Broken shoulders in dairy heifers in New Zealand: Investigating the relationship between live weight and bone morphology in the bovine forelimb.

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Abstract

Spontaneous humeral fractures in first lactation dairy heifers are a significant economic and welfare issue in the New Zealand dairy industry. With approximately 4% of dairy farms being affected and, within farm, up to 25% of replacement heifers lost, the cost to the dairy industry is estimated to be over $10 million. Preliminary observations from Dittmer et al. (2016), showed the presence of growth arrest lines in bones from affected heifers, indicating a period of malnutrition in the heifer's life. However, with little known about how heifer's bones grow within the New Zealand pastoral system, it is difficult to identify developmental time periods that have the greatest impact on long bone growth and, therefore, greatest potential to affect the risk of fracture. Therefore, the aim of this thesis was to investigate the relationship between live weight and bone morphology in the bovine forelimb to identify potential risk periods for spontaneous humeral fractures in first-lactation dairy heifers.

In this thesis, the metacarpus and humerus were opportunistically collected from cattle from a variety of experiments carried out at Massey University and from samples submitted from farms throughout New Zealand. Measures of bone morphology in the metacarpus were highly correlated with the same measure in the humerus (R^2=0.89-0.98). Measures of the humerus and metacarpus were also highly correlated with live weight at all ages. However, the relationship between bone and live weight changed over time demonstrating differences in bone maturity. Distal regions of the forelimb such as the metacarpus matured by one year of age as indicated by a different relationship between live weight and pQCT measures of the humerus at 24 months compared with 8-12 months

of age. However, proximal regions of the forelimb such as the humerus continued to grow after a year of age. Differences in proximal and distal bone growth were reflected in the pattern of stature growth. A cohort of dairy heifers (Holstein Friesian, Jersey, and Holstein-Friesian cross Jersey) were measured for live weight, height, wither-rump length, girth, and distal and proximal forelimb length (stature) from birth to 15 months of age. As live weight increased, the contribution of the distal limb to increases in height decreased, which is consistent with knowledge that physeal closure in cattle occurs earlier in distal limb bones, such as the metacarpus, than in proximal limb bones, such as the humerus.

Finally, bones collected from heifers affected and unaffected by spontaneous humeral fractures were compared and the effect of copper status on bone measures was examined. The mid diaphysis of the humerus of the affected group had reduced cortical bone mineral density ($p<0.05$) and a trend for reduced cortical content and total bone content which contributed to a reduced stress-strain index. A trend for reduced bone length in affected humeri provides additional support for the hypothesis of inhibited humeral growth. Heifers with low liver copper concentration had reduced humeral length and reduced cortical bone mineral density ($p<0.05$). Growth and fracture data presented in this thesis support the hypothesis that the most likely developmental period in which inadequate bone growth occurs in heifers subsequently affected by humeral fracture is in the months leading up to the event, which coincides with the animal's second winter.

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List of abbreviations

AN- Anorexia nervosa

DEXA- Dual-energy X-ray absorptiometry

F- Holstein-Friesian

FJ- Holstein-Friesian-Jersey Crossbred

FSH- Follicle stimulating hormone

GnRH- Gonadotrophin releasing hormone

FX- Holstein-Friesian Crossbred

GH- Growth hormone

IGF-I- Insulin-like growth factor

J- Jersey

JX- Jersey Crossbred

LH- Luteinizing hormone

pQCT- Peripheral quantitative computed tomography

General introduction

Spontaneous humeral fracture in first lactation dairy heifers is a condition affecting at least 4% of all dairy farms in New Zealand. It is estimated that approximately 5,000 heifers per year are lost to this syndrome, resulting in significant economic loss to the New Zealand dairy industry as well as significant animal welfare implications (Dittmer et al., 2016, J. Hunnam, unpublished data). Currently, there is no method to identify heifers that are at risk of humeral fractures because the bone morphology that predispose a heifer to fracture are not known.

Humeral fractures in cattle tend to be spiral fractures that originate from the proximal end of the diaphysis (Rakestraw, 1996). In cattle with normal cortical bone thickness, humeral fractures are rare (5% of all fractures in cattle and 18% of all long bone fractures) due to the protection provided by the biceps brachii, brachiocephalicus, and brachialis muscles that surround the humerus caudally, laterally and cranially (Rakestraw, 1996). Post-mortem examination of fractured humeri have shown an association with lower cortical bone thickness in affected bones contributing to a decrease in bone strength (measured by stress strain index) (Dittmer et al., 2016). Growth arrest lines have also been observed in the contra-lateral humeri from heifers with spontaneous humeral fractures indicating a period of malnutrition at some point in the heifer's life (Dittmer et al., 2016). The combination of weaker bones and sudden draw of calcium due to the onset of lactation is likely to contribute to an increased risk of spontaneous fractures in the humerus.

Identification of heifers at risk of humeral fracture is difficult due to the location of the humerus. The ability to examine the bone is restricted to post-mortem examination. Therefore, a herd can only be identified as being at risk of a high number of animals having humeral fractures after multiple heifers have been affected, at which time there is limited opportunity to increase bone strength.

