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# A history of lameness is associated with reduced proportions of collagen type I relative to type III in the digital cushions of dairy cattle

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## ABSTRACT

Hoof horn lesions (HHL) are a highly prevalent and recurrent causes of lameness in dairy cattle globally. The digital cushion is composed of 3 cylinders of adipose tissue embedded in a system of collagenous connective tissue, which are designed to reduce the risk of HHL onset. Previous research has identified that animals with a history of lameness and HHL are more likely to have a reduced digital cushion volume in their lateral digits, but the impact on the histological structure remains unknown. Collagen is an important fibril related to adipose tissue structure and function, but its role in the digital cushion is poorly understood. Our study aimed to examine the proportions of type I and type III collagen within the digital cushions of dairy cattle at cull, and to investigate associations with digital cushion volume, lameness, and HHL occurrence throughout the animal's life. This retrospective cohort study resulted in 599 digital cushions being dissected from the hind feet of 54 animals. Digital cushion tissue underwent picrosirius red staining, combined with systematic random sampling and collagen content analysis. The results described the relative proportions of type I and type III collagen. The proportion of type I collagen was used as the outcome variable in multivariable linear regression models. The median (minimum–maximum) proportion of collagen that was type I contained within the lateral and medial digits was 56.2% (23.6%–83.8%) and 59.6% (13.3%–92.7%) respectively. The proportion of type I collagen was lower in animals that had a history of HHL and lameness throughout their lives. Animals with a lower BCS at cull or that were culled at a later parity had less type I collagen in their lateral digits at cull. Animals with a higher digital cushion volume also had an increased proportion of type I collagen in their lateral digits at cull. Our results have highlighted the histological impact that HHL have on the structure of the digital cushion. We hypothesized that localized inflammation associated with HHL was associated with a remodeling of the adipose tissue within the digital cushion, which would predispose the individual to a future of lameness and HHL.

**Key words:** dairy cow, collagen, lameness, hoof horn lesion, digital cushion

## INTRODUCTION

The digital cushion is a key structure in hoof horn anatomy which functions a as force and pressure dissipater in the digit, reducing the risk of hoof horn lesions (**HHL**) such as sole hemorrhage, sole ulceration, and white line disease. These lesions are highly prevalent and recurrent in nature (Leach et al., 2012; Solano et al., 2016) and present a challenge to both animal welfare (Whay and Shearer, 2017) and the economic viability of dairy businesses (Huxley, 2013). The digital cushion has been described as 3 parallel cylinders of connective tissue containing adipose depots that extend beneath the distal phalanx dorsally from the bone's most caudal aspect (Räber et al., 2004). This structure exists to protect the horn-producing germinal epithelium by transferring pressure away from the flexor tuberosity during locomotion and standing (Räber et al., 2004; Newsome et al., 2017a). Animals with a thinner digital cushion have been shown to be at an increased risk of lameness, and digital cushion thickness has been associated with the BCS of the individual animal (Bicalho et al., 2009; Newsome et al., 2017a; Griffiths et al., 2020). Previous research suggests that thinner digital cushions are less able to dissipate the concussive forces of hoof strikes, meaning that contusions within the hoof capsule are more likely to occur, resulting in the disruption of

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

horn production and leading to the formation of HHL (Bicalho et al., 2009; Newsome et al., 2016, 2017a,b). Indeed, this has been supported by longitudinal studies demonstrating that BCS loss precedes lameness (Green et al., 2014; Randall et al., 2015).

Laboratory studies have dissected the digital cushions from hooves of cadaver dairy cattle to relate histological measurements of the structure to animal variables (Hiss-Pesch et al., 2019; Newsome et al., 2021). Those studies found that the digital cushions of lower BCS animals contained smaller adipocytes than those of higher BCS animals. Furthermore, Räber et al. (2004, 2006) identified variations between the digital cushions of older animals compared with their younger counterparts. Notably, the digital cushions of heifers contained mainly loose connective tissue, whereas animals in their second or third parity contained significantly more fat, and animals in their fourth or greater lactation had large amounts of connective tissue and little adipose tissue (Räber et al., 2004). Räber et al. (2006) also identified differences between the fatty acid composition of the digital cushions across parities; heifers had more arachidonic acid than animals in their second or greater parity. In relation to fatty acid composition, Newsome et al., (2021) showed lower C10:0 capric acid, C14:0 myristic acid, C15:0 pentadecanoic acid, and C20:0 arachidic acid in animals with BCS 1.50 to 2.50 compared with BCS 3.00 to 4.00, and C22:1n-9 erucic acid was lower in cows aged 2 to <4 yr compared with those aged ≥4 to 6 yr.

A history of lameness caused by HHL has also been linked to a compromised digital cushion structure. Our previous work identified that animals experiencing lameness or HHL throughout their lives were more likely to have a reduced digital cushion volume in their lateral digits when culled (Wilson et al., 2021). We hypothesized that the inflammatory processes associated with the onset and development of HHL utilized the fatty acids contained within the digital cushion, leading to the development of highly fibrotic scar tissue (Newsome et al., 2016). Newsome et al. (2016) suggested that the deposition of connective tissue identified by Räber et al. (2004) may be linked with fat being mobilized from the digital cushion to facilitate the inflammation associated with HHL pathogenesis, but this is yet to be proven. These studies suggest that an animal's lameness history, age, parity, and BCS are linked to the structure and function of the digital cushion. It is hypothesized that changes to the digital cushion structures that result from lameness and HHL will predispose the individual to future HHL and lameness events.

It has already been demonstrated that there are key genetic (Oikonomou et al., 2014; Stambuk et al., 2020) and developmental (Gard et al., 2015) elements influencing the structure of the digital cushion. The potential increase in occurrence of HHL driven by this predisposition could lead to further reduction of the digital cushion volume by redistributing fatty acids into inflammatory pathways. This utilization of fatty acids from adipose tissue has been shown to lead to the remodeling of the cellular structure of adipose tissue (Contreras et al., 2017a,b). The extent of this remodeling in the digital cushion remains to be observed, and the implications that this remodeling may have on functionality of the digital cushion are yet to be elucidated. Collagen fibrils are thought to be essential in the structure and function of adipose tissue (Ojima et al., 2016), but their role in the digital cushion is poorly understood.

Despite the importance of the digital cushion, little research has been conducted investigating the histology of this structure, especially in relation to the impact of lameness and HHL on its structure and function. Previous work has identified anatomical structures and key differences in the digital cushion structures of different animals (Räber et al., 2004, 2006), particularly in relation to collagenous connective tissue. Our present study aimed to compare lameness, the occurrence of treatment for HHL, and BCS history on collagen composition within the digital cushion to further understand the mechanisms by which lameness increases the risk of future lameness events.

