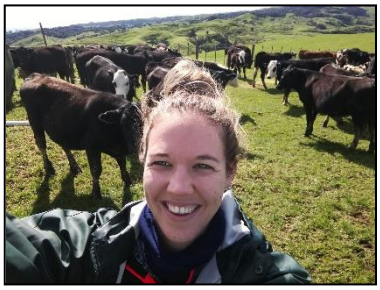

The unsolved mystery of Aotearoa: Spontaneous humeral fractures in first lactation dairy heifers

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Prefactory note

Within the Master of Science in Veterinary Medicine at Utrecht University, students have to fulfil a Research Traineeship. This paper is the final report of the Research Traineeship carried out by Eva de Jong at the department of Agriculture and Environment of Massey University in Palmerston North, New Zealand. Research was conducted into bone growth in heifer calves and the influence of nutrition during the early weaning period on optimising peak bone mass. To this end, Peripheral Quantitative computer tomography (pQCT) was conducted. Cross-sectional pQCT scan slices were acquired in the diaphysis of the third and fourth metacarpal bones of 24 heifer calves at 50% of the MC3/4 length from the carpometacarpal joint space.

Abstract

In New Zealand spontaneous humerus fracture in first lactation dairy heifers has been reported. This is a recent syndrome, with increase in the number of cases over the last seven to eight years.
The syndrome leads to an estimated loss of 5000 dairy heifers every year which represents major economic and welfare loss. It is hypothesised that spontaneous humeral fractures are occurring as a result of skeletal fragility that may be due to one or more growth checks during the rearing period rather than a specific traumatic event in early lactation. This study examined the effect of age and different feeding regiments on morphological measures of bone and measures of bone development and the correlation between measures of bone development. Peripheral quantitative computer tomography scanning was conducted on the mid-diaphyseal region of the third and fourth metacarpal bones of the right forelimb of 24 KiwiCross calves. The results of this research indicate that restricted feeding during the first 12 weeks of the life of a calf does not cause a difference in bone strength. This pilot study addressed only one aspect of this multifactorial problem. The implications and suggestions for future research are discussed.

Introduction

In New Zealand spontaneous humeral fracture in first-calving dairy heifers in early lactation has been reported, with an apparent sudden increase in the number of cases over the last seven to eight years. This spontaneous humeral fracture appears to be unique to New Zealand. The condition has first been described in 2008, when six of 200 crossbred heifers from a dairy farm in the Manawatu region had a spontaneous fracture of the humerus within the first couple of months of lactation (Weston, 2008). Copper levels were determined in serum or liver of several of the affected animals. It was reported that the dairy heifers had a copper deficiency because of a missed routine copper injection and after treatment no more spontaneous humeral fractures occurred. In the past years there have been several reports of cases of humeral fractures in dairy heifers (Weston et al., 2012). In 2014-15 a national survey was held among 505 dairy farmers from all through New Zealand. It was found that spontaneous humeral fracture occurred in first- and second lactation heifers in 9.7% of the 505 dairy herds. Within the affected herds a prevalence of 2.1% was detected (Hunnam J, unpublished data). Another survey conducted at the same time in the Waikato region found a prevalence of 2.7% among first- and second lactation dairy cows that were slaughtered by a pet food company (Hunnam J, unpublished data). Humeral fractures occur infrequently and often remain untreated because of economic constraints. Euthanasia is frequently recommended because of the poor prognosis (Rakeshaw, 1996). The syndrome leads to an estimated loss of 5000 dairy heifers every year which represents an economic loss of approximately 10 million New Zealand Dollars per year (Rogers et al., unpublished data). Above all, however, there is a welfare cost. In order to address this problem, it is important to investigate the pathophysiology and possible causes of humeral fractures in dairy heifers. It is hypothesised that spontaneous humeral fractures are occurring as a result of skeletal fragility that may be due to growth check during the rearing period rather than a specific traumatic event in early lactation. Therefore, the present study focuses on bone growth in calves.

Dairy production in New Zealand

In New Zealand, dairy production is based on the efficient conversion of pasture into milk by grazing cows (Back, 2017). Grazing pasture as a main source of feed is one of the main differences between New Zealand and other major dairy industries in the world. The temperate climate allows pasture to grow and be grazed by cows throughout the year and makes housing and feeding the dairy herd in expensive buildings redundant. This enables New Zealand’s dairy farmers to produce milk at a lower

cost than other major dairy industries in the world. The dairy season in New Zealand is closely synchronised with pasture growth, which is much greater in spring than in winter. Therefore, the dairy season runs from June to May each year. Pasture usually comprises ryegrass and white clover species.

Payments to farmers are based on kilograms of milksolids, which is the combined yield of fat and protein in the milk with adjustments for milk volume (Back, 2017; Holmes et al., 2007). In 2017/18 the New Zealand Dairy Industry processed 20.7 billion litres of milk containing 1.84 billion kilograms of milksolids (LIC & DairyNZ, 2017-18). This is equivalent to about 3 percent of the total world milk production. However, New Zealand accounts for approximately 30 percent of all dairy products traded internationally since over 95 percent of the milk produced in New Zealand is manufactured and exported (Back, 2017). This is worth around 14 billion New Zealand Dollars a year. In the 2017/2018 season the total cow population was 4.99 million. The regional distribution is presented in Figure 1 (LIC & DairyNZ, 2017-18). Holstein-Friesian, Jersey and Holstein-Friesian/Jersey crossbreed are the three dominant types of dairy cattle in New Zealand. The national dairy herd was dominated by the Jersey breed until the late 1960s. By 1970, Holstein-Friesian was the dominant dairy breed in New Zealand. Ayshire, Milking Shorthorn, Guernsey and Brown Swiss are the other breeds of dairy cattle present in smaller numbers in New Zealand. At present, Holstein-Friesian/Jersey crossbreed, known as KiwiCross, is the prevalent breed category in all regions except the Manawatu/Wairarapa (LIC & DairyNZ, 2017-18). Abovementioned numbers state clearly that New Zealand will benefit from retaining their dairy cows in order to control the amount of replacement heifers.