Therefore, the aim of this thesis was to investigate the relationship between live weight and bone morphology in the bovine forelimb to identify potential risk periods for spontaneous humeral fractures in first-lactation dairy heifers.

The main objectives of the work presented in this thesis were:

- To evaluate whether computed tomography scans of the metacarpus can be used as a predictor for humeral bone structure.
- Describe the relationship of pQCT-derived measures of bone strength and morphology within two different bones, in the proximal and distal limb, with gross measurements of size and growth.
- Examine the effect of sex and age on bone morphology post puberty.
- Describe proportional changes in stature with weight in Holstein Friesian, Jersey and Holstein-Friesian Jersey crossbred calves from birth to 15 months.
- Compare and contrast pQCT derived measures of bone morphology of bones collected from 2-year-old dairy heifers affected and unaffected by humeral fractures.

Foreword to thesis chapters

Some of the chapters in this thesis have been published in refereed scientific journals, resulting in overlap in the information presented in the chapter introductions, to allow them to appear standalone as published papers. Additionally, some formatting has been made post-publication, to ensure consistency among the chapters. These changes are considered typographical and make no change to any outcomes of the paper.

Chapter 1

Review of Literature

1.1 Animal growth

1.1.1. Proximal vs distal growth

Pre-pubertal live weight gain is mainly the result of increases in height and length. In cattle, 80% of mature height is achieved by 7 months of age but only 35-45% of mature weight has been attained. Longitudinal growth after 7 months of age slows and 90% of mature height is achieved by one year of age so, further increases in live weight gain are the result of increases in frame size (Hammack and Gill, 2009).

The rate of growth is affected by the mature size of an animal. At a young age, growth is rapid until an animal approaches the growth asymptote when growth slows. As the asymptote is approached, lean growth decreases and fat deposition increases (Berg and Butterfield, 1968). The age that this occurs is younger in animals with a smaller mature size such as Jersey breed cows (Handcock et al., 2019b). Therefore, at a comparable weight, animals with a heavier mature weight will be leaner and less mature than animals with a light mature weight.

1.1.2. Skeletal growth

The first wave of growth after birth begins with the region of the cranium, facial region and lumbar area (Pomeroy, 1978). Secondary growth occurs in the limbs at the metacarpal and metatarsal regions before it radiates down to the digits and upwards in the limbs into the trunk and lumbar region. The lumbar region is the last part of the body to attain maximum growth rates (Pomeroy, 1978). When comparing proximal and distal limb growth, proximal ends of limbs have a higher growth trajectory

compared to the distal ends of bones (Berg et al., 1978). Distal bones such as the third and fourth metacarpal (MC3/MC4) have earlier bone maturity than proximal bones such as the humerus (Bartosiewicz, 1984). Berg et al (1978) examined bone growth patterns and distribution in joints of carcasses. As the size of the carcass increased, the proportion of bone weight in the metacarpus decreased in relation to total bone weight whereas the proportion of bones in the shoulder such as the humerus remained the same (Berg et al., 1978). Humeral growth is therefore constant in response to the size of the animal whereas distal limbs such as the MC3/MC4 have a decreased growth trajectory as the animal gets larger.

1.2. Bone

1.2.1. Function

Bone is a rigid organ that makes up a significant part of the vertebrate skeleton and contributes essential functions to the body. Bone supports the body by protecting organs such as the brain and spinal cord, as well acting as a site for muscle and tendon attachment (Weiner and Wagner, 1998). The skeletal system also provides a means of locomotion through the use of joints and ligaments for stabilisation and movement (Sinclair et al., 2014). The shape and size of individual bones is determined by genetic and functional factors to carry out a specific function with minimal risk of failure but without excessive energy expenditure (Sinclair et al., 2014). Bone is also responsible for calcium and phosphorus homeostasis where the resorption of bone results in an increase in plasma calcium and phosphorus concentrations (Weiner and Wagner,

1998). Bone also contains haematopoietic tissues for blood cell formation (Hall, 2005). The following section will describe bone structure and type to provide an understanding for subsequent discussion of bone turnover and response to loading which is important for understanding bone strength.

1.2.2. Gross structure

Long bones are divided into three parts: diaphysis, metaphysis and epiphysis (Figure 1.1). The diaphysis is the tubular shaft of the long bone designed to resist the functional loading patterns of bending and torsion (Sinclair et al., 2014). The epiphysis is at either extremity of the bone and consists of mainly trabecular bone with a thin outer coating of articular cartilage (McFadyean, 1908). The diaphysis and epiphysis are joined by the metaphysis that contains the growth plate of hyaline cartilage called the physis. After growth is completed, this cartilage mineralises and is replaced with an epiphyseal line (Mackie et al., 2008). The internal surfaces of bone such as the medullary cavity of long bones and trabecular bone are lined with endosteum. The surfaces of the bone that are in contact with each other in a joint are covered in a hyaline cartilage called articular cartilage (Craig et al., 2016). Lining the exterior of bone not covered by articular cartilage, is a fibrous sheath consisting of dense irregular connective tissue called the periosteum.