The current study was designed to investigate the associations between animal variables (including lameness history) and the collagen content of the digital cushion in adult dairy cattle at culling, using picrosirius red (**PSR**) staining of digital cushion histology. The null hypothesis stated that collagen type content contained within the digital cushions within the hind hooves of adult dairy cows at cull was not associated with a history of lameness or HHL during the animal's lactating lifetime.

## MATERIALS AND METHODS

### *Study Design*

A retrospective cohort study was designed and conducted to investigate the association between animal-level variables and the proportions of types I and III collagen contained in the digital cushion at cull, and is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines (von Elm et al., 2008; STROBE, 2020). The study was conducted with permission from the University of Nottingham School of Veterinary Medicine and Science ethics committee (reference no. 1913 161208). The population described within this study was the same as described in Wilson et al. (2021), and we refer the reader to that publication for a more detailed description of herd management and data collation.

### Study Herd and Management

Our study recruited Holstein cows that were culled from the Scotland's Rural College (**SRUC**) Langhill research herd, based at the Crichton Royal Farm in Dumfries, Scotland. To be eligible, animals also needed to have been recruited to our previous study (Wilson et al., 2021). Our study collected the hind feet from cull dairy cows at slaughter, stored locally at −20°C before transportation to the University of Nottingham. Once thawed, they were scanned using magnetic resonance imaging (**MRI**) to collate digital cushion volume and fat content, then refrozen before dissection (see Wilson et al. 2021 for details).

The Crichton Royal research herd is extensively documented and has been described previously, and we encourage the reader to refer to Pryce et al. (1999), Chagunda et al. (2009) and Randall et al. (2016) for a comprehensive description of the facility and its management. Briefly, animals were housed continuously and milked 3 times daily in one of 2 herds (Langhill or Acrehead). The Langhill herd was more intensively monitored than the Acrehead herd in all aspects of data pertinent to this study. At Langhill, the herd was managed on a long-term 2 × 2 factorial study, where animals belonged to either a control or select genetic line and were managed on one of 2 ration types from 3 trials as described by Wilson et al. (2021).

Routine hoof care at the Langhill herd took the format of weekly or fortnightly mobility scoring (using the 5-point mobility score system of Manson and Leaver, 1988) and routine hoof inspections, as described in our previous paper (Wilson et al., 2021). Animals would be identified as lame and presented for treatment following a single score 4 or 5 (described as "obvious lameness affecting behavior" or "severe lameness with extreme difficulty walking," respectively) or 2 consecutive scores of 3 (described as "lameness that does not affect behavior"). This treatment (for HHL or other causes of lameness) was conducted by either a veterinarian or trained technician and recorded on the farm management software. Body condition score was captured weekly using the system described by Mulvany (1977). Animals were moved to the Acrehead herd after their fourth lactation ended, unless they had been moved there for management purposes beforehand.

### Selection Criteria

Animals were eligible for study enrollment if they were included in our previous research and MRI scans of both hind feet were available (Wilson et al., 2021). To be eligible for dissection, animals had to have been culled directly from the Langhill herd and have a comprehensive history of lameness scoring (and thereby lameness causing HHL treatment records), BCS, and milk yield until 7 d before culling.

### Freeze-Thaw Protocols

Once transported, the hooves were thawed at room temperature for 24 ± 3 h before scanning and then refrozen at −20°C within 4 h of the scanning taking place. Scans were conducted as previously described (Wilson et al., 2021).

### Dissection Protocol

Tissue sections were cut from the sole of the still frozen hoof along the coronal plane using a bandsaw until the digital cushion was visible for further dissection, (as seen in Figure 1). After the digital cushion was exposed, the specimens were left at room temperature for 12 ± 4 h to thaw. Individual cushions were removed by dissection, and cut into cuboidal pieces measuring ~5 mm × 10 mm × 10 mm (height, width, length) and placed into scintillation vials containing 4% paraformaldehyde (**PFA**) for 12 h ± 3 h at 5°C. Vials were labeled with the following information: (1) cow ID, (2) limb ID (L = left, R = right), (3) hoof capsule ID (L = lateral, M = medial), (4) cushion ID (1 = abaxial, 2 = middle, 3 = axial), (5) sample number (which varied for each animal and cushion depending on the cushion size).

### Sample Processing

Once removed from the 4% PFA fixative, the samples underwent the following fixation/processing protocol: the samples were (1) submerged in 1× PBS and stored for 7 to 14 d (minimum 7 d, maximum 14 d) at 5°C; (2) dehydrated in increasing concentrations of ethanol (70% for 1.5 h at room temperature, 90% for 72 h at 5°C, 100% for 3 h at room temperature [each ×2]); (3) immersed in xylene for 3 h [×2]; and (4) embedded in paraffin wax at 60°C.

### Sectioning Protocol

Serial tissue sections were cut at 9-µm thickness using a microtome (Leica Microsystems Ltd., UK) with a N35 long duration stainless steel microtome blade (FEATHER, Japan) and mounted onto polysilinated glass microscope slides (Fisher Scientific Ltd., UK). All sections were cut on the sagittal plane. The slides were stored at room temperature before staining.

### Picrosirius Red Staining

Tissue sections were de-paraffinized in xylene for 4 min, and then rehydrated through an ethanol series (100%, 90%, 70%; 5 min each). Next, the PSR solution (Abcam, UK, ab150681) was applied for 1.5 h. The sec-

**Figure 1.** Photograph showing the coronal cut taken to expose the digital cushion, enabling further dissection. Digital cushions were then sectioned and stained with PSR, allowing quantification of collagen types I and III within the digital cushions (n = 108 hind feet from 54 dairy cattle). Analysis was undertaken against animal-level variables to understand the effects of lameness and BCS on the digital cushion structure.