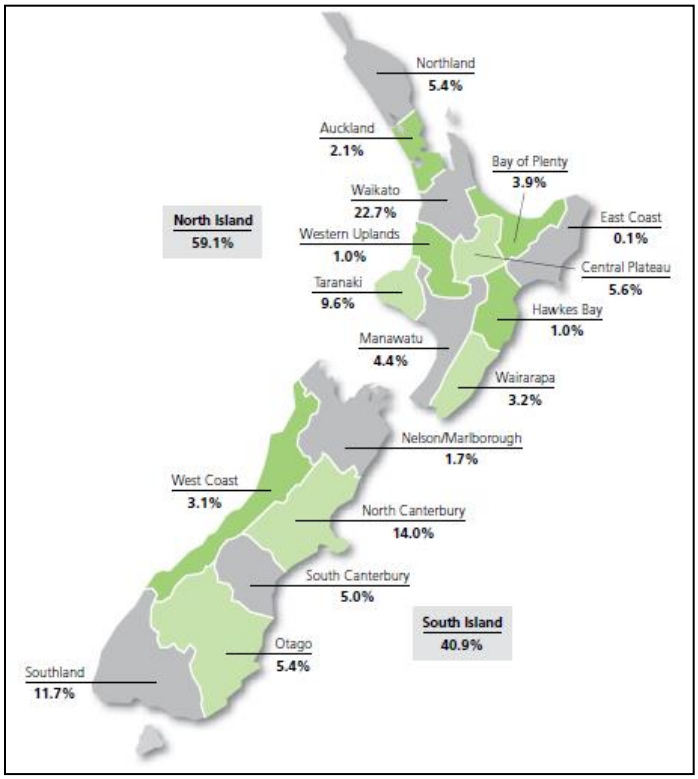

Figure 1: Regional distribution of dairy cows in 2017/18 (LIC & DairyNZ, 2017-18).

Calves

This study focuses on calves. Calving usually takes place in spring. Within 24 hours of birth, the calf is separated from its mother and placed in the calf shed with other calves of the same age. A proportion of the heifer calves are kept as replacement. Bull calves or unwanted heifer calves are sold as bobby calves and will be slaughtered as veal or sold to commercial calf-rearers. A small portion of the bull calves will be reared as breeding bulls. It is very important that the newborn calf receives colostrum as soon as possible, at least 2 litres but preferably

10% of their body weight (Back, 2017). As long as the calf is fed milk, this is digested and absorbed through the fourth stomach compartment alone (the abomasum). As soon as the calf starts to nibble grass or hay/concentrates the major stomach compartment (the rumen) starts to develop. The amount of milk, the type of feed offered, the frequency of feeding, the number of calves in a pen and the amount of time before the calves are put outside differ between calf-rearing systems. Most farms offer a minimum of 4 litres of milk per day to small calves, feeding them twice a day. The calves are also offered a hard feed and hay in the pen, in order to get them used to this feed, to be able to reduce the amount of milk later on and to stimulate rumen development. After spending their first few weeks in the calf shed, the calves are put outside to graze. Between eight and twelve weeks of age the calves reach approximately 20 per cent of mature body weight. This corresponds to 90 kg at 8-12 weeks for a Holstein Friesian x Jersey cross, also known as KiwiCross, calf. At this time the calves are weaned off milk and concentrate feeds. The growth rate of calves has to be sufficient to ensure that the calves weigh enough to reach puberty by 12 months of age, to be able to have several oestrus cycles before being mated at 15 months of age. Successful breeding at 15 months is required for the heifer to calve at 24 months of age (Back, 2017). Providing calves with proper nutrition is of utmost importance for bone strength. Conversely, malnutrition leads to a greater fragility of the calf bone. This could possibly lead to spontaneous humeral fractures later on in life. Enough ground to start investigating these fractures.

Spontaneous humeral fractures

Spontaneous humeral fracture is an emerging syndrome in New Zealand that is mainly found in dairy heifers early in their first lactation, but sporadically preceding to calving or in second lactation heifers. Generally, it is a mid-shaft spiral fracture, illustrated in Figure 2, that presents itself as acute lameness of the forelimb and there is usually no history of trauma (Weston et al., 2012). It appears that the mostly highly productive cows (based on estimated breeding worth) that are most susceptible. Peripheral Quantitative Computer Tomography (pQCT) scan analysis of the mid diaphysis of humerus from affected cows shows a reduced cortical bone area, a lowered bone mineral content and a decreased periosteal circumference. At a histopathology level an increase in osteoclastic resorption, a reduction in the porosity of trabecular bone and in some cases even growth arrest lines are present. The findings of pQCT scan analysis and histopathology of the fractured humerus are consistent with osteoporosis, which is the suspected primary problem in the pathogenesis of spontaneous humeral fracture (Dittmer et al., 2016; Weston et al., 2012). Osteoporosis is characterized by a negative balance between bone formation and bone resorption in favour of resorption, which leads to a reduced quantity of qualitatively normal bone (Bonucci & Ballanti, 2014). Spontaneous humeral fractures appear to be a multifactorial problem. The sections below describe three possible causes that might be interrelated.

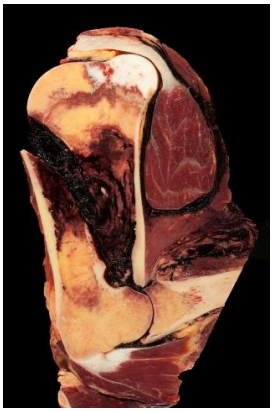

Figure 2: Spiral fracture through the diaphysis of the humerus of a 2-year-old dairy heifer (Dittmer et al., 2016).

Copper deficiency

Earlier investigations into humeral fractures focused on copper deficiency. Copper is involved in forming cross-links between collagen molecules. Copper deficiency can be a predisposing factor for humeral fractures in dairy heifers, since a shortage of copper may result in increased fragility of the bone. This increased fragility remains until osteoblasts replace the bone. The exact role of copper deficiency remains unclear and it is definitely not the sole reason for humeral fractures. However, when copper deficiency exists next to osteoporosis in the bone, it likely increases the tendency to fracture (Craig et al., 2016; Weston et al., 2012).

Malnutrition

It is believed that during periods of malnutrition, less bone is formed and laid down, making the bone fragile. Growth arrest lines are usually found with inadequate feed intake or starvation, which stops growth from the physis and causes formation of a bone layer sealing the physis. This layer of bone moves into the metaphysis when animals return to adequate nutrition and growth continues (Craig et al., 2016). Monitoring of nutrition and trace element status in dairy heifers in order to make sure they are optimally fed during the early weaning period is of utmost importance, since this enables them to establish sufficient bone mass prior to pregnancy and lactation.This indicates that the cause of insufficient bone deposition in the humerus of affected heifers possibly is a nutritional challenge that occurred during growth and development resulting in skeletal fragility (Craig et al., 2016; Weston et al., 2012).

