3 Development of the digital cushion

Incidence of lesions and lameness were much higher in heifers on Farm 2 than on Farm 1; this difference appears not to be fully explained by associations between body fat and sole soft tissue thickness, and other components of the digital cushion could be impacting upon lameness in heifers. Gard *et al.* (2015) tested the effect of raising dairy bull calves on different terrains, with different amounts of exercise. Between 2 and 6 months of age, calves were either kept in grass paddocks (control group) or on a 0.8 km lane of dirt, stones and grass, and fed at alternate ends of the lane to entice exercise (exercise group). The control calves walked a mean of 1.1 km daily on grass, compared with the exercise group of 3.2 km daily on rougher terrain, and at 6 months old the digital cushion had a volume 37 % greater than the control group. Perhaps, using stimuli such as underfoot conditions and exercise to improve growth and development of the digital cushion in early life could protect cows from the harsh forces experienced during adult life. This could have a prolonged beneficial effect on the function of the digital cushion in protecting the germinal epithelium from contusion. In the current study, the sole soft tissues of heifers on Farm 2 were thinner than those on Farm 1, and there were some differences in rearing systems: heifers on Farm 2 were kept at pasture or loose-housed on straw throughout rearing, compared with the much firmer concrete floors of the rearing sheds on Farm 1 (Table 4.1). The soft surfaces on which Farm 2 heifers were reared could have resulted in poorly developed digital cushions and hooves that were ill-adapted for the hard flooring surfaces of the milking herd sheds. Improving the structure of the digital cushion in the first two years of life could develop the structure and non-fat components of the digital cushion and limit lesion incidence later in life. This is an exciting area for future research.

6.7.2.4 Repeat lameness and digital cushion function

Hirst *et al.* (2002) found that lameness from claw horn disruption lesions during first lactation strongly predicted lameness incidence in subsequent lactations. This suggests that either a first lameness event increases risk of future lameness, or animal-level factors that cause a heifer to go lame persist throughout life and predispose lameness in subsequent lactations, too. Both Lischer *et al.* (2002) and Tsuka *et al.* (2012) found that the digital cushions of cows that had sole ulcers at slaughter were thinner than those without lesions. Further, Räber *et al.* (2006) suggested that inflammation that occurs during claw horn disruption lesions could cause depletion of fats within the digital cushion and lead to scarring, leaving a thinner digital cushion. In the current study, thin sole soft tissue thickness in parity >1 animals that preceded lesions could have been a result of claw horn lesions in previous lactations.

To address this, lesion incidence in heifers was studied; heifers were assumed to have experienced fewer previous lesions and digital cushions that have experienced less trauma. The only lesion data available for animals pre-first calving on either

farm was that collected at AP-8, where no heifers on Farm 1 had sole ulcers or severe sole haemorrhages, whilst 6 heifers on Farm 2 did (1 had a sole ulcer and 5 had severe sole haemorrhages). In heifers, thin sole soft tissue thickness increased the odds of experiencing a lesion, in addition to significant effects of back fat thickness and differences between claw and farm. This suggests that even if lesion incidence in cows affects the structure and function of the digital cushion that has knock-on effect in future lactations, having a thin digital cushion increases the odds of first claw horn disruption lesions in heifers, too. Future work should assess whether raising young-stock on different terrains and improving the structure of the digital cushion before first calving relates to differences in lesion incidence in lactating animals (Gard *et al.*, 2015).

6.7.3 Lameness incidence

Thin sole soft tissue on the *lateral* claw was associated with higher odds of subsequent lameness (measured by mobility score), but thin sole soft tissue on the medial claw did not influence future lameness incidence. It is a well reported finding that cows predominantly go lame due to pathology on the lateral claw (e.g. Murray *et al.*, 1996). The sole soft tissues were thicker on the lateral claw in this and previous work (Kofler *et al.*, 1999; Bicalho *et al.*, 2009; Toholj *et al.*, 2013), which could be an adaptation to cope with the greater forces that the lateral claw experiences (van der Tol *et al.*, 2003); perhaps this adaptation was insufficient to adequately dissipate the additional forces placed on the lateral claw.

Having a thinner sole soft tissue thickness on the lateral claw 90-150 days prior to a mobility score increased the odds of becoming lame, yet if the measurement was taken in the 30-90 days prior to a mobility score, a thinner sole soft tissue did not increase the odds of lameness. Perhaps, the soft tissues in some cows were becoming inflamed and were already abnormally thickened when taken 30-90 days prior to a mobility score, with lameness only being observed later when a lesion became severe. Since an underlying theory of the pathogenesis of claw horn disruption lesions is that lesions lead to poor horn growth (or cessation of growth), which increases the risk of ulcers and painful lesions later, the time between a lesion forming and lameness being observed could be large. Other work found effects of lameness before a lesion was observed on the foot or clinical lameness was seen: Amory *et al.* (2008) found that milk yield decreased prior to lameness diagnosis. The time delay between thin sole soft tissue and lameness being observed could resemble tissue-level pathology that causes disruption in horn growth that is later associated with clinical lameness. Alternatively, the reduction in milk yield prior to lameness could point to a wider cow-level effect that was not captured in this study, such as metabolic influences on milk yield that also predispose lameness.

Thin cows appeared to have been more likely to go lame, although this effect was correlated with sole soft tissue thickness and was not significant when sole soft tissue

thickness was included in the models. No associations were found between loss of body condition and lameness in the current study, despite cows undergoing a large body condition loss: the mean difference between back fat thickness at AP-8 and AP+17 was 8 mm (Table 5.1), corresponding with almost a 1-point decrease in body condition score (Figure 5.3). Back fat thickness correlated with sole soft tissue thickness. Given the large decrease in back fat thickness observed, had loss of back fat predisposed lameness with any meaningful effect size, an effect of change in back fat thickness on lameness incidence ought to have been detected using the analytical techniques described. Loss of back fat was associated with higher odds of lesion incidence as mentioned previously, which might simply have been a more sensitive measure of claw horn disruption than lameness. The sample size in the current study may not have been large enough to detect effect of back fat thickness, in addition to the effects of other significant variables.