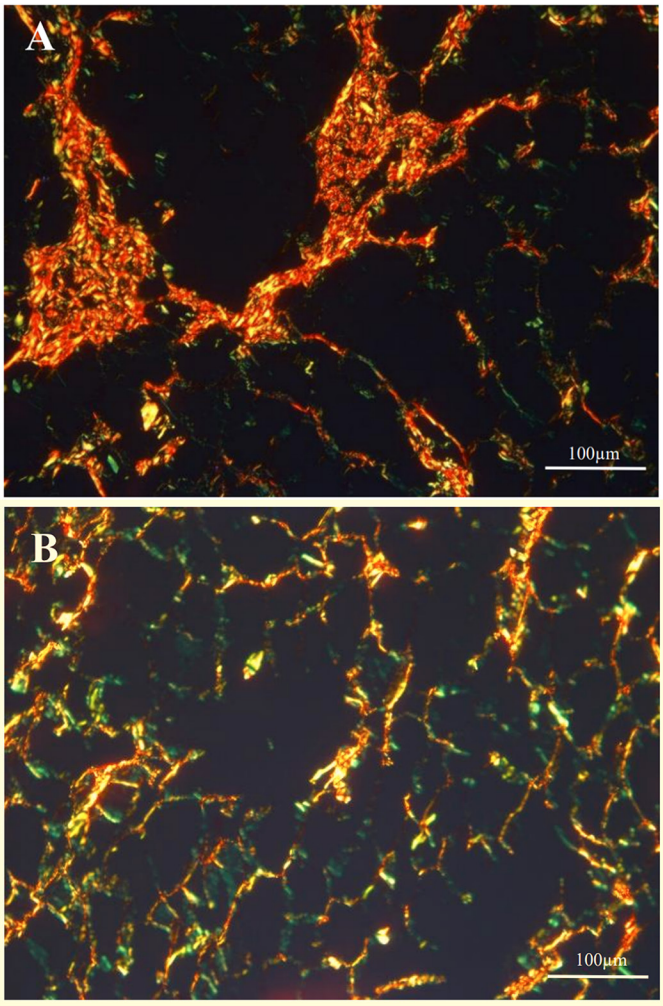


**Figure 2.** Photomicrographs illustrating collagen in the digital cushion in sound (A) and lame (B) animals. (A) Middle cushion of a left lateral digit from an animal with a mean lameness score of 1.8 in the 4 wk before cull. Type I collagen content (red fibrils) was greater than the type III collagen (green fibrils). (B) Middle cushion of a left lateral digit of an animal exhibiting a mean lameness score of 4.3 in the 4 wk before cull. This photomicrograph indicates more type III collagen compared with type I.

tions were then rinsed in 2 changes of acetic acid solution, followed by one rinse with 100% ethanol. Sections were dehydrated in 2 changes of 100% ethanol for 5 min each, placed in xylene for 4 min, and coverslips were then mounted using distyrene, plasticizer, and xylene mounting media. All staining was conducted by the same operator (AA).

### *Microscopy and Sampling Techniques*

Systematic random sampling (Mayhew and Burton, 1988) was used to capture images of one tissue section per slide from every cushion. This method ensured coverage of each specimen without area duplication. Samples were viewed and photographed under polarized light microscopy (DM5000 B, Leica, Germany) with 5 images captured. Images were exported in .tiff format with a 100-µm scale bar. All microscopy was carried out by a single, blinded trained operator (JPW). Example photomicrographs are shown in Figure 2.

### *Image Analysis*

Images were processed using Fiji software (Schindelin et al., 2012). Two macros were designed to analyze the amount of red and green color in the image. These macros are shown in Supplemental Figure S1 (see Notes), and the color thresholds described in these macros are the same as those described by (Zerbinati and Calligaro, 2018). The macros identified and quantified the number of pixels relating to each of the 2 wavelength ranges identifying green- or red-stained collagen.

Once the total pixels of each respective color had been collated, the proportion of pixels defined as red or green was calculated by dividing the total area of pixels for

**Table 1.** Total number of digital cushions made available for staining and microscopy following dissection and processing[1]

| Digit identification | Cushion identification | | |
|---|---|---|---|
| | Abaxial | Middle | Axial |
| Left lateral | 52 | 48 | 53 |
| Left medial | 54 | 45 | 53 |
| Right lateral | 48 | 44 | 48 |
| Right medial | 46 | 44 | 47 |

[1]Samples were dissected from a cohort of 54 animals culled from the Langhill herd based at SRUC's Crichton Royal Farm, selected based on their comprehensive lameness history.

either red or green by the total area of colored pixels. The proportions of red and green compared with each other were also calculated. Data were collated and stored into Microsoft Excel 2016 files (Microsoft Corp.).

### Data Handling and Manipulation

Available herd data captured the time spent within the milking herd since first calving (i.e., no dry-period or pre-first calving data were available). Animal data were exported from the farm database into Microsoft Excel files for the period of April 10, 2010, to September 27, 2018. All data handling and analysis was carried out using Microsoft Excel 2016 and RStudio V1.2.5033 (RStudio Team 2019, RStudio: Integrated Development for R, RStudio Inc.). Data handling and screening included exploration of the data to identify missing values or errors. The timeframes used in the analysis were based on the date of an animal's lameness score giving a "week of score" to which other data would be appended. Data collected between lameness scores were adjoined to the week of score at the beginning of the respective 7- or 14-d period that existed between the scores. Descriptive statistics were used to investigate the distribution of datapoints and assist in the construction of explanatory variables for statistical models.

### Statistical Modeling

Multivariate linear regression models were constructed to investigate the associations between lameness history, digital cushion volume, and collagen content within the digital cushions. The outcome variable was the proportion (%) of collagen that could be attributed as type I within the lateral digits of both hind hooves at cull, which was calculated by averaging the proportion of type I collagen within the axial, middle, and abaxial cushion contained within the lateral digits of left and right hind hooves. This, as a function, also described the proportion of type III collagen, as it was the proportion of type I relative to type III that was described. Linear regression models were constructed in R Studio, taking the following format:

$$Y_i = \beta O + \beta_i X_i + \cdots + eO_i,$$

$$\left[eO_i\right] \sim N\left(0, \sigma_e^2\right),$$

where $Y_i$ is the outcome variable of the $i$th cow, $X_i$ represents the explanatory variables for the $i$th cow, $\beta_i$ is the coefficient for the respective explanatory variables for the $i$th cow, $\beta O$ is the intercept value, and $eO_i$ is the residual error term, with an assumed normal distribution with mean = 0 and variance = $\sigma_e^2$. All explanatory variables were tested within the model initially using univariate linear regression models to explore the associations between individual variables and the outcome variable. Final models were constructed with a forward stepwise approach with variables being retained in the model when $P < 0.05$.

Model fit was assessed by visual assessment of residuals and the removal of outlying datapoints (large influence or high leverage) to check their impact on model parameters. Histograms of residuals and Cook's D plots were used for this visualization (Cook, 1977). If coefficients remained statistically similar (<5% difference) upon outlier removal with the same biological interpretation, then model fit was deemed adequate. Associations between the proportions of collagen, digit, and hoof location were investigated using Mann–Whitney U tests, scatterplots, and histograms.