Lactation

After calving the bone is further weakened when bone tissue is mobilised to supply calcium for milk production. A study in pregnant and lactating dairy goats and sheep followed the changes in skeletal tissue during their first and second lactation. A decrease was found in the bone mineral content during pregnancy and the first week of lactation (Liesegang et al., 2007). This is the result of an accelerated bone resorptive phase in order to supply the demand for calcium. This process is uncoupled from the process of bone formation. During lactation, the mineral decrease in bone of these animals is reversed, since the two phases of bone remodeling are now tightly coupled. The decrease in bone mass was greater in the first compared to the second pregnancy and lactation, which proves that the animal is better adapted to the circumstances during the second lactation, even though the milk production is increased in the second lactation (Liesegang et al., 2007). The reduced area of cortical bone, lowered mineral content and sparse, thin metaphyseal trabeculae of fractured humeri of dairy heifers indicate that resorption of calcium from the surface of trabeculae is inadequate to supply lactation, especially in the first lactation. As a consequence, an increase in osteoclastic resorption is seen, which results in further weakening of the bone and contributes to the likelihood of humeral fracture (Craig et al., 2016). Previous research concerning the humerus was carried out on living animals, in which it was impossible to scan the humerus. Therefore, a comparable alternative was chosen: the metacarpal bone. The following section will further elaborate on this.

The metacarpal bones

The humerus is a short, thick bone that is surrounded by the biceps brachii, brachiocephalicus and brachialis muscles and bordered by the thoracic wall (Rakestraw, 1996). Its protected position makes it very difficult to approach the humerus in the living animal, as shown in Figure 3. This is the reason for choosing the metacarpal bone (MC3/4) instead. The third medial metacarpal (MC3) and the fourth lateral metacarpal (L4) bones, the weight bearing metacarpal bones, merged into one single bone. The fusion is complete apart from dorsal and palmar longitudinal grooves, a distal canal and an intercapital notch between the two separate distal heads, as illustrated in Figure 4 (Budras et al., 2011).
For the understanding of this study, it is important to create better knowledge of bone itself. Therefore, different aspects of bone are described next.

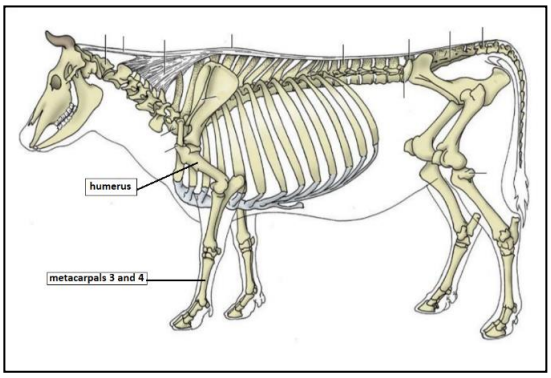

Figure 3: Skeleton of a ruminant showing the humerus and the metacarpal bones (Singh et al., 2018)

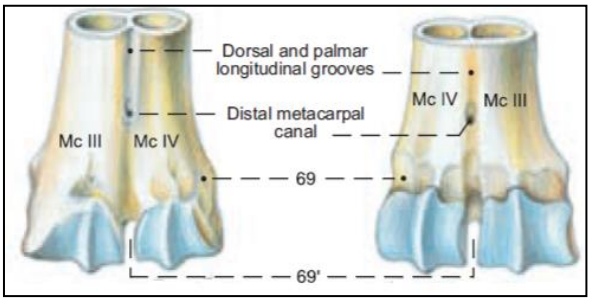

Figure 4: The weight-bearing main metacarpal bones (MC III and MC IV). 69' indicates the intercapital notch between the two separate distal heads (69) (Budras et al., 2011).

Bone

Function of bone

Bone is a rigid organ with a wide range of functions. It provides mechanical support for the entire body and attachment sites for the muscles and tendons to allow locomotion. It protects key organ systems from external harm and contains the bone marrow within its medullary space, where hematopoiesis takes place. Furthermore, bone plays an important role in calcium homeostasis. Although bone seems a rather rigid structure, it is in fact a dynamic organ undergoing remodeling throughout the life of all mammals. Due to continuous bone remodeling, bone maintains its strength and mineral homeostasis (Craig et al., 2016; Eurell & Van Sickle, 2006). Bone is a connective tissue that is made up of different types of bone cells and fibers that are embedded in a hard, mineralized substance called the bone matrix (Goff, 2015; Sinowatz, 2009). Bone minerals provide the bone with hardness. Moreover, the organic component of bone provides toughness and tensile strength.

Bone cells

Bone tissue consists of four cell types: osteoblasts, osteocytes, lining cells and osteoclasts (Craig et al., 2016). Bone shape and structure is regulated by the formation of bone from osteoblasts and resorption of bone from osteoclasts. These are controlled by a variety of growth factors found in the

matrix. Bone lining cells are inactive osteoblasts where no formation or resorption occurs. Osteocytes are osteoblasts that are entrapped within the bone matrix (Clarke, 2008; Craig et al., 2016; Goff, 2015).

Long bones

The skeleton of an animal consists of four different types of bone: flat bone, long bone, short bone and irregular bone (Craig et al., 2016). The humerus and the metacarpal bone are both long bones. Long bones consist of cortical bone and trabecular bone, as illustrated in Figure 5. Cortical bone is very dense bone found at each end of the bones under the articular cartilage and at the outer edges of long bones. Trabecular bone is lightweight bone that is found within the marrow cavity where hematopoietic cells reside. It forms a strong scaffolding that allows the bone to bend slightly. The spongy or trabecular bone reduces weight of the bone without greatly compromising strength and allows some flexibility that would not be possible if the entire bone was compact bone (Clarke, 2008; Craig et al., 2016; Goff, 2015).

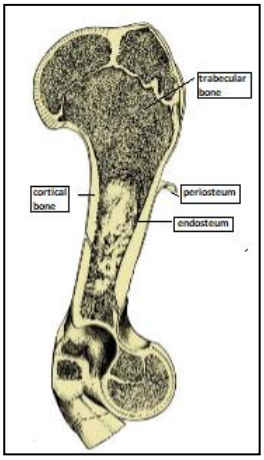

Figure 5: A bovine humerus, illustrating the organization of a long bone (Singh et al., 2018).