A cow-level random effect improved the fit of Model 3, suggesting that if one leg of a cow went lame, the other leg was more likely to go lame. This could point to a cow level fixed effect that have been missed, or suggest that once a cow had gone lame on one leg, more weight was placed on the contralateral leg and she subsequently went lame on that leg. This latter possibility provides more reason for the first lameness to be detected early and treated promptly.

6.7.3.1 Lameness in heifers

As with lesion incidence, there was a large difference in lameness incidence in heifers between farms: heifers on Farm 2 were much more likely to go lame (OR: 4.2). In heifers, thin sole soft tissue thickness prior to a mobility score tended to increase the odds of lameness at a mobility score. However, there were differences between farms: heifers on Farm 2 had thinner sole soft tissues and were more likely to go lame. No effect of sole soft tissue thickness on lameness was detectable beyond the effect of Farm.

Previous authors have suggested that the digital cushion does not become fully mature until second lactation (Räber *et al.*, 2006) or found that the relationship between body condition and lameness was different in heifers than in parity >1 animals (Randall *et al.*, 2015). The latter work had a much larger dataset than in the current study and found that whilst body condition loss predisposed lameness in parity >1 animals, body condition loss did not predispose lameness in heifers. These results might be replicated in Model 1a in the current study (outcome: lesion incidence), if an assumption is made that lesions were a more sensitive indicator of claw horn disruption than clinical lameness itself. Further, Randall *et al.* (2015) found that if an animal had not become lame during first lactation, body condition loss increased the odds of lameness in the second lactation. The authors reasoned that the digital cushion is immature in first lactation (Räber *et al.*, 2006), or that body condition scores were designed for adult cows and perform less well in heifers. From

the results of the current study, there does appear to be a different relationship between body fat and subsequent lameness in heifers.

6.7.4 Lesions versus lameness

Visual presence of lesions appears to have been a more sensitive indicator of claw horn disruption than lameness (identified by mobility score). Many previous works have found difficulty linking lesions with clinical lameness, finding lesions on claws without clinical lameness (e.g. Whay *et al.*, 1997; Manske *et al.*, 2002b; Tarlton *et al.*, 2002; Knott *et al.*, 2007; Maxwell *et al.*, 2015). This could be because cows are stoic animals and hide pain where possible; given their ancestral heritage as prey animals, displaying signs of pain is an outward sign of weakness and could identify them as an easier target for predation (Lin, 2014). Alternatively, mild disruption to claw horn production associated with mild lesions simply may not be painful. Or, by the time lesions are seen at the sole, disease in the sole soft tissues may have resolved. The tenet of this work regarded whether sole soft tissue thickness lead to perturbation of sole horn production, which can lead to lameness, and it achieved this: the lesion models demonstrate that thin sole soft tissue increased the odds of lesion formation (in addition to other variables), and the lameness models demonstrate that thin sole soft tissues increased the odds of subsequent lameness. Whilst the current work did not attempt to identify only lameness that was associated with a claw horn disruption lesion (although infectious causes of lameness were well controlled on both farms models differed little with the exclusion of cows that developed infectious causes of lameness), it appears that lesions were a more sensitive indicator of disruption of horn growth than lameness. Further, to be considered "lame", a leg had to be lame at >1 mobility score, therefore mild or short-term lameness could have been missed.

The models found no overall differences in lameness levels between farms, except in heifers. However, there were differences between mobility scorers, as outlined in 4.3.4.2; the Observer 1 (on Farm 1) identified cows as more lame than Observer 2 (on Farm 2). Further, the walking surfaces on the two farms were different: Farm 1 had rubber matting in the passageways, whilst Farm 2 had concrete slats. Subjectively, underfoot conditions on Farm 2 were slippery and cows walked poorly on the concrete slats, but when removed to the assessment crush and walked across flat, dry and even concrete, mobility was often much improved. All cows were mobility scored on either the rubber matting (Farm 1) or concrete slats (Farm 2) as it was impractical to remove all cows from the sheds for mobility scoring on a fortnightly basis. These factors were all incorporated into the farm effect, but the differences made it difficult to define what a "lame" cow was. Mobility scorers had shown higher concurrence between more severely lame scores (Table 4.5) and thus the lameness outcome used incorporated both 2B scores and the milder 2A scores. The primary aim of the lameness definition was to help identify cows with lameness associated with claw horn disruption. One-off lame scores could have been

artefactual and might not have truly been lameness. A high threshold for lameness ensured that few non-lame cows were considered to be lame.

6.7.5 Lesion scoring system

All photographs were analysed in random order by the same observer, who was blind to cow, farm and assessment point (4.3.2.3). This negated issues with inter-observer and inter-farm differences that were apparent with the mobility scoring system. The lesion scoring system was sensitive compared with other systems described (4.3.2.1). This was intended, but in accordance with previous work, mild lesions were not significant in the final models. Still, the system enabled the detection of severe sole haemorrhage lesions being predicted by thin sole soft tissue thickness, which other work has not reported. The lesion scoring system appears to have performed well as a marker of disruption of claw horn growth.

6.8 Conclusions

This work found that having thin sole soft tissues, but not thinning of the tissues, increased the odds of sole ulcer or severe sole haemorrhage lesions being present on claws later in lactation. This association was detected in heifers and in parity >1 cows. Loss of back fat increased the odds of lesion presence in cows, but only being thin at the previous assessment point increased the odds of a lesion being present in heifers. With regards to lameness (measured by mobility score), having thin sole soft tissue on the lateral claw, or having thin back fat thickness, increased the odds of lameness incidence in parity >1 animals, but in heifers there were large differences between farms and such associations were not found. The finding that thin sole soft tissue predisposed lameness and lesions, rather than *thinning* of the sole soft tissues, suggests that it is ultimately the thickness of the sole soft tissue (a component of which is the digital cushion) that protects the germinal epithelium of the sole from disruption. This absolute thinness could be influenced by many factors, including parturition, fat content with can change with body fat, stature, parity and external stimuli such as young-stock management.