The explanatory variables used were identical to those described in Wilson et al. (2021). Variables were constructed to explore the impacts of first lactation, last lactation, and the entire productive life ("the period described") of the individual and were calculated as described, as follows: Number of HHL treatments recorded (as either a continuous or categorical [0–1, 2–6, 7+] variable for the period described); proportion of lameness scores as "sound" (i.e., score 0–2 using the Manson and Lever system; proportion of lameness scores recorded as ≤2 for the period described); proportion of BCS >3 (an average of all BCS recorded for the individual for the period described); average live weight (in kg; an average of the recorded live weights for the period described); average daily milk yield (in L), calculated by averaging the daily milk yield for the period described); age at cull (in mo); DIM at cull; the animal's genetic line according to herd management (control or select); the feed regimen to which the animal belonged throughout its lifetime (higher or lower level of nutrition); the DIM at which an animal first became lame; average BCS during the 4 wk before cull; and average lameness score during the 4 wk before cull. Animals

**Table 2.** Descriptive statistics of the proportion of type I collagen within the digital cushions of the lateral and medial digits[1]

| Item | Digit | Mean | Median | Interquartile range | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Proportion (%) of type I collagen | Lateral | 56.2 | 54.9 | 6.5 | 23.6 | 83.8 |
| | Medial | 59.6 | 57.7 | 8.1 | 13.3 | 92.7 |

[1]The proportion of type I collagen in each hoof capsule was derived from the average collagen content across the 3 cushions within each capsule. Samples were dissected from 54 animals culled from the Langhill herd based at SRUC's Crichton Royal Farm, selected based on their comprehensive lameness history.

would be included in the models if a complete dataset for all variables was present up to 7 d before cull.

## RESULTS

### Study Denominators

A total of 599 individual digital cushions were dissected from the hind hooves of 54 animals. A description of the cushion locations is provided in Table 1. Cushions were classified by digit location (left or right, medial or lateral) and cushion type (abaxial, middle, axial). A total of 49 digital cushions were deemed irretrievable from the 54 animals selected (i.e., no digital cushion could be dissected out from the digits; this was cross-examined against the MRI images to confirm no digital cushion presence), and 17 were lost due to handling and processing errors, meaning that a total of 582 cushions were dissected from 216 digits from 108 hind hooves belonging to 54 animals. Of the animals dissected, the mean parity at cull was 2.4 and the mean age at slaughter was 53 mo. From the 582 digital cushions, 2,915 photographs were taken for the purpose of analysis.

### Descriptive Statistics

Table 2 describes the results derived from the analysis of all 108 hind hooves. The median (minimum–maximum) proportion of collagen that was type I contained within the lateral and medial digits was 56.2% (23.6%–83.8%) and 59.6% (13.3%–92.7%), respectively (Figure 3 $P = 0.03$). The median (minimum–maximum) proportion of type III collagen contained within the lateral and medial digits was therefore 43.8% (16.2%–76.4%) and 40.4% (7.3%–86.7%), respectively. The collagen content of the digital cushions in the lateral digits consisted of more type I collagen than type III collagen in 35 of the 54 animals (Figure 4). Additionally, the volume of digital cushion, as derived from the mDIXON Quant sequence described in our previous paper (Wilson et al., 2021), was not strongly correlated with the proportion of type I collagen contained within the respective cushions ($R^2 = 0.05$; Figure 5). The median (minimum–maximum) proportion of type I collagen contained within the left and right hoof was 59.3% (11.7%–92.7%) and 57.4% (30.0%–82.01%), respectively ($P > 0.05$).

Histological data from the 54 animals with a complete history of lameness and BCS data until a maximum of 7 d before cull was available to construct all models. Of these 54 animals, 22 had at least one treatment for an HHL recorded during their lactating lifetime. A total of 8 recordings of sole ulceration were recorded among 4 cows, 78 recordings of sole hemorrhage were recorded among 18 cows, 26 recordings of white line disease were recorded among 8 cows, 54 recordings of infectious causes of lameness were made among 18 cows, and 30 recordings of "other" lesions were recorded among 11 cows. Table 3 describes the prevalence of hoof lesions (both infectious and noninfectious) throughout our population of cattle. Table 4 describes the explanatory variables constructed for animals retained within the models.

### Statistical Modeling

All of our models are summarized in Table 5.

***Model A. Associations Between Lameness and BCS in the 4 Wk Before Cull and Collagen Content of the Digital Cushion.*** Animals with an elevated mobility score in the 4 wk before culling had a lower proportion of type I collagen in their lateral digit digital cushions. For every 1-point increase in the average mobility score in the 4 wk before cull, the proportion of type I collagen contained within the digital cushion was lowered by 3.97% (95% CI: −6.87 to −1.07, $P = 0.01$). Animals with an increased average BCS in the 4 wk before cull had a higher proportion of type I collagen contained within their digital cushions. For every 1-point increase in the average BCS in the 4 wk before cull, the proportion of type I collagen contained within the digital cushions was higher by 6.49% (95% CI: 2.69 to 10.29, $P = 0.003$).

***Model B. Associations Between a Lifetime History of Lameness and Collagen Content of the Digital Cushion.*** Animals with an increased proportion of their life spent as sound had a higher proportion of type I collagen in the digital cushions within their lateral digits. For every 1% increase in the time spent sound, the proportion of type I collagen contained within the digital cushion was higher by 0.16% (95% CI: 0.03 to 0.29, $P = 0.03$).