Parts of bone

The regions at the end of long bones are named the epiphyses. They are connected by a long compact shaft called the diaphysis, as shown in Figure 6. The ends of the epiphyses are covered by a thin layer of articular cartilage, the remainder of the external surface of the bone is covered by the periosteum (Eurell & Van Sickle, 2006). The periosteum is a thin layer of connective tissue outside of the bone that gives rise to cells essential to bone growth and repair, the osteoprogenitor cells. The internal surface of bone trabeculae within the marrow cavity is lined with the endosteum, a thin layer of connective tissue that is very similar in morphology and function (Craig et al., 2016; Goff, 2015). In a growing animal, the physis or growth plate and the metaphysis separate the diaphysis and the epiphysis. The diaphysis is primarily composed of dense cortical bone with spongy bone in the medullary space, whereas the metaphysis and epiphysis are composed of trabecular meshwork bone surrounded by a relatively thin shell of dense cortical bone. The physis is responsible for longitudinal growth of the animal's bones. During the growth process, temporary trabeculae with cartilage spicules are formed in the metaphysis (primary spongiosa) and later remodeled and replaced by permanent bony trabeculae (secondary spongiosa) (Clarke, 2008; Craig et al., 2016). Once growth is finalised, proliferation of the physeal cartilage cells stops and the physis converts into an epiphyseal scar. However, bone formation on the metaphyseal site of the physis continues (Eurell & Van Sickle, 2006).

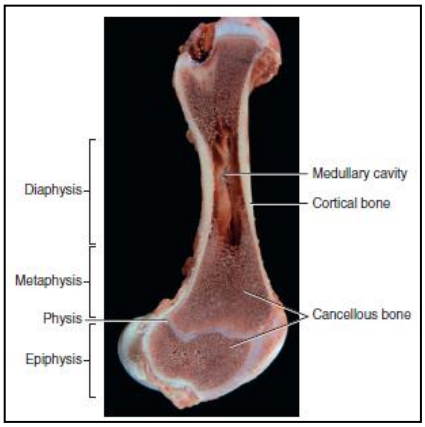

Figure 6: The femur from a newborn calf, illustrating the different parts of the bone (Craig et al., 2016).

Aim of the study

Returning once again to the main problem, data on humeral fractures in dairy heifers is limited and observational / descriptive in nature. The present study provides a starting point for further investigation into this syndrome. It is hypothesised that spontaneous humeral fractures are occurring as a result of skeletal fragility that may be due to growth check during the rearing period rather than a specific traumatic event in early lactation. A growth check is a temporary reduction of growth in a young animal which is usually the result of protein or calorie malnutrition. Several predisposing factors have been described. However, it is likely that inadequate bone mass is the primary problem in humeral fractures (Dittmer et al., 2016; Weston et al., 2012). There is limited data on bone growth in heifer calves and the influence of nutrition during the early weaning period on optimizing peak bone mass (the amount of bony tissue present at the end of the skeletal maturation). That is why the following research questions are proposed:

1. Do bone parameters[1] measured with pQCT scan analysis at the mid-diaphyseal region of the MC3/4 change within the first 12 weeks of the life of a KiwiCross calf in New Zealand?
2. Do different feeding regiments (5L vs. 5LR vs. 10L) within the first 12 weeks of the life of a KiwiCross calf in New Zealand have an influence on bone parameters measured at the mid-diaphyseal region of the MC3/4 with pQCT scan analysis?
3. Is there a correlation between the different bone parameters?

The hypothesis tested was that (1) measures of bone size and strength increase with age, (2) a period of poor nutrition (as in the 5L treatment) during the pre-weaning period decreases bone deposition of heifer calves, thereby, leading to lower measures of bone strength, relative to well-fed calves. (3) The strength strain index, a measure of bone strength, is positively correlated with the abovementioned bone parameters.

In order to answer these questions an experimental cohort study was conducted. Data from analysis of pQCT scans were used to quantify different bone parameters in osteoporosis (Stagi et al., 2016). Since osteoporosis is suspected to be the primary problem in the pathogenesis of spontaneous humerus fracture it is useful to use pQCT scan analysis in this research (Dittmer et al., 2016; Weston et al., 2012).

[1] Bone parameters that are measured: BA, BMC, BMD, CSC-BMD, Peri C, Endo C, SSI and CortTh.

Methods

Study design

This is a blinded prospective experimental cohort study that uses pQCT scan analysis in order to determine different bone parameters in MC3/4 of the right forelimb. The treatment groups were randomly allocated.

Setting and subjects

The study was conducted in New Zealand at a commercial dairy farm between the 6th of August and the 26th of October 2018. Data were collected from 24 heifer calves that were enrolled in a larger ongoing experiment investigating the growth and development of heifers on three different conventional feeding systems. A power analysis was carried out to determine the smallest possible sample size. It was a pilot trial and since the trial was euthansing the calves it needed to be a low number for animal ethics as well. The calves are KiwiCross calves born at a commercial dairy farm between August 8th and September the 2nd of 2016. At birth, weight, rump height, length (crown to rump), leg length (elbow to hoof), leg cannon length (fetlock to hoof) and heart girth, were measured. The calves were initially fed *ad libitum* first milking colostrum until 3 days of age. In order to collect the MC3/4 of the right forelimb, the animals were euthanised. From 6 calves the MC3/4 were harvested at 5 days of age. No measures were done at birth for these 6 calves and they were not assigned to a treatment group. Eighteen heifer calves were randomly allocated into three cohorts at 4 days of age. Each cohort, consisted of 6 calves, were offered either: 1) 5 liters of milk per day as two feeding regiments with free access to pasture (5L), 2) 5 liters of milk per day as two feeding regiments that had ad libitum access to a balanced ration[2] (5LR), or 3) 10 liters of milk per day as two feeding regiments with free access to pasture (10L). These amounts of milk were based on calf rearing rations from the guidelines of Dairy New Zealand (LIC & DairyNZ, 2017). The milk fed to the calves was milk that was not suitable for the milk tank, also called waste milk. The calves were fed milk until approximately 3 weeks old when they also were provided free access to pasture or a balanced ration. At 6 weeks[3] old (half way through weaning age), the MC3/4 of the right forelimb of 8 calves (3 from both cohort 5L and 5LR and 2 from cohort 10L) were harvested. One of the calves from cohort 10L was euthanised at 10 days of age due to illness. At 12 weeks[4] old (weaning age), the MC3/4 of the right forelimb of the remaining 9 calves (3 from each cohort) were harvested. One of the calves from cohort 5L died at 71 days of age due to illness. Depending on whether treatment has a significant effect on bone growth, this calf will be included or excluded from the research. The bones were labelled and stored in the freezer (-20°C) at Massey University NZ until the moment of scanning.

[2] ~0.5 kg/calf; 20% High Protein Calf Pellets, Sharpes Stock Feeds, Carterton, New Zealand).

[3] ~40 days.