7 General Discussion

7.1 Introduction

In the 1990s, Vermunt and Greenough (1994) stated that the disease process behind the formation of the claw horn disruption lesions was not fully understood. Over 20 years later, much literature has been published regarding risk factors and possible underlying mechanisms behind different presentations of the disease. It is perhaps safe to say that the underlying mechanism behind claw horn disruption lesion development is through disruption to claw horn growth. However, complex interactions between risk factors at different stages of life or lactation, and how they predispose lesion formation, are not fully understood. Additionally, lameness levels do not appear to have reduced much since the 1990s. Proven interventions are needed to implement lameness control measures on farm, and understanding of the disease process can guide such interventions.

Green (2015) used the term "paradigm" to describe the theories surrounding lameness in sheep, and how beliefs surrounding lameness stand to be challenged and progressed upon. The body of knowledge surrounding lameness in dairy cattle can also be considered as a paradigm, and this chapter discusses the relevance of the work presented in the current thesis to the paradigm of lameness in dairy cattle.

7.2 Post-publication opinions on claw length work

Foot trimming is a common component of lameness prevention and treatment. However, over-zealous trimming can damage the foot (Bell, 2015). Step 1 of the Dutch Method of foot trimming states to trim the dorsal wall to 75 mm (Toussaint-Raven, 1985). In Chapter 3, the appropriateness of this length as a set measure for foot trimming was assessed. The study measured the length of the dorsal wall in contact with the dermis that must be avoided during foot trimming, and added two constant adjustments to allow for a minimum sole thickness of 5 mm and a wall thickness of 8 mm. The work demonstrated that one set length cannot be suitable for all claws, and found that cutting the dorsal wall to 75 mm and leaving a 5 mm step at the toe (as is commonly reported during foot trimming) would cut 55 % of claws too short. Few variables were found to explain variation in foot trimming length; only age, carcass weight (the only available measure of cow size) and lateral or medial claw. These variables explained 22 % of the null model variance. Since cow-side measures gave little indication of the *minimum* dorsal wall length to be applied during foot trimming, it seems imperative that the commonly recommended measure of "75 mm" be updated to a longer length. If foot trimming operators adhere to recommended lengths, this could reduce the proportion of claws that would be over-

trimmed. The study showed that trimming to 86 mm and 93 mm would have been suitable for all animals <4 years and ≥4 years old respectively when trimming the toe to a point; if leaving a 5 mm step at the toe, 7 mm can be taken from each of these measurements.

The work in Chapter 3 was published as a paper in Veterinary Record in July 2015 (Archer *et al.*, 2015). The author of this thesis has also presented it multiple times nationally and internationally, at "Livestock Show", Birmingham, UK, July 2015; "Bovi-Bond Meeting", Billerbeck, Germany, October 2015 and "Lameness in Ruminants", Valdivia Chile, November 2015. The work has received much interest from both the foot trimming and veterinary professions. In a letter to Veterinary Record following its publication, Blowey (2015) highlighted that 5 mm may not be an appropriate *minimum* sole thickness and mentions the dangers of trimming to a *minimum*, rather than an optimal thickness. If always trimming to a *minimum* sole thickness, over-trimming will likely occur in practice. In response to this, there is no good evidence to suggest that a particular set sole thickness is appropriate in all cases, and if a greater sole thickness or a different toe angle is desired, the recommended *minimum* dorsal wall length can be calculated from the description of the methodology in 3.2.5.

From presenting this work to farmers, foot trimmers, vets and researchers, it has become clear that many people do not use a set claw length when foot trimming. This therefore reinforces the importance of updating the *guidelines*, as perhaps persons being trained in foot trimming are more likely to adhere to guidelines. Refreshingly, many professionals have agreed that 75 mm is not appropriate as a set length and in another letter to Veterinary Record, Burnell *et al.* (2015) of the Cattle Lameness Academy (an enterprise that offers lameness management services, training and consultancy in alliance with veterinary practices) have updated the guidelines that they teach to farm staff, based on this work. Conversely, Pieter Kloosterman (an instructor of the Dutch Method of foot trimming) maintains that the differences in lengths presented in this work are due to differences in size of the cow and landmarks used (personal communications, October and November 2015). The only data on cow size available in Chapter 3 was carcass weight, which did correlate with minimum dorsal wall length, adding some evidence to the former reasoning. However, a consistent landmark was used in the current work and still demonstrated great *variation* in length of the dorsal wall, even in the final model after accounting for the significant explanatory variables. Whilst the measurements recommended in the current work will vary slightly with landmark used, the work conclusively demonstrated that no one size would fit all claws and the work did not find cow-side measures to explain the majority of the variation in *minimum* dorsal wall length.

In an editorial published alongside the paper in Veterinary Record, Bell (2015) highlighted that the differences in landmarks used, breeds, size of cow, size of step left at the toe and measurement error, could mean that foot trimming operators who

consider themselves to be trimming to the correct 75 mm are not over-trimming. Bell (2015) highlighted that transferring knowledge and understanding of the internal structures of the foot and the variation in them to farmers, foot trimmers and veterinarians constitutes a key step in managing foot health on farm.

7.3 The suspensory apparatus; how important is "idiopathic laminopathy"?

A longitudinal study assessed the thickness of the sole soft tissues (i.e. the digital cushion and corium) beneath the base of the foot; the primary aim was to assess how sole soft tissue thickness related to changes in body condition. However, the analysis revealed some unexpected findings, one of which was that the sole soft tissues were thinnest at approximately 1 week (4-10 days) after calving; cows had not lost body condition by this stage. Later in lactation, at the nadir of back fat thickness, sole soft tissue thickness was thicker. The thinness of the sole soft tissues immediately after calving could have been a result of laxity within the suspensory apparatus associated with calving (Tarlton *et al.*, 2002; Knott *et al.*, 2007). This could demonstrate the importance of the suspensory apparatus on the position of the distal phalanx within the hoof and on thickness of the sole soft tissues beneath the distal phalanx; laxity of the suspensory apparatus was not assessed in this study. This finding highlighted that sole soft tissue thickness is in part dependent on the integrity of the suspensory apparatus. If physiological changes associated with calving can have an effect on the suspensory apparatus and sole soft tissues, other physiological events throughout the study period that were not captured may have also had an effects on both structures.