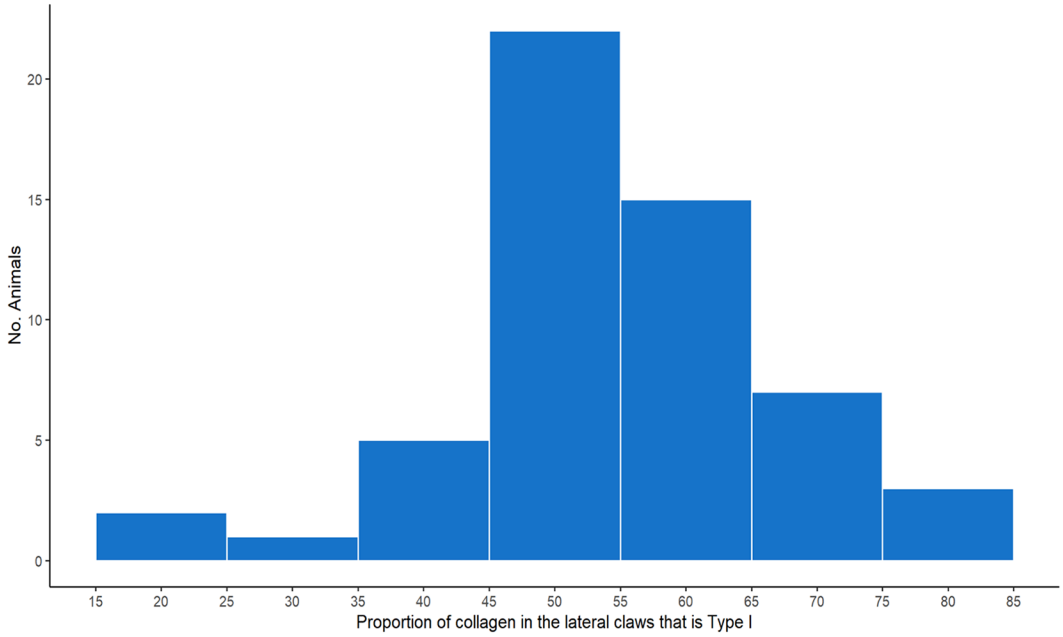


**Figure 3.** Histogram showing the proportion of type I collagen within the digital cushions of 108 lateral digits from the hind feet of 54 culled dairy cows. Animals were from the Langhill herd at Scotland's Rural College's Crichton Royal Farm (Dumfries, Scotland) and selected for analysis if a complete mobility history was present. Collagen content was determined through histological staining with PSR, and the proportion of type I collagen was calculated as a relative measurement to the proportion of type III collagen, as per Zerbinati and Calligaro (2018).

In our study, we observed a range of effects in line with parity at cull; an increased parity number at cull may be associated with a substantial reduction in type I collagen content, or it may be associated with a biologically unimportant increase. For every additional lactation the animal lived for, the proportion of type I collagen contained within the digital cushion of the lateral digits was lowered by 2.62% (95% CI: −5.34 to 0.1, $P = 0.06$). Animals with a greater volume of digital cushion in their lateral digits at cull had a greater proportion of type I collagen. For every 1-mL increase in digital cushion volume, the proportion of type I collagen increased by 0.52% (95% CI: 0.02 to 1.01, $P = 0.04$).

***Model C. Associations Between Hoof Horn Lesion Treatments and Collagen Content of the Digital Cushion.*** Animals with an increased number of HHL recorded during the lactating lifetime had a lower proportion of type I collagen in their digital cushions. For every increase of 1 in the number of HHL recorded the proportion of collagen type I was reduced by 1.60% (95% CI: −2.40 to −0.80, $P = 0.002$).

## DISCUSSION

Animals with a history of lameness were more likely to have a lower proportion of type I collagen in the digital cushion of their lateral digits at cull. The outcome measure for our study was the proportion of type I collagen contained within the lateral digits of dairy cattle at cull, and this was calculated as a proportion relative to type III collagen. This lower proportion of type I collagen conversely resulted in a higher proportion of type III collagen (due to the proportion of type I collagen being calculated as a function of the sum of type I and type III collagen).

The retrospective nature of this study design means that the direction of causality cannot be established from our results. There are 2 clear mechanisms through which the differences in collagen content may be associated with lameness. First, the study animals experienced lameness, which is underpinned by localized inflammatory mechanisms occurring within the hoof capsule. These inflammatory mechanisms could lead to a turnover of collagen tissue and the remodeling of the adipose tissue supported therein. Alternatively, or additionally, alterations to the collagen content of the digital cushion may have been caused by non-lameness or HHL-associated events, which predisposed the animals to lameness, thereby creating the observed association. Regardless of the direction of causality, there was a link between a lifetime history of lameness and the proportions of type I and type III collagen within the lateral digits of the hindlimbs at cull.

Type I collagen is essential for the organization and competence of many key structures throughout the body, irrespective of species. It is organized into regular fibril