[4] ~83 days.

Data collection

In order to collect the data the stored bones of the 24 calves of 2016 were assembled. Bone length was measured at the lateral side of the bone using a ruler measuring from the distal to the proximal side of the bone, as can been seen in Figure 7. The calves were weighed at birth and at slaughter. At birth, height, leg length, body length, girth and leg cannon length were also measured. This data had already been collected by researchers in 2016.

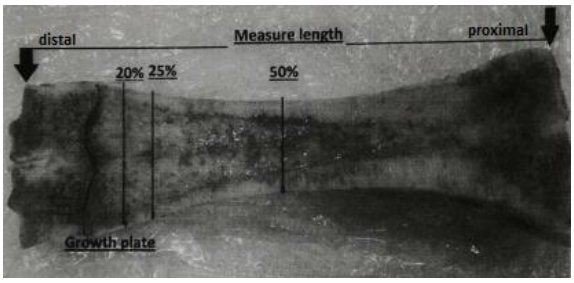

Figure 7: Illustration of the bone length measurement and of the scan site at 50% of the longitudinal bone length.

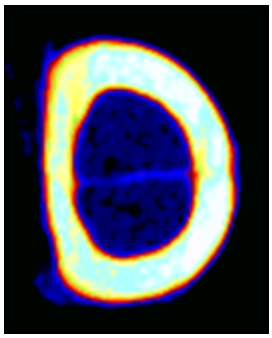

Figure 8: Example of a pQCT scan at the centre of the diaphysis.

pQCT scanning was conducted using a Stratec XCT 2000 bone scanner (Stratec Medizintechnik GmbH). This technique is a validated tool for estimating long-bone strength in animals (Ferretti et al., 1996). Each bone was placed in the machine with the proximal end clamped as if it was attached to the knee. Scanning was performed at 50% of the longitudinal bone length to approximate the centre of the diaphysis. A single tomographic slice was taken at a voxel size of 0.30 mm, of which an example is presented in Figure 8.

The contour mode defines the way the outer contour of the bone is detected and was set at mode 1: TRESHOLD ALGORITHM. This allows the operator to select a threshold value which will be used to separate the soft tissue from the outer edge of the bone. The contour threshold was set at 280 mg/cm^3, meaning that voxels with this value and above defined the outer border of the bone. The internal threshold for cortical bone was 710 mg/cm^3, meaning that tissue with density greater than this was defined as cortical.

The peel mode defines the way (sub)cortical and trabecular bone are separated and was set at mode 1: CONCENTRIC PEEL. This algorithm concentrically peels away a defined percentage of the outside area of the bone. The outside 55% of the bone is peeled away, leaving a 45% inner region which corresponds to trabecular area.

Using the pQCT manufacturer's software, the following bone parameters were determined: total bone area (BA)**,** total bone mineral content (BMC), total bone mineral density (BMD), cortical and subcortical density (CSC-BMD), periosteal circumference (Peri C), endosteal circumference (Endo C), cortical thickness (CortTh) and polar moment of inertia of the total bone area (SSI) (Table 1).

Table 1. Overview of bone parameters used for this research.		
BA	Total bone area [mm^2]	Cross sectional area of the bone after the soft tissue has been peeled off.
BMC	Total bone mineral content [mg/mm]	The mineral content of the total bone within a 1mm slice. Gives an estimate of the mineral (mainly calcium and phosphorus) content of bone, the mineral providing mechanical stiffness and load bearing strength for the bone (Clarke, 2008).
BMD	Total bone mineral density [mg/ccm]	The mean density of the total bone. This is the ratio of bone mineral content to bone area.
CSC-BMD	Cortical and subcortical density [mg/ccm]	The mean density of the cortical and subcortical bone.
Peri C	Periosteal circumference [mm]	The circumference at the height of the periosteum.
Endo C	Endosteal circumference [mm]	The circumference at the height of the endosteum.
CortTh	Cortical thickness [mm]	The mean cortical thickness, defined by the distance between outer and inner edge of the cortical shell.
SSI	Polar moment of inertia of the total bone area [mm^3]	A measure of bone strength. The strength strain index is particularly influenced by bone area, the further bone tissue is from the neural axis the better it is able to resist bending.

Data Analysis

Data were initially examined using simple descriptive statistics and plots in Excel. Linear models were fitted to describe the relationship of the pQCT derived bone parameters with bodyweight and total bone area. Statistical analyses were conducted using SPSS software version 25 (IBM Corp., Armonk NY, USA) with a significance level set at $P < 0.05$. Prior to analysis, data were checked for normality using a Q-Q plot. Data were examined using a general linear model, also known as a two-way ANOVA. In each model the treatment (5L vs. 5LR vs. 10L) and age at slaughter (five days, six weeks and twelve weeks) were fitted as a fixed factors to examine if treatment altered the regression slope co-efficient concerning the bone parameters (dependent variables). Values were expressed as least square means ± standard error of the mean (SEM). This was followed by a secondary analysis in which the direct relationship between the individual bone parameters was investigated using the Pearson Correlation.

Results

Characteristics of the sample

There were no significant differences ($p > 0.05$) in any of the parameters measured at birth as shown in Table 2.

Table 2. Weight, height, length, leg length, left and right leg cannon length and girth of 18 KiwiCross calves at birth categorized in 3 different feeding regiments: 5L, 5LR and 10L (least squares mean ± SEM).

Variables (N = 18)	**5L** (N=6)	**5LR** (N=6)	**10L** (N=6)	***P* Value**
weight [kg]	35.5 ± 1.8	35.2 ± 1.8	36.1 ± 2.3	0.946
height [cm]	75.3 ± 1.2	74.3 ± 1.8	74.6 ± 2.3	0.850
length [cm]	87.4 ± 1.4	86.8 ± 1.9	89.6 ± 2.9	0.613
leg length [cm]	48.8 ± 0.7	47 ± 0.6	49.0 ± 1.7	0.309
left leg cannon [cm]	10.8 ± 0.3	10.9 ± 0.3	11.0 ± 0.2	0.825
right leg cannon [cm]	10.7 ± 0.3	11 ± 0.4	11.1 ± 0.2	0.664
girth [cm]	75.4 ± 1.3	76.8 ± 1.4	76.4 ± 1.7	0.768

Primary analysis

The first step that was taken in the primary data analysis was to visually inspect the data and determine if there were potential relations between age and bodyweight, age and bone length and age and SSI. Moreover, if there were potential relations between treatment and bone length and treatment and SSI. For this purpose, scatter plots were made. They are presented in Figure 9. The scatterplots show a visual relation of bodyweight, bone length and SSI with age. A relationship of bodyweight, bone length and SSI with treatment was dubious. All of these relations were used in the general linear model.