In Chapter 1 (1.3.1) the bovine and equine literature surrounding laminitis was briefly reviewed. Use of the term "laminitis" to describe pathology of the laminar region in horses appears to have shortcomings since laxity in the suspensory apparatus may not be inflammatory in origin; laminopathy might be a more appropriate term since it does not imply inflammation induces the disease (Katz and Bailey, 2012). Laminopathy in horses appears to occur through different routes, which can be grouped as inflammatory and metabolic; metabolic causes include laminitis with hyperinsulinaemia, with insulin appearing to have an effect on the integrity of the laminae. In bovine physiology "classical laminitis", which has been defined as "inflammation of the laminae within the claw" (Vermunt and Greenough, 1994), was historically considered to be behind the formation of claw horn disruption lesions. However, this hypothesis has largely been rejected as explaining the majority of these common lesions (Mulling and Greenough, 2006). As is the case in the equine field, changes that occur in the laminae of the bovine hoof are still not fully understood, although changes in the laminae do occur and predispose to lesions (Tarlton *et al.*, 2002; Knott *et al.*, 2007). The role of insulin in bovine laminar pathology has not been explored and it would be interesting to investigate whether hyperinsulinaemia influences laminar integrity in bovids as it appears to in horses.

Perhaps these changes that predispose to claw horn disruption lesions could be referred as "idiopathic laminopathy". This term simply suggests that there are changes in the laminar region of the foot that have a role in pathology of the claw horn disruption lesions, which are not fully understood. After all, if physiological effects associated with calving could have implications on the suspensory apparatus of the foot, other physiological alterations throughout lactation could also play a part, and this could highlight a component of lameness onset that ought to be explored further.

7.4 Does body condition loss really cause lameness?

Several studies have demonstrated that being thin and body condition loss are risk factors for lameness (Hoedemaker *et al.*, 2009; Machado *et al.*, 2011; Green *et al.*, 2014; Lim *et al.*, 2015; Randall *et al.*, 2015; Solano *et al.*, 2015), and this might occur through thinning of the digital cushion and greater forces being transferred through the germinal epithelium of the sole (Bicalho *et al.*, 2009). Previous work associating thickness of the sole soft tissues with body condition has been cross-sectional, and the study described and analysed throughout Chapters 4, 5 and 6 explored associations between measures of body fat and thickness of the digital cushion in a longitudinal manner; lesion and lameness incidence was also recorded. Measures of digital cushion and corium thickness (termed "sole soft tissue thickness") did change with body condition throughout lactation, but the effect was much smaller than presented in previous work (Bicalho *et al.*, 2009). Additionally, many other variables had an effect on sole soft tissue thickness, and only "absolute thinness" of the digital cushion, not *thinning* of the digital cushion, predisposed to lesions and lameness.

In studies of pressure distribution on the plantar surface of the hind claws, van der Tol *et al.* (2002) found that pressure was greatest on the abaxial region of the sole surface (zone 3 of the foot map, a site for the formation of white line disease) when standing, but was greatest axially, over the sole ulcer site, when walking (van der Tol *et al.*, 2003). For the digital cushion to have a biomechanical role in reducing peak pressures in these regions and preventing lesions, it must either dissipate forces during foot strike or it must help balance them over the structures that are designed to bear weight, enough to reduce pressure over the sole ulcer site sufficiently to protect the germinal epithelium in sites where high pressures occur. For the mechanism by which body condition loss predisposes lameness to be through thinning of the digital cushion, the difference in size of the digital cushion with body condition change would have to be large enough to result in biomechanical differences in digital cushion function. The differences in size of the digital cushion seen with changes in body fat were very small in the current study, and whether these small differences could relate to biomechanical differences was not shown. However,

it appears that in some circumstances, body condition could have contributed to the "absolute thinness" of the digital cushion that did predispose lesion formation.

Further, no work has demonstrated under what conditions cows mobilise fat from the digital cushion; work in live animals could explore this with biopsies of the soft tissues of the sole, which has been described by MacCallum *et al.* (2002). Some very preliminary work to support the hypothesis that cows can mobilise fat from the digital cushion is presented in Appendix 1. Briefly, digital cushion samples of 16 cows that had a body condition score measurement taken within 1 week of slaughter were studied histologically (cows originated from the SRUC dataset described in Chapter 2). Adipocytes were circumscribed using stereological techniques and results showed that fatter cows at slaughter had adipocytes within the digital cushion with larger total area than those of thinner cows (Appendix Figure 1). This could suggest that cows mobilise fats from the digital cushion as they do from adipose tissues elsewhere in the body.

Body condition loss occurs in early lactation in part due to insulin resistance, which helps partition energy to the mammary gland in order to maintain milk production. This is exacerbated in the highest producing cows (Waltner *et al.*, 1993) and high producing cows are also more likely to go lame (Amory *et al.*, 2008; Bicalho *et al.*, 2008). Body condition loss has been demonstrated as a risk factor for subsequent lameness, but not as a cause of lameness. Even small losses in body condition can increase the risk of lameness (Lim *et al.*, 2015; Randall *et al.*, 2015) and these losses could be part of the homeorhetic response that is not affected by caloric intake, which might be difficult to prevent. Intervention studies must assess whether minimizing body condition loss during early lactation does reduce lameness incidence and therefore serves as a realistic control point for lameness. Alternatively, up-stream events prior to body condition loss could be causing both body condition loss and lameness, as is explained in the next section.

7.5 Metabolic inflammation in the aetiopathogenesis of claw horn disruption lesions

An exciting study assessed innate immunity reactants in cows throughout the dry period and during the first 4 weeks of lactation, and retrospectively pair-matched cows that had become lame with cows that had remained sound (Zhang *et al.*, 2015). They found that pro-inflammatory cytokines (specifically, interleukin-6 and tumor necrosis factor α: **TNF-α**) and acute phase proteins (haptoglobin and serum amyloid A) were higher in the lame cow group before they became lame. Previous works had found acute phase proteins to be raised during lameness (Smith *et al.*, 2010; Tadich *et al.*, 2013), which could be a result of pain and stress associated with lameness, but the work of Zhang *et al.* (2015) implies that lameness could be predisposed by a more generalized inflammatory state.