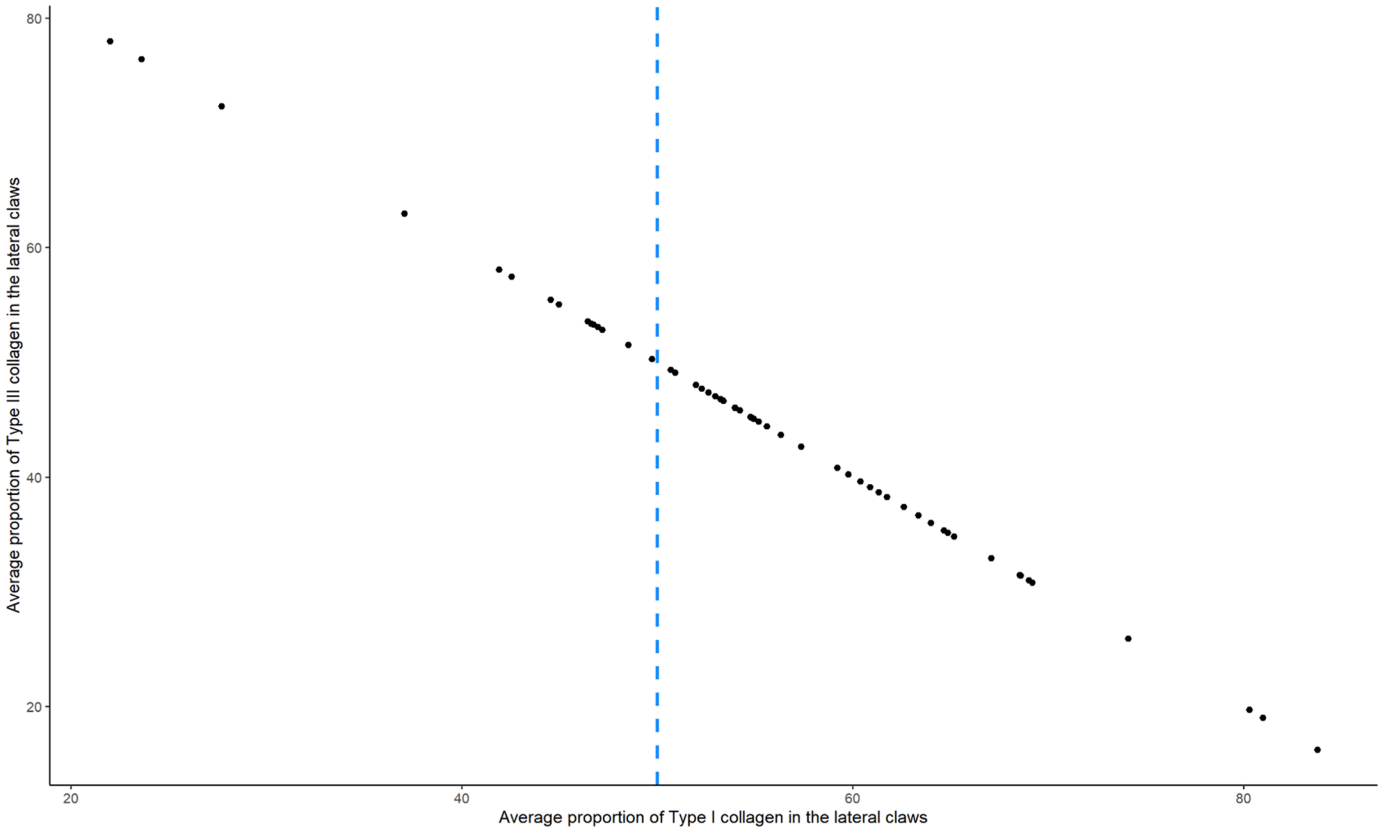


**Figure 4.** Scatterplot showing the proportion of type I versus type III collagen within the digital cushions of 108 lateral digits from the hind feet of 54 dairy cows. Animals were culled from the Langhill herd at Scotland's Rural College's Crichton Royal Farm (Dumfries, Scotland) and selected for analysis if a complete mobility history was present. Collagen content was determined through histological staining with PSR, and the proportion of type I collagen was calculated as a relative measurement to the proportion of type III collagen, as per Zerbinati and Calligaro (2018). Animals represented to the right of the vertical dashed line had more type I collagen in their digital cushions at cull than type III.

bundles, which can undergo post-transcriptional changes to optimize functionality in situ (Charvolin and Sadoc, 2019). Type III collagen exists as single fibrils and is essential in the healing of wounds (Volk et al., 2011). Typically, type III collagen is deposited first, and the proportion of type I collagen increases as wound healing progresses (Clore et al., 1979). Furthermore, type III collagen is typically deposited by mesenchymal cells (particularly fibroblasts), which means it has a substantial role to play in various inflammatory related conditions (Ku et al., 2006). It is understood that the matrix metalloproteinases (**MMP**) have a specific role to play in the degradation of collagen (Van Doren, 2015), which is typically not affected by proteolytic enzymes and processes. Matrix metalloproteinases are expressed as inflammatory mediators (Van Doren, 2015), highlighting a potential inflammatory pathway through which the proportion of collagens may be altered due to pathology. If higher levels of MMP are present within the hoof capsule, then this may degrade the collagen contained within the suspensory apparatus and the digital cushion, and the functional anatomy of the hoof will have a lower tolerance for the concussive and compressive forces associated with locomotion and standing, respectively, meaning that contusions within the corium are more likely to occur, leading to the disruption of horn production and HHL onset. The degradation of collagenous structures driven by MMP may lead to the permanent compromising of the digital cushion's ability to adequately protect the germinal epithelium from the excessive forces associated with locomotion and standing. If animals experience repeated systemic inflammatory events, such as calvings and transition periods (Bradford et al., 2015), then degradation and compromise of the digital cushions structure and function may accumulate over several lactations. Our previous work has highlighted the importance of both local and systemic inflammation in the etiology of HHL (Wilson et al., 2022). The inflammatory nature of HHL may explain this degradation of collagenous structures contained within the hoof capsule. Alternatively, systemic inflammation occurring during the transition period (Bradford et al., 2015) may lead to an increase in MMP