The periosteum acts as an attachment point for lymphatic and blood vessels to attach to, which supplies nutrients to the bone. Tendons and ligaments are also bound to the fibrous layer of the periosteum by Sharpey's fibres that extend into the bone (Hall,

2005). The innermost layer of the periosteum is called the cambium layer and contains osteoprogenitor cells (Buckwalter and Cooper, 1987).

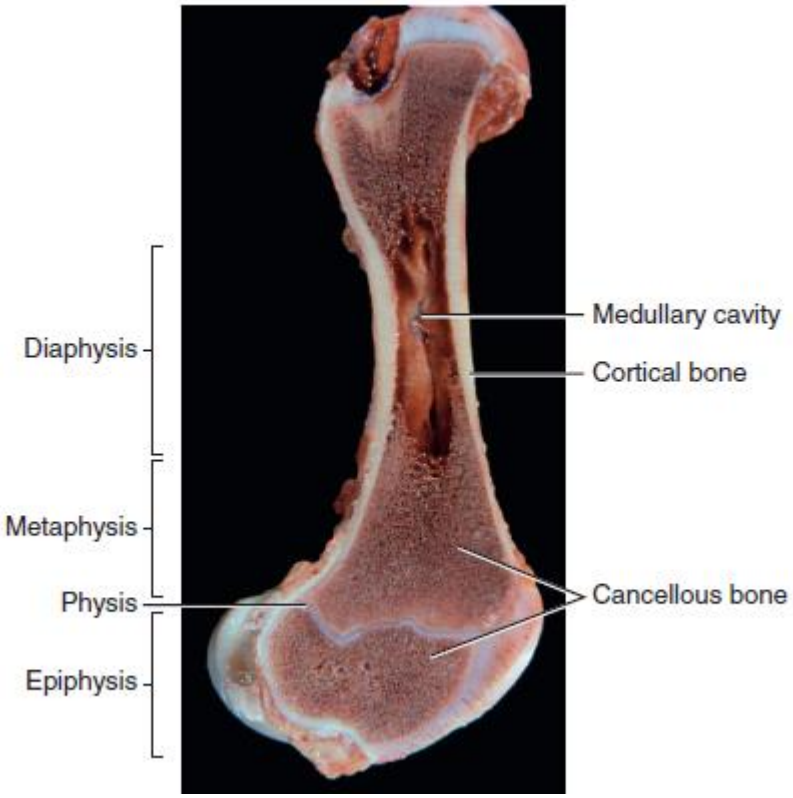

Figure 1.1 Cross section of a bovine humerus labelled with bone types and bone sections (Craig et al., 2016).

Bone is organised in a hierarchal manner consisting of cortical (compact) bone and trabecular (cancellous) bone (Rho et al., 1998). The relative proportions of cortical and trabecular bone differ in bones and is based on shape and function. Each region of bone contains different proportions of bone types, which is defined by the arrangement of lamellar bone. In mature lamellar bone, collagen fibres of the bone matrix are oriented in parallel layers with osteocytes present between the layers (Craig et al., 2016).

The structural unit of cortical bone is an osteon containing 3-20 concentric lamellae of bone matrix. Bone matrix surrounds the central osteonal canal that runs parallel to the long axis of the bone (Rho et al., 1998, Weiner and Zaslansky, 2005). The osteonal canal contains the vascular and nerve supply of the osteon and a second group of canals that run at right angles to the long axis of the bone (called perforating canals). The role of the canals is to connect the blood vessels and nerves to the periosteum (Craig et al., 2016).

Trabecular bone is comprised of a network of trabeculae with spaces between filled with haemopoietic tissue or fat (Craig et al., 2016). In contrast to cortical bone, the lamellae are arranged parallel to the surface. This type of bone is found at the epiphysis and metaphysis of long bones and is arranged in a manner that optimises load transfer (Mellon and Tanner, 2012).

1.2.3. Cell types

Bone turnover is carried out by four cell types that interact to optimise bone morphology in relation to changes in mechanical demands (Sinclair et al., 2014). Osteoblasts, lining cells and osteoclasts are present on bone surfaces and osteocytes permeate the interior of bone (Marks Jr and Popoff, 1988). Osteoblasts are derived from a type of mesenchymal stem cells called osteoprogenitor cells. Osteoblasts then differentiate into osteocytes when encased in osteoid of bone lining cells when bone formation is complete (Florencio-Silva et al., 2015). Osteoprogenitor cells are located in the inner cellular layer (cambium layer) of the periosteum, the endosteum that lines

the marrow cavity, and the lining of the vascular passageways in the bone matrix. However, osteoclasts are sourced from haemopoietic stem cells of the monocyte series (Harada and Rodan, 2003).

Bone shape and structure is dependent on the formation of bone by osteoblasts and resorption of bone by osteoclasts. The combination of osteoblasts, osteoclasts, osteocytes, bone lining cells and the capillary blood supply make up the basic multicellular unit (BMU) (Florencio-Silva et al., 2015). The BMU allows for a coupling of bone formation