The claw horn disruption lesions could be part of a wider, systemic inflammatory process; this idea is presented in light of discussions surrounding consequences of "subacute" inflammation in cows post-partum, recently reviewed by Bradford *et al.* (2015). Acute inflammation occurs during particular diseases, whilst a more general, systemic, "subacute" or "metabolic" (Hotamisligil, 2006) inflammatory state has been described in cows around calving. This inflammation appears necessary for a cow to recover from calving and might drive the adaptation of metabolism to lactation, yet the degree to which systemic inflammation occurs varies, and a high degree can have detrimental effects on tissue function and predispose diseases during lactation (Qu *et al.*, 2014). Bradford *et al.* (2015) hypothesize that moderating the inflammatory response post-partum will facilitate the transition to lactation. In a general sense (and not relating to lameness), early work has supported this: blanket administration of meloxicam or sodium salicylate after calving increased whole-lactation milk production compared to a control group, and tended to increase survival within the herd (Carpenter *et al.*, 2015). Further, Yuan *et al.* (2013) had reported complementary results via a different experimental design: administering TNF-α decreased milk yield, increased the odds of health disorders and in some cases increased plasma eicosanoid concentrations (total sample size: 9 parity 1 and 24 parity >1 cows). The work of Zhang *et al.* (2015) suggests that lameness could be another disease that is predisposed by a generalized inflammatory state in the peri-parturient period.

One route by which systemic inflammation could predispose lameness is through insulin resistance: systemic inflammation induces insulin resistance, which further induces body condition loss (Bradford *et al.*, 2015), which has been shown to predispose lameness. Metabolic factors such as inflammation or even a genetic predisposition to increased insulin resistance in high producing cows could have predisposed cows to both body condition loss and lameness in previous studies (Green *et al.*, 2014; Lim *et al.*, 2015; Randall *et al.*, 2015). Another route could be that inflammation has an effect on the suspensory apparatus of the hoof (although this is perhaps a more tenuous suggestion). Tarlton *et al.* (2002) and Knott *et al.* (2007) suggested that relaxin could be the cause of the distal phalanx to sitting lower in the hoof capsule around calving, although they did not show that weakened suspensory apparatus was a result of relaxin. The mechanisms that cause weakening of the suspensory apparatus around calving have not been fully explored, and perhaps a systemic inflammatory state was also having an effect on the suspensory apparatus. Inadequacies of the suspensory apparatus via this postulated mechanism could also be considered as "idiopathic laminopathy".

Researchers of equine laminitis (or rather, *laminopathy!*) recently held a workshop on laminitis pathophysiology, which brought together laminitis researchers and experts in human sepsis and metabolic syndrome. The purpose was to discuss how predisposing factors such as sepsis and metabolic syndrome might lead to laminar

pathology. A review of the workshop explains that the immune system is considered to have an important role in the disease process (Moore and Belknap, 2009). In cows, only very early work has outlined a possible role of innate immunity reactants as predisposing lameness (Zhang *et al.*, 2015), but the immune system may be playing a larger role than is currently understood. It seems that effects of metabolic alterations and the immune system on the suspensory apparatus of the bovine foot ought to be explored more, and perhaps an idiopathic laminopathy is contributing towards the claw horn disruption lesions that has not yet been defined.

7.6 Chronic lameness and degeneration of internal foot anatomy

The digital cushion and flexor tuberosity of the distal phalanx appear to be damaged with age and with claw horn disruption lesion incidence (Lischer *et al.*, 2002; Räber *et al.*, 2004; Tsuka *et al.*, 2012). However, risk of lameness, claw horn disruption lesions and abnormal bone modelling all increase with age (Benjamin *et al.*, 2000; Sanders *et al.*, 2009) and previous work did not distinguish whether changes observed were associated with age only, or whether there was an additional effect of lameness. The work presented in Chapter 2 took the hind feet of cows culled from a research herd and measured bone modelling on and around the flexor tuberosity. The work demonstrated that bone modelling was linked with poor locomotion preceding slaughter *in addition to* effects of age, and that bone modelling was specific to incidence of claw horn disruption lesions.

Further, the bone modelling has previously been given a variety of different terms, but histological study demonstrated that the bone appeared to be heterotopic ossification. This could also be described as exostosis and is a common sequela to both arthroplasty and arthritis, likely occurring as a result of instability in the joint and inappropriate force transfer through the bone (Romano *et al.*, 2004; Saudan *et al.*, 2007). Alternatively, such bone modelling could occur as a result of trauma to and proliferation of osteoprogenitor cells within the periosteum, resulting in periosteal hyperostosis and new bone being laid down (Long *et al.*, 1993).

This work, that demonstrates damage to foot anatomy associated with lameness, could help explain why early detection and treatment of clinical lameness cases is an effective component of lameness management. The inflammatory response to claw horn disruption lesions could damage the digital cushion and additionally utilize local fat reserves (Räber *et al.*, 2006), causing the digital cushion to become thinner and further predispose lesions (Machado *et al.*, 2011). To stimulate discussion around the poorly defined sequence of events during lameness onset and perpetuation, a possible pathogenic pathway is proposed as follows: (1) inflammation occurs during an active claw horn disruption lesion, (2) fat is utilized locally, (3) the digital cushion becomes depleted or is replaced with scar tissue and future cushioning capacity is impeded, (4) both local inflammation and trauma to the

periosteum with inappropriate cushioning of forces stimulate bone modelling, which exerts greater point-forces on the dermis and the sole-producing germinal epithelium of the foot and (5) this self-perpetuating cycle whereby a claw horn disruption lesion damages the foot and predisposes the cow to further lameness consigns the cow to a lifetime at greater risk of lameness. Maintaining the integrity of the digital cushion could be vital, and if prolonged pathology occurs within it, both the sole soft tissues and the surrounding structures such as the flexor tuberosity could begin to degenerate and enter this viscous cycle of decline.