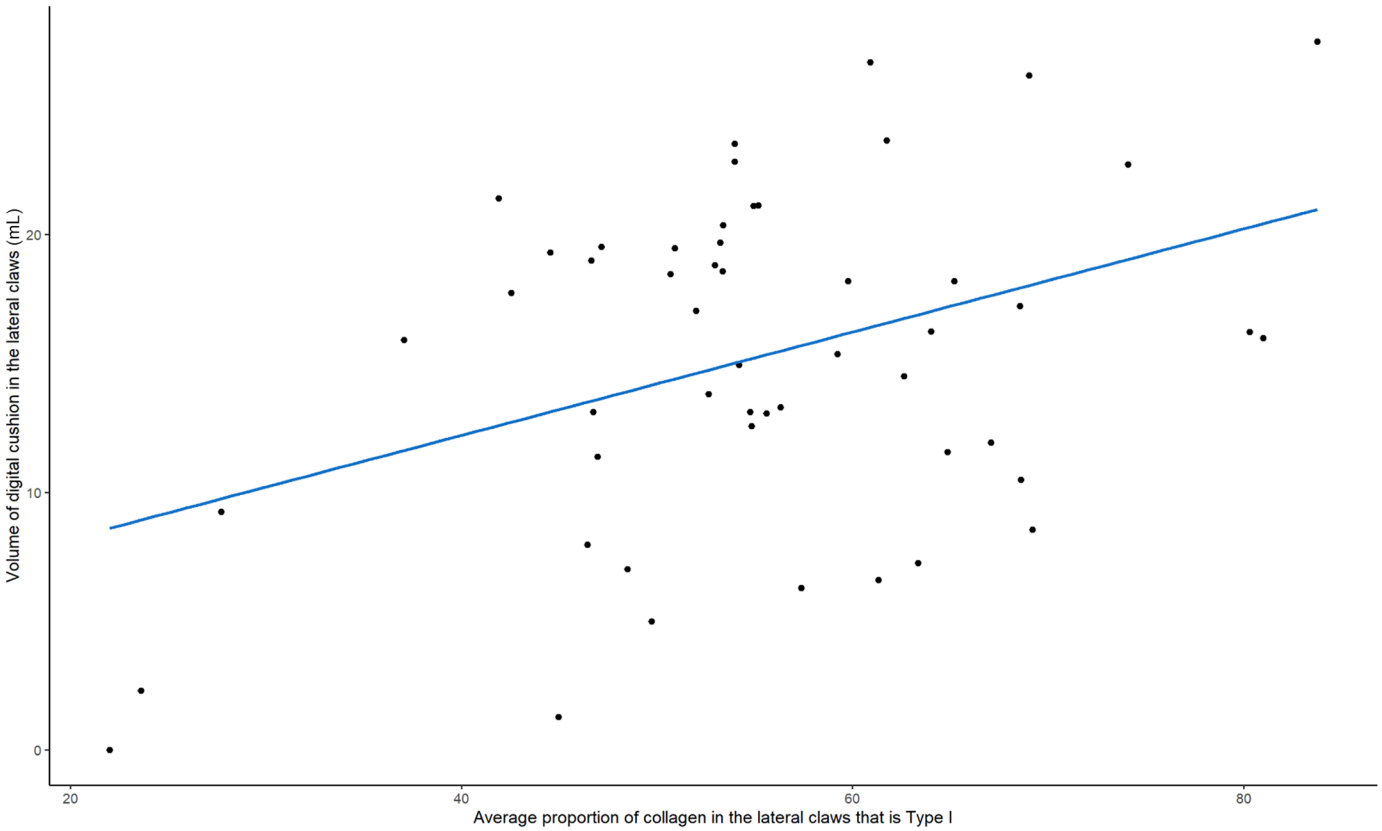


**Figure 5.** Scatterplot showing the proportion of type I collagen against the digital cushion volumes within 108 lateral digits from the hind feet of 54 dairy cows. Animals were culled from the Langhill herd at Scotland's Rural College's Crichton Royal Farm (Dumfries, Scotland) and selected for analysis if a complete mobility history was present. Collagen content was determined through histological staining with PSR, the proportion of type I collagen was calculated as a relative measurement to the proportion of type III collagen, as per Zerbinati and Calligaro (2018), and the digital cushion volume was measured using MRI scans, as per Wilson et al. (2021). Blue line represents $R^2 = 0.05$.

circulation leading to a degradation of the collagenous structures within the hoof capsule, in turn predisposing the individual to HHL. Matrix metalloproteinases and their potential involvement represent a potential area of future investigation in this field.

Previous studies have identified how the digital cushions of older animals appeared to contain more dense collagenous tissue than those of heifers (Räber et al., 2004). Those authors suggested that changes occurred within the first lactation, which led to differences in the digital cushion structure. They went on to discuss how HHL pathogenesis and calving and transition may have a role to play in the alteration of digital cushion structure. The proposed inflammatory pathogenic mechanisms underpinning HHL etiology (Newsome et al., 2016) may directly affect the digital cushion via other means, in addition to purely utilizing fatty acids as mediators in biochemical processes. If the inflammatory processes described by Newsome et al. (2016), and Wilson et al. (2022, 2025) are occurring within the hoof capsule, then it is likely that these could have an impact on the proportions of type I and type III collagen contained within the digital cushion, as shown in the present study. The impacts of this change in collagen proportions are unknown, apart from the associations with lameness, but it could be hypothesized that an increase of type III collagen (which is often indicative of scarring) may inhibit the digital cushion's ability to dissipate the concussive forces associated with locomotion and standing. This could be due to 2 mechanisms; first, the irregularity in structure of type III collagen may mean that it is less structurally sound than type I collagen (i.e., it may offer less support and structure to the adipocytes contained within the digital cushion; Clore et al., 1979). Additionally, the deposition of more type III collagen will likely mean that the proportion of collagen to adipocytes is also likely to change. This may, once again, impede the functionality of the digital cushion in its ability to protect the corium from excessive compression. Newsome et al. (2016) also found that exostoses developed on the most caudal aspect of the pedal bone, and the extent of these bony proliferations was correlated with the lameness history of the ani-

**Table 3.** Summary of the hoof horn lesions recorded across 54 animals culled directly from the Langhill herd at the Royal Crichton research dairy herd (SRUC Dumfries, Scotland)[1]

| Hoof lesion recorded | Total number of recordings | Number of cows affected |
|---|---|---|
| Sole ulceration | 8 | 4 |
| Sole hemorrhaging | 78 | 18 |
| White line disease | 26 | 8 |
| Infectious causes (e.g., digital dermatitis) | 54 | 18 |
| Other (e.g., abscesses and double sole) | 30 | 11 |

[1]Animals were selected based on the presence of a complete lameness history. Hind hooves were passed through a 3 Tesla MRI scanner based at the Sir Peter Mansfield Imaging Centre (University of Nottingham, Nottingham, UK) using a mDIXON Quant sequence before having all retrievable digital cushions dissected. Variables were constructed based on animal data made available from the farm database and encapsulated the time spent within the milking herd since first calving.

mal. In a subsequent publication (Newsome et al., 2021), the authors went on to explain how the development of these exostoses could degrade the structure of the digital cushion, as they both exist at the same site, so interaction is highly likely. Indeed, in our work, we found that dairy cows that experienced a history of lameness throughout their lifetime had a significantly reduced (−0.89 mL in combined lateral digital cushion volume with an increase in HHL recordings of 1) digital cushion volume in their lateral digits at cull (Wilson et al., 2021). Both Newsome et al. (2021) and Wilson et al. (2021) suggested that the histology of the digital cushion may change due to the trauma exerted on it by the excessive development of new bone and reduced digital cushion volume, thereby predisposing the animal to future HHL due to compromised digital cushion function. These changes to the structure of the digital cushion could potentially increase the susceptibility of the horn producing germinal epithelium to compressive and concussive forces. This could mean that HHL are much more likely to occur due to this compromised functionality of the digital cushion. The ability of the digital cushion to act as a metabolically active tissue may also change as the differences in structure differ or change. One mechanism through which this could occur is via fatty acids becoming less readily available locally within the hoof capsule due to adipose tissue remodeling, as described by Contreras et al. (2017a,b). This could lead to other localized changes to anatomy and metabolic function within the hoof capsule, which may predispose the animal to future HHL.

It could be hypothesized that the histological structure of the digital cushion may vary between animals due to their genetic potential and the rearing system in which they were managed as youngstock. Recent research describing the role of genetics in the onset of HHL has yielded substantial impact (Oikonomou et al., 2014; Barden et al., 2022), and relating this to measurements of digital cushion histology may aid in understanding the variation within our population more effectively. However, from our control and select genetic lines, we observed no impact of the proportions of collagen contained within the digital cushion. Additionally, further understanding of the medial digit digital cushion, its structure, and the potential role of genetics is required. In our population, <2% of hoof lesions were recorded on the medial digit, and we therefore did not investigate the outcomes on collagen structure. No previous research in this field has examined the effects of either of genetic predispositions or