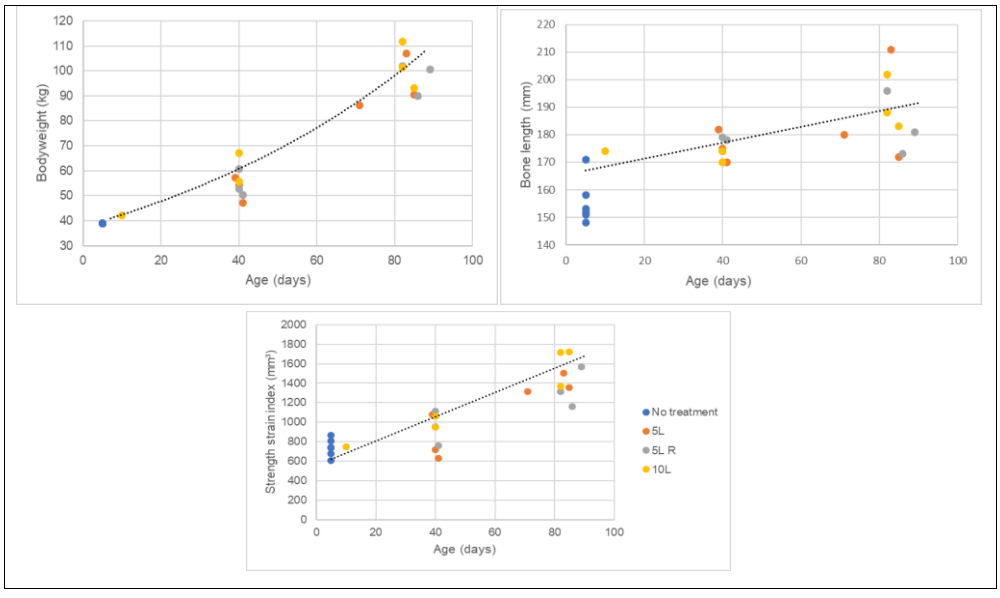

Figure 9: Scatterplots of body weight, bone length and strength strain index of the MC3/4 of the right forelimb in 24 dairy calves at age of scanning and per treatment group.

Quantile-quantile plots showed a normal distribution of the residuals. There was no significant effect ($p > 0.05$) of the interaction between treatment and age and of treatment alone on slaughter weight, bone length and the different bone parameters. This means the interaction variable is redundant. For this analysis, the interaction variable was deleted from the model. After this, there was still no significant effect ($p > 0.05$) of treatment on slaughter weight, bone length and the different bone parameters. Therefore, the treatment variable was deleted as well. After this, it was decided to take all 24 calves into consideration for statistical analysis looking at the effect of age on bone parameters. There was a significant effect ($p < 0.05$) of age on slaughter weight ($F(2,24) = 184.262$, $p < 0.05$), bone length ($F(2,24) = 16.289$, $p < 0.05$), BA ($F(2,24) = 12.398$, $p < 0.05$), BMC ($F(2,24) = 56.436$, $p < 0.05$), BMD ($F(2,24) = 11.463$, $p = 0.203$), CSC-BMD ($F(2,24) = 23.425$, $p < 0.05$), Peri C ($F(2,24) = 11.828$, $p < 0.05$), CortTh ($F(2,24) = 31.452$, $p < 0.05$) and SSI ($F(2,24) = 31.452$, $p < 0.05$). There was no significant effect of age on Endo C ($F(2,24) = 1.583$, $p = 0.229$). This is presented in Table 3.

Table 3. The effect of age on slaughter weight, bone length and mid-diaphyseal pQCT measurements of the metacarpal bone of the right forelimb of 24 KiwiCross calves (least squares mean ± SEM).

Variables (N = 24)	**Agegroup**	**Mean ± SEM**	**F (2,24)**	***P* Value**
slaughter weight [kg]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	39.300 ± 2.417 55.631 ± 2.261 98.933 ± 2.132	184.262	p < 0.05
bone length [mm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	158.143 ± 3.837 175.250 ± 3.589 187.333 ± 3.384	16.289	p < 0.05
total bone area [mm^2]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	303.786 ± 15.582 298.938 ± 14.575 387.667 ± 13.742	12.398	p < 0.05
total bone mineral content [mg/mm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	175.030 ± 8.742 210.451 ± 8.178 293.434 ± 7.710	56.436	p < 0.05
total bone mineral density [mg/ccm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	580.343 ± 28.882 711.825 ± 27.017 761.489 ± 25.472	11.463	p < 0.203
cortical and subcortical density [mg/ccm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	952.986 ± 11.981 1058.500 ± 11.207 1102.656 ± 10.566	23.425	p < 0.05
periosteal circumference [mm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	61.717 ± 1.536 61.108 ± 1.437 69.710 ± 1.536	11.828	p < 0.05
endosteal circumference [mm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	45.808 ± 1924 41.357 ± 1.799 44.629 ± 1.696	1.583	p = 0.229
cortical thickness [mm]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	2.532 ± 0.140 3.143 ± 0.131 3.992 ± 0.123	31.452	p < 0.05
strength strain index [mm^3]	~age = 6 days (N=7) ~age = 40 days (N=8) ~age = 83 days (N=9)	740.009 ± 62.505 907.083 ± 58.468 1447.126 ± 55.124	41.184	p < 0.05

Secondary analysis

The first step that was taken in the secondary data analysis was to visually inspect the data and determine if there were potential correlations between SSI and the other bone parameters. For this purpose, scatter plots were made. They are presented in Figure 10. The scatterplots seemed to show a visual relation and were used in the Pearson Correlation.