7.7 Possible targets for the use of NSAIDs in lameness control

Lame cows are in pain. Cyclo-oxygenase enzymes (COX-1 and COX-2) play a role in the onset of the inflammatory cascade and prostaglandin formation. The enzymes are induced by the inflammatory process and their inhibition both provides analgesia and has anti-inflammatory effects (Whay *et al.*, 2005). Hyperalgesia has been demonstrated to occur in lame cows; that is, when cows are lame, they have a heightened response to nociceptive stimuli elsewhere on the body (Whay *et al.*, 1998; Tadich *et al.*, 2013). Ketoprofen (an NSAID) non-selectively inhibits both COX-1 and COX-2 enzymes (Williams and Higgs, 1988), and the administration of ketoprofen as part of a combined treatment for claw horn disruption lesions has been shown to moderate hyperalgesia. The administration of ketoprofen to lame cows reduced responses to noxious stimuli for prolonged periods: responses were reduced at 3, 8 and 28 days after administration, demonstrating prolonged effects of the drug on hyperalgesia (Whay *et al.*, 2005). Additionally, the administration of ketoprofen to newly lame cows being treated for claw horn disruption lesions has been associated with improved resolution of lameness, which could have acted to reduce inflammation in the soft tissues of the sole, as well as moderate the pain response (Thomas *et al.*, 2015a).

The linear regression model presented in Chapter 5 (Table 5.2) demonstrated that cows with a sole ulcer had thickened sole soft tissues. This could be a sign of inflammation in these tissues, which would be a plausible target for NSAID therapy. Other studies have also detected foot lesions to be hotter, using infrared thermography: Nikkhah *et al.* (2005) identified increased temperature at the coronary band with sole ulcers and sole haemorrhage, which they postulated was a result of increased blood flow to the foot in response to the lesion, whilst Stokes *et al.* (2012) used an infra-red camera and correctly identified 80 % of foot lesions and 73% of lesion-free claws by setting a single threshold temperature; i.e., feet with lesions were hotter. Rodríguez *et al.* (2016) reported that locomotion score ≥3 cows had hotter feet, but concluded that the poor sensitivity and specificity of their measurements hindered infrared thermography from being used in detection of early lameness. In Chapter 6, the models demonstrated that having a thinner sole soft tissue thickness during the 4-10 days after calving increased the odds of lesion

development later in lactation, and Oikonomou *et al.* (2014b) demonstrated that thin sole soft tissues during the same period were hotter; this could be a sign of early contusions in the corium and early inflammation in the region. Perhaps, administration of NSAIDs during this period would moderate the inflammatory response and help maintain claw horn growth. Thomas *et al.* (2015a) found that the administration of NSAIDs to cows with claw horn lesions only significantly improved recovery rates (compared to a functional trim only) when combined with the application of a block to the non-lame claw. This could suggest that analgesia provided by NSAIDs caused cows to put more weight on the lesion, damaging the soft tissues further and negating anti-inflammatory effects of the drug. Conversely, when NSAIDs were administered in conjunction with the application of a block to the non-lame claw, the pressure relief provided by the block may have enabled the anti-inflammatory action of the NSAID to promote healing of the lesion, as this combination significantly improved recovery rates.

The previously discussed research into the effects of peri-parturient inflammation on disease incidence in early lactation suggests that systemic (or "metabolic") inflammation might predispose lameness (7.5). If this is correct, then the administration of NSAIDs in the peri-partum period might reduce risk of lameness during lactation, in a manner alluded to by Carpenter *et al.* (2015). This remains an exciting area for future research.

Finally, there could be a role of NSAIDs in the reduction of bone modelling. The histological results presented in Chapter 2 demonstrated that the bone modelling was heterotopic ossification, seemingly occurring as a sequela to trauma to the bone (for example through direct trauma or as a result of an inflammatory response in the adjacent soft tissues). Prostaglandins play an important role in both osteoblast and osteoclast function, and inhibition of prostaglandins slows bone growth (Vuolteenaho *et al.*, 2008); this can be beneficial if the new growth is pathological. Heterotopic ossification is a common sequela to arthroplasty and arthritis in human patients (Romano *et al.*, 2004; Saudan *et al.*, 2007) and several NSAIDs (both non-selective, such as ibuprofen, and COX-2 selective, such as celecoxib) have been shown to reduce and prevent this new bone modelling; the NSAIDs antagonize cyclo-oxygenase enzymes involved in bone growth (Romano *et al*., 2004; Saudan *et al*., 2007; Zhang *et al*., 2013). Perhaps, administering NSAIDs to cows with an active claw horn disruption lesion could not only act to reduce inflammation in the soft tissues of the sole, but additionally have a direct inhibitory effect on heterotopic ossification.

It must be remembered that bone modelling could be occurring as a result of weakness at this site on the distal phalanx, and whether or not trying to prevent bone modelling at this site is safe requires further work; O'Connor and Lysz (2008) highlight issues with suppressing bone healing using NSAIDs. Further, NSAIDs can be nephrotoxic and the author cautions care with their use. It has for a long time,

however, been considered that use of analgesics in dairy cattle is insufficient (O'Callaghan, 2002). Their use has increased over the last 10 years (Huxley *et al.*, 2014), which is promising for the welfare of dairy cattle. Whay *et al.* (2005) point out that non-selective NSAIDs might be optimal for anti-inflammatory and analgesic properties in lame cows, and only non-selective NSAIDs are available for use in dairy cows in the UK (NOAH, 2016). It therefore appears that there are plenty of non-steroidal anti-inflammatory drugs available for the treatment of lameness in dairy cattle, and there is good evidence to support their use.

7.8 The paradigm of lameness in dairy cattle

As stated previously (7.1), the body of knowledge surrounding lameness in dairy cattle can be considered as a paradigm. The intricate associations of risk factors with anatomical and physiological alterations have been further studied in this thesis, but are still not fully understood. For illustration, a simple model by which work in the current thesis fits into the complex array of mechanisms behind the formation of the claw horn lesions is outlined in Figure 7.1, and is based on work discussed throughout this chapter. Understanding the mechanisms behind links displayed in Figure 7.1 can guide interventions to manipulate the disease processes. Interventions to tackle lameness are greatly needed in order to reduce disease incidence on farm.