**Table 4.** Summary of explanatory variables constructed for 54 animals culled directly from the Langhill herd at the Royal Crichton research dairy herd (SRUC Dumfries, Scotland)[1]

| Item | Mean | Median | Maximum | Minimum |
|---|---|---|---|---|
| Lactation number at cull | 2.4 | 2.0 | 5.0 | 1.0 |
| Age at cull (mo) | 52.8 | 51.0 | 92.0 | 27.0 |
| Proportion of scores submitted as sound (%) | 73.8 | 79.1 | 100.0 | 0.00 |
| Proportion of scores submitted as BCS >3 (%) | 2.6 | 0.0 | 28.1 | 0.0 |
| Average daily weight (kg) | 584.1 | 585.7 | 680.1 | 443.2 |
| Average daily milk yield (L) | 27.7 | 27.7 | 40.6 | 16.2 |
| Number of HHL treatment records | 1.9 | 0.0 | 18.0 | 0.0 |
| Average lameness score 4 wk before cull | 2.3 | 2.1 | 5.0 | 0.0 |
| Average BCS 4 wk before cull | 2.3 | 2.2 | 3.5 | 1.4 |

[1]Animals were selected based on the presence of a complete lameness history. Hind hooves were passed through a 3 Tesla MRI scanner based at the Sir Peter Mansfield Imaging Centre (University of Nottingham, Nottingham, UK) using a mDIXON Quant sequence before having all retrievable digital cushions dissected. Variables were constructed based on animal data made available from the farm database and encapsulated the time spent within the milking herd since first calving.

**Table 5.** Outputs from linear regression models investigating the association between lameness and the collagen content of digital cushions in the lateral digits of the hindlimbs at slaughter[1]

| Item[2] | Coefficient | 95% CI | *P*-value |
|---|---|---|---|
| Model A | | | |
| Intercept | 51.28 | | |
| Mean lameness score[3] 4 wk before cull | −3.97 | −6.87 to −1.07 | 0.01 |
| Mean BCS[4] 4 wk before cull | 6.49 | 2.69 to 10.29 | 0.003 |
| $R^2$ | 0.21 | | |
| Model B | | | |
| Intercept | 41.7 | | |
| Proportion of life spent sound | 0.16 | 0.03 to 0.29 | 0.03 |
| Parity at cull | −2.62 | −5.34 to 0.1 | 0.06 |
| Volume of digital cushion in the lateral digits (mL) | 0.52 | 0.02 to 1.01 | 0.04 |
| $R^2$ | 0.29 | | |
| Model C | | | |
| No. of hoof horn lesion treatments | −1.60 | −2.40 to −0.80 | 0.002 |
| $R^2$ | 0.22 | | |

[1]The outcome variable was the proportion of type I collagen, relative to type III cushion in the lateral digits. n = 582 cushions from 216 digits from 108 hind hooves from 54 animals. Animals were culled from the Langhill herd at the SRUC Crichton Royal Farm (Dumfries, Scotland) and selected for analysis if a complete mobility history was present. Explanatory variables were constructed based on animal data made available from the farm database and encapsulated the time spent within the milking herd since first calving.

[2]Model A explored the associations between lameness in the 4 wk before cull and the proportion of type I collagen in the digital cushion at cull. Models B and C explored the association between the proportion of lameness scores submitted as sound and the number of hoof horn lesions recorded throughout the animal's lactating life on the proportion of type I collagen in digital cushion at cull, respectively.

[3]Animals were lameness scored using a 5-point scale (Manson and Leaver, 1988). Animals presenting as a single score 4 or 5 (described as "obvious lameness affecting behavior" or "severe lameness with extreme difficulty walking," respectively) or twice consecutively as a score 3 (described as "lameness that does not affect behavior") were considered lame and received treatment. Animals were considered sound if they were not scored as 4 or 5, or were scored twice consecutively as 3.

[4]Body condition score was captured weekly, adopting the scoring system described by Mulvany (1977) using a 5-point scale with increments of 0.25.

rearing period on the histological structure of the digital cushion, nor on the lameness outcomes potentially generated from such factors. Future research is warranted in this area, given the large amount of unexplained variation described in the current study.

There are several limitations which should be considered when interpreting the findings of our study. First, as previously stated, the direction of causality cannot be attributed from a retrospective study such as this. The associations described should be interpreted with care, and it is recommended that they be used to inform hypotheses for future prospective intervention studies. Second, the study population examined was based on a convenience sample of 54 animals from a single dairy research herd in the United Kingdom. These animals were selected for further analysis because they had a comprehensive lameness history up to the point of cull, which may have created a bias in the population studied. It is not possible to comment on the extent of any potential bias or the impact of that potential bias on the results; however, no factors have been identified which would suggest that the animals studied are not representative of intensively managed dairy cattle. The use of cadaver material should also be considered when interpreting our results, with the observations of the current study representing an end-of-life effect that is the culmination of all experiences the animal may have had. Additionally, the method of using a lameness score as an explanatory variable means that some animals may have been misdiagnosed or not treated for an HHL that was present and causing subtle gait changes. We feel that the risk of this is minimal due to the rigorous management of this herd (particularly in relation to lameness scoring). Having a more robust quantification of mild sole hemorrhaging (which may be present, but not identifiable on lameness score alone) may reduce some of the unexplained variation within our population. It is also important to note that models A and B both use a lameness score that would encompass both HHL and other sources of lameness that do not affect the digital cushion (such as digital dermatitis or upper limb injuries). We feel that the overall impact of this is low due to the relative prevalence of HHL in comparison to digital dermatitis within our population.

The proportion of type I collagen in the lateral digits of the hind limbs varied substantially (23.6%–83.8%) between adult dairy cattle at cull. Animals with a history of lameness or HHL had a significantly reduced proportion of type I collagen when compared with their sounder counterparts. We encourage the reader to acknowledge the higher levels of sole hemorrhage recording in com-

parison to sole ulceration recording in our population. This farm aimed to intervene in early-stage lameness through regular and sensitive lameness scoring and appeared to achieve this given the low levels of sole ulceration recorded. We observed associations between HHL recordings and lameness scores on the collagen composition of the digital cushion. This could highlight that sole hemorrhage (which is an early stage of the sole ulcer etiology) has potentially important detrimental impacts on digital cushion histology. Our models explained a small proportion of variation in type I collagen content ($R^2$ ranging from 0.21 to 0.29). A large proportion of the observed variation remains unexplained. Further research is needed to understand the source of this variation between animals and what effects this variation has on future lameness risk. Evaluating novel medical imaging techniques and connecting them to histological measures may support our understanding of the dynamic structure and function of the digital cushion in both cadaver material and in live cows. This would provide insight to optimize future treatment and prevention strategies for HHL to reduce the burden of lameness on animal health, welfare, and productivity.

## NOTES

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**Nonstandard abbreviations used:** HHL = hoof horn lesion; MMP = matrix metalloproteinase; MRI = magnetic resonance imaging; PFA = paraformaldehyde; PSR = picrosirius red; SRUC = Scotland's Rural College.

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