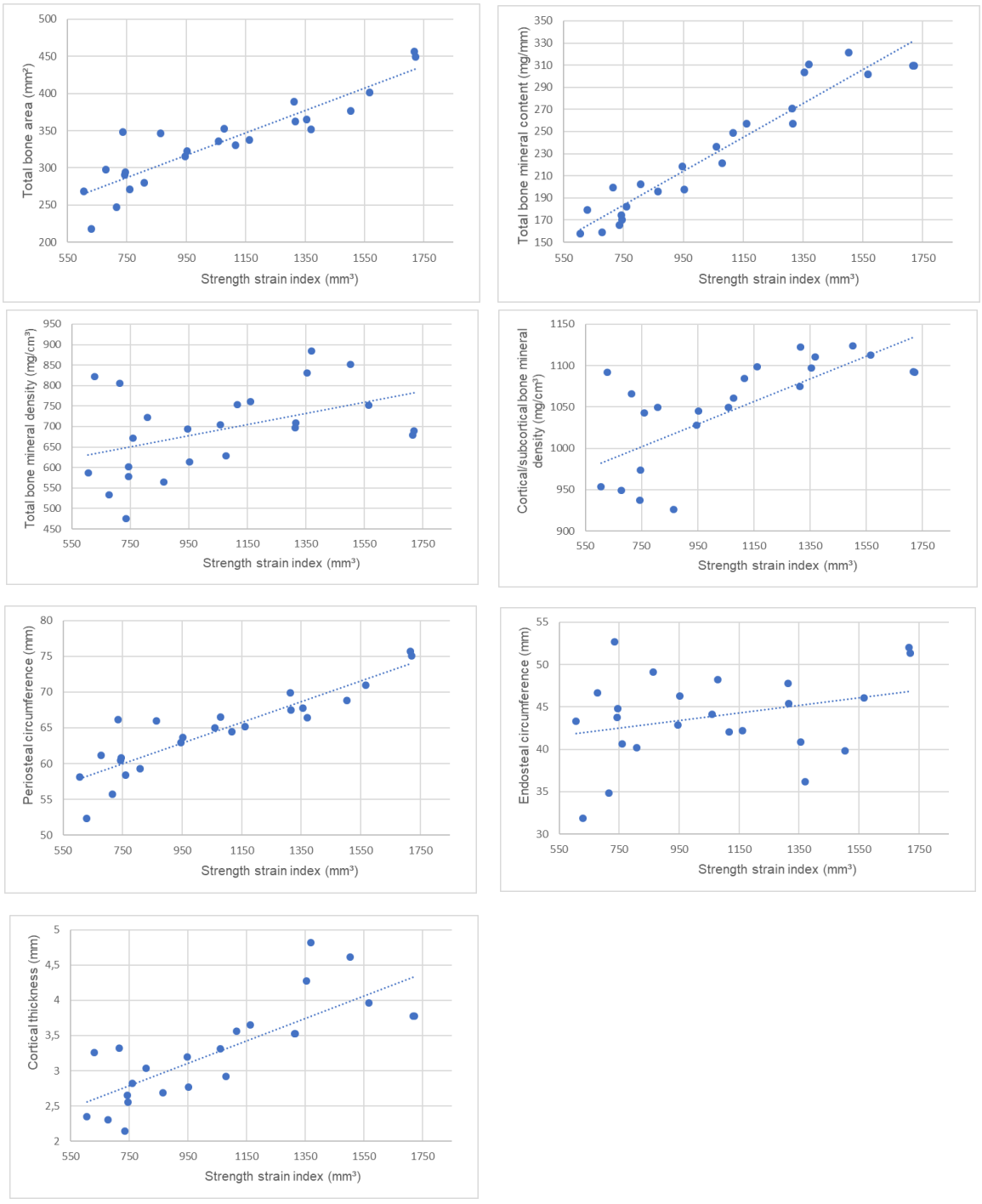

Figure 10: Scatterplots that show the correlation of strength strain index (mm^3) with total bone area (mm^2), total bone mineral content (mg/mm), total bone mineral density (mg/cm^3), cortical/subcortical bone mineral density (mg/cm^3), periostal circumference (mm), endosteal circumference (mm) and cortical thickness (mm) measured at mid-diaphysis of the metacarpal bone of the right forelimb of 24 KiwiCross calves (least squares mean ± SEM).

Tertiary analysis

Bone area showed a significant correlation with BMC ($r(23,24) = 0.786$, $p < 0.05$), Peri C ($r(23,24) = 0.998$, $p < 0.05$), Endo C ($r(23,24) = 0.661$, $p < 0.05$) and CortTh ($r(23,24) = 496$, $p < 0.05$). There was a significant correlation of bone mineral content with BMD ($r(23,24) = 0.657$, $p < 0.05$), CSC-BMD ($r(23,24) = 0.776$, $p < 0.05$), Peri C ($r(23,24) = 0.779$, $p < 0.05$) and CortTh ($r(23,24) = 0.922$, $p < 0.05$). There was an observable correlation of bone mineral density with CSC-BMD ($r(23,24) = 0.851$, $p < 0.05$), Endo C ($r(23,24) = -0.702$, $p < 0.05$) and CortTh ($r(23,24) = 0.880$, $p < 0.05$). Periosteal circumference showed an observable correlation with Endo C ($r(23,24) = 0.669$, $p < 0.05$) and CortTh ($r(23,24) = 0.489$, $p < 0.05$). Strength strain index had a significant correlation with BA ($r(23,24) = 0.905$, $p < 0.05$), BMC ($r(23,24) = 0.960$, $p < 0.05$), CSC-BMD ($r(23,24) = 0,690$, $p < 0.05$) Peri C ($r(23,24) = 0.896$, $p < 0.05$) and CortTh ($r(23,24) = 0.789$, $p < 0.05$). These results are presented in Table 3.

Table 4. Correlation between mid-diaphyseal pQCT measurements of the metacarpal bone of the right forelimb of 24 KiwiCross calves.

Bone parameters	**BA**	**BMC**	**BMD**	**CSC-BMD**	**Peri C**	**Endo C**	**CortTh**	**SSI**
BA	X	r = 0.786 p < 0.05	r = 0.057 p = 0.790	r = 0.345 p = 0.099	r = 0.998 p < 0.05	r = 0.661 p < 0.05	r = 0.496 p < 0.05	r = 0.905 p < 0.05
BMC		X	r = 0.657 p < 0.05	r = 0.776 p < 0.05	r = 0.779 p < 0.05	r = 0.060 p = 0.781	r = 0.922 p < 0.05	r = 0.960 p < 0.05
BMD			X	r = 0.851 p < 0.05	r = 0.044 p = 0.838	r = -0.702 p < 0.05	r = 0.880 p < 0.05	r = 0.050 p = 0.849
CSC-BMD				X	r = 0.329 p = 0.116	r = -0.363 p = 0.082	r = 0.845 p < 0.05	r = 0.690 p < 0.05
Peri C					X	r = 0.669 p < 0.05	r = 0.489 p < 0.05	r = 0.896 p < 0.05
Endo C						X	r = -0.322 p = 0.125	r = 0.300 p = 0.155
CortTh							X	r = 0.789 p < 0.05

Discussion

The objective of this study was to begin investigating the possible causes for spontaneous humeral fractures in first-lactation New Zealand dairy heifers. The first hypothesis was met as this study showed a significant effect of age on several bone parameters. The second hypothesis was not met, given that no effect of treatment was found on the different bone parameters. The third hypothesis was confirmed by the positive influence of the bone parameters on the strength of the bone.