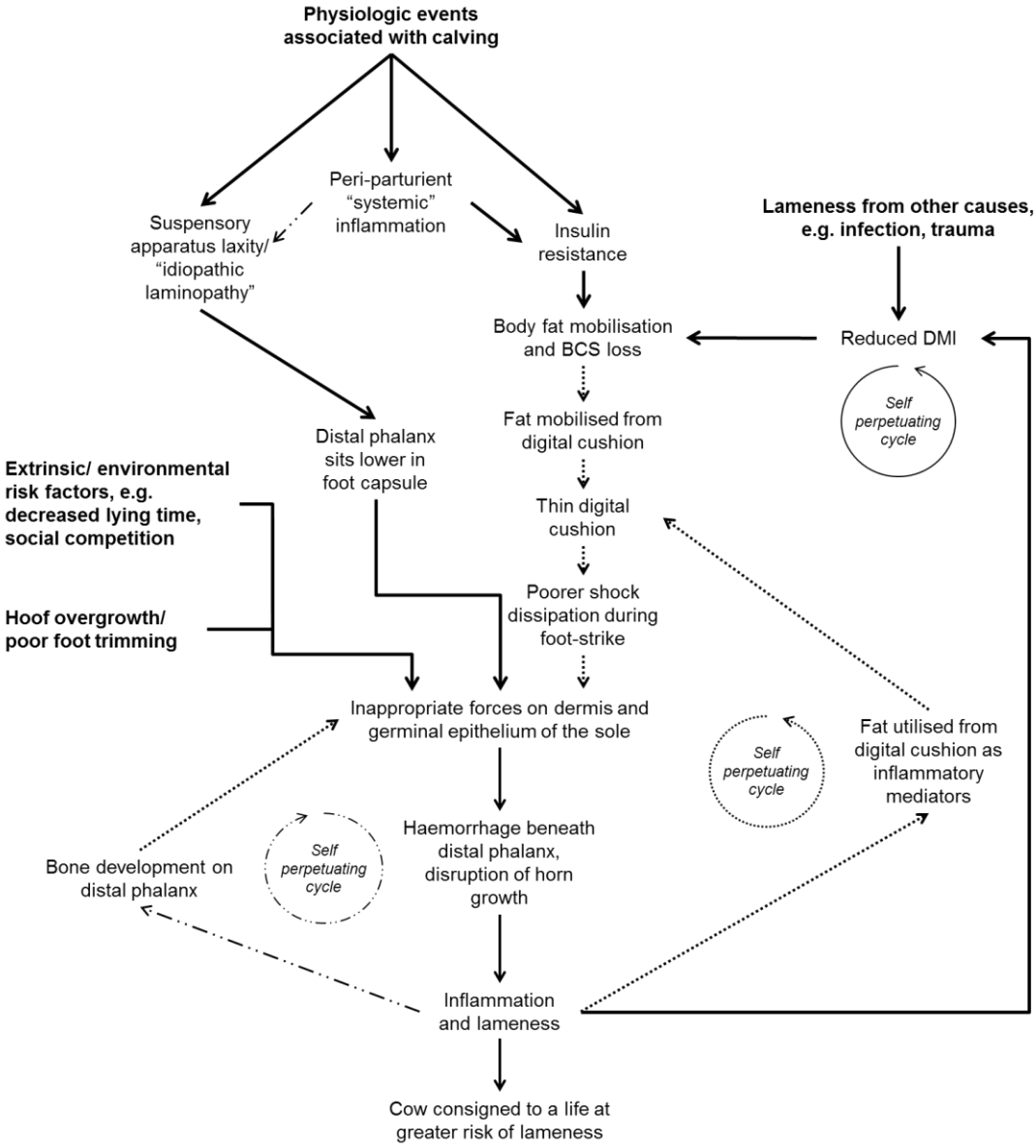

Figure 7.1: The paradigm of lameness in dairy cattle. Proposed sequences of events involved in the pathogenesis of claw horn disruption lesions, demonstrating how the work in this thesis sits within the body of literature surrounding the claw horn disruption lesions in dairy cattle. Self-perpetuating cycles are suggested, with inflammation being a key factor in lesion recurrence. Line styles highlight the levels of evidence supporting the links displayed: (1) data in peer-reviewed literature supports this link (solid line), (2) some evidence exists to substantiate this link, but the link has not been confirmed (dotted line) and (3) the present thesis hypothesises this link (dotted-dashed line).

7.9 Conclusions

Foot trimming is commonly used for the prevention of lameness, and it is important that it does not cause further damage. The foot trimming work presented in Chapter 3 demonstrated large variation in dorsal wall length of hooves, the majority of which was not accounted for using available cow-side measures. The minimum lengths that would have been suitable for all claws to be trimmed to were 93 mm for cows aged ≥4 years and 86 for cows aged <4 years, although 7 mm could be taken from these measurements if a step is left at the toe. Recommendations for foot trimming, which perhaps are most tightly adhered to by new foot trimming operators, should therefore be based on the proportion of claws for which a measurement is suitable, rather than being based on population means.

The longitudinal study of the sole soft tissues beneath the distal phalanx (i.e. digital cushion and corium thickness) described and analysed throughout Chapters 4, 5 and 6 presented a previously un-utilized method for assessing how sole soft tissue thickness changes with measures of body condition. The study found sole soft tissue thickness to be positively correlated with measured of body fat, but the effect size was much smaller than reported in cross-sectional work, and the correlation was only present under certain conditions; there was no positive association between the two variables when a lesion was present or shortly after calving. Further analysis demonstrated that having a thin sole soft tissue thickness predisposed lameness and lesions later in lactation, whilst *thinning* of sole soft tissue thickness did not influence future lameness or lesion incidence. Many variables were associated with sole soft tissue thickness and the reasons for this are not fully understood. Measures of body fat appeared to be one component that could contribute to having a thin digital cushion and, under some circumstances, could have contributed to the "absolute thinness" of the digital cushion that did predispose lesion formation.

The work in Chapter 2 addressed pathology seen within the foot with recurrent lameness, and found that bone modelling was associated with lameness from claw horn disruption lesions during life. The bone modelling resembled hypertrophic ossification. Once bone modelling is present on the flexor tuberosity, it is hypothesized that they cause greater point forces on the germinal epithelium of the sole and help perpetuate lesion formation and lameness. Their presence highlights the need for early and effective treatment of lame cows in order to reduce the degeneration of hoof architecture and a predisposition to further lameness.

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Appendix 1: Relationship between adipocyte size in the digital cushions of *post mortem* specimens and body condition score at slaughter

Aim

The aim of this preliminary work was to investigate whether adipocytes in *post mortem* specimens of digital cushions were larger if cows had higher body condition score at slaughter.