A strength of this study is that KiwiCross calves were chosen for the study population, which is the most prevalent dairy breed in New Zealand. The average birthweight of the KiwiCross calves in this study was similar between treatment groups and when compared to their ancestors a bit under average birth weight reported in Holstein-Friesians (Jasper & Weary, 2002; Davis Rincker et al., 2011; Korst et al., 2016; Geiger et al., 2016) and a bit over average birthweight in Jerseys (Quigley & Martin, 1995; Quigley,1996; Uys et al., 2011).

To our knowledge, no studies have been done on finding out when calves reach their peak bone mass. The calves in this study had not yet reached to this point either. However, this study showed that with age, body weight increases and with that measures of bone size and strength increased. In other words, the metacarpal bone gains strength in the first twelve weeks of the life of a calf.

Since the slaughter weight did not differ significantly between groups, it is suggested that different feeding regiments did not influence the weight gain of the calves. A study by Rosenberger et al. (2017) looked at different milk allowances and found that providing higher amounts of milk resulted in higher weight gains before weaning. This finding was consistent with previous studies (Diaz et al., 2001; Cowles et al., 2006). In the current study, restricted amounts of milk did not result in undernutrition of the calf, though guidelines recommend over 5L of milk/day for optimal growth of the calf. Meanwhile, providing 10L of milk/day, which is considered as *ad libitum* feeding, did not lead to excess weight.

Moreover, growth with regards to bone length and bone diameter was not influenced by the different milk feeding regiments either. Length of the bone is a more fixed variable whereas the bone diameter is affected by stress exerted on the bone (Clarke, 2008; Goff, 2015). The growth in diameter of the shaft of long bones is due to deposition of new bone on the outside surface by cells within the periosteum. Strain exerted on the bone can increase the amount of mineral and thickness of the bone matrix deposited during bone remodeling (Reece et al., 2015).

Increasing the diameter of the bone increases the bending strength (Dittmer et al., 2016; Clarke, 2008). Therefore, this research focused on the influence of nutrition on bone parameters. It was hypothesised that higher growth rate was more easily sustained in groups with unlimited feeding as compared to restricted, resulting in greater bone deposition. However, there was no significant effect of nutrition on the bone parameters up to 12 weeks of age in this trial. This could be explained by the fact that the different feeding groups gained approximately the same amount of weight.

Thereupon, the question arises if there is a correlation between bone parameters. The emphasis was on bone parameters that are important for bone strength. Amongst these was SSI, an expression of resistance to lateral, dorso-palmar and torsional deformation (Ferretti et al., 1996). The SSI is mainly influenced by bone area. Resistance to bending is augmented mostly by bone furthest away from the neural axis (Leonard et al., 2004; Pearson & Lieberman 2004). Thus, primarily increase in cortical width and density improves bone strength. This is confirmed by a study of Dittmer et al., who found that in animals with humeral fractures, BMD was slightly higher compared to unaffected animals. This was explained by the fact that an adequate mineralisation of the bone does not naturally implicate a good strength, since a thinner area of cortical bone reduces the overall mineral content of the bone (Dittmer et al., 2016). This was supported by on the one hand a significant correlation between cortical and subcortical density plus cortical thickness plus bone mineral content and SSI and on the other hand a non-significant correlation between total bone mineral density and SSI found in the present study. Furthermore, the augmentation of cortical thickness is perfectly explained by a stronger relation between SSI and periosteal circumference compared to endosteal circumference. Since these results are derived from calves, it is an addition to the previously mentioned study on cows (Dittmer et al., 2016). In conclusion, significant correlations were found between different bone parameters and SSI. This is an important finding for future studies that examine development of bone strength.

In this study, the treatment groups were randomly allocated, after which the investigator that performed the intervention on the living animals was not blinded for treatment. The investigator that performed data collection and analysis was blinded for treatment, but not for age. The method used to determine bone strength and predict fracture risk, is a validated method that is used in osteoporosis in humans (Augat et al., 1998; Sheu et al., 2011; Dennison et al., 2014; Crockett et al., 2015; Stagi et al., 2016; Jiang et al., 2018). Before performing pQCT scan analysis, a protocol was drawn up and this protocol was strictly applied by the investigator. Statistical analyses were conducted using SPSS software version 25 (IBM Corp., Armonk NY, USA) with a significance level set at $p < 0.05$.

The sample size of this study is limited due to the fact that it was a pilot study, enforced by ethical reasons of slaughtering the animals. This also led to the limitation that repeated measurements and follow-up were not possible. No comparable studies have been carried out, leading to the restriction of not being able to compare the results. The results of this study need to be interpreted with care and are generalizable for New Zealand, KiwiCross only. The occurrence of spontaneous humeral fractures appears to be unique to New Zealand, which is attributed to the fact that New Zealand dairy farms are predominantly pasture-based and supplementary feed is only used to fill feed deficits so that cows maintain energy intake and production (LIC & DairyNZ, 2017). There is no advantage to replacing good quality pasture with an alternative feed source; therefore, supplements should only be used to provide energy when there is insufficient pasture available. The apparent sudden increase in the number of cases over the last seven to eight years is thought to be due to selection for higher milk production (LIC & DairyNZ, 2017-18).

The pilot study presented here addressed only one aspect of this multifactorial problem and has a number of limitations. Since the bone parameters that are of influence on bone strength can also be measured in living animals, this would be recommended to further investigate the effect of nutrition not only on a larger scale but also in a period of time by repeating measurements in the same animal. In addition, bone parameters are not only affected by nutrition. In addition, it would be interesting to compare feeding regiments with and without supplementation. Moreover, other suggestions for intervention and strain factors that can be investigated are exercise (Barneveld & Van Weeren, 1999), genetics (Rauch, 2005), illness and environmental conditions. Finally, it would be interesting to take first lactation dairy heifers as the study population.

Conclusion

Research about the occurrence of spontaneous humeral fractures in dairy heifers in New Zealand is scarce. It seems to be a national problem only and finding a cause to prevent thousands of dairy cows from death is of high state value. The present study is a pilot study, that addressed only one aspect of this multifactorial problem. Future studies should focus on bigger sample groups with higher level of evidence that look into this syndrome in the broadest sense, before and after the moment of occurrence. Exercise, genetics, illness and environmental conditions can be taken into consideration as possible factors.

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