Study samples

Samples available for study were from the digital cushions of feet in the dataset described in Chapter 2: cows that had been culled from the SRUC Dairy Research and Innovation Centre between November 2014 and August 2014. The digital cushions were dissected for histological study if a body condition score measurement was available for the cow within the 7 days preceding slaughter.

Cuboidal tissue samples were cut from the middle pad beneath the distal phalanx: site E, as described by Räber *et al.* (2004). The dimensions of the cuboid were width (**W**) from axial to abaxial, length (**L**) from dorsal to plantar and height (**H**) vertically. W was always the greatest dimension, followed by L, and H was the smallest dimension; this ensured that the tissue could be orientated for histology after processing and always cut in the frontal plane. Samples were washed in phosphate-buffered saline (PBS) for 10 minutes under constant agitation, repeated twice, and then processed for histology.

Histology

To optimize the fixation protocol, test samples for histology were sectioned into three and underwent three different methods of fixation. The three protocols were:

1. Placed in 10% neutral buffered formalin (NBF) for 24 hours.
2. Placed in PBS for 16 hours, followed by NBF for 8 hours.
3. Placed in PBS for 24 hours (i.e. tissues were not fixed).

The tissues were embedded, cut and stained, to assess the effect of the three fixation protocols. The first method caused the tissue to shrink and distorted tissue architecture. The third did not fix the tissues and made cutting difficult. The second, where samples were in NBF for 8 hours, fixed the tissue and preserved tissue architecture, and was used for the experiment.

A sample taken from site E of each claw underwent fixation, using the second protocol described above. Tissues were processed overnight in an automatic

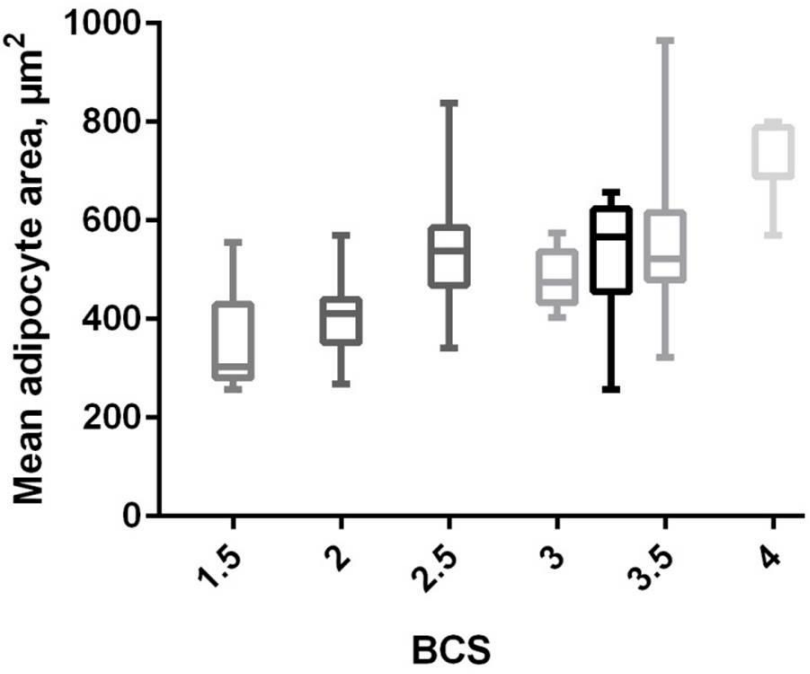
processing machine, using a protocol similar to that described in 2.3.3.2, and stained with haematoxylin and eosin, as described in 2.3.4.1.

Systematic random sampling (Mayhew and Burton, 1988) was utilised to analyse histological sections and one tissue section on every 10^{th} slide was photomicrographed using a light microscope (DM5000 B, Leica, Germany). Regions of the photomicrographs were randomly chosen for study. Adipocytes within the area were circumscribed manually using ImagePro Plus (Media Cybernetics, USA) by a blinded anatomist and histologist, aiming to measure 200 adipocytes per claw. The process was systematic to ensure that no cells were counted twice. Only whole cells were measured and the photomicrograph boundary acted as a forbidden line. The surface area of each cell was calculated by the image analysis software, from which diameter of the cell was estimated using the equation: Diameter = $2 \times \sqrt{(\text{Surface Area}/ \pi)}$.

Results

Body condition score was only recorded within the last 7 days for 16 cows. Some tissue samples were unavailable as they had been used in other preliminary experiments (not described in this work), and the final dataset consisted of 55 claws of 16 cows. No cells were observed in 4 samples, after 4 slides were checked, which were incrementally 240 µm apart within each sample. Cell dimensions were therefore based on 60 claws, of which 34 were lateral and 26 were medial. A total of 11,853 cells were measured; in 6 samples, too few adipocytes were found to reach the cell count of 200, although >120 cells were still counted from each sample. Very large appeared to be damaged cells, and were excluded from the analysis. Further, some cells were very small and were thought to belong to a different tissue type; these were also excluded from the analysis. 206 cells were counted with an area >2,000 μm^2, the average area of this subset was 25,055 μm^2 and predominantly occurred within 3 tissue samples. 419 cells with an average area <70 μm^2 were counted. It must be noted that where "normal" large adipocytes were present, these particularly small and particularly large cells were preferentially not counted as they were suspected to be a different subset of cells. Ratios of the different classifications of cells therefore in no way represent the relative abundance within the tissue.

The final dataset compiled from histology that was used for analysis consisted of 11,228 cells from 51 claws of 16 cows, and the mean number of adipocytes counted per sample was 220. Cell area followed a chi-squared distribution, with a median of 457 μm^2 (IQR: 265 to 698) and cell diameter was approximately normal, with a mean of 24.5 µm (SD: 8.0). Appendix Figure 1 shows a box-plot of mean adipocyte area plotted against body condition score at slaughter. Cows with higher body condition score at slaughter appeared to have fatter cells. No further statistical analysis was performed on this dataset.



Appendix Figure 1: Box-plot of mean digital cushion adipocyte area plotted against body condition score within 7 days of slaughter. Samples from 3, 6, 20, 6, 4, 7 and 5 digital cushions are included in each group.

Appendix 2: Examiners

Many thanks to Prof Christoph Mülling and to Dr John Burford for having examined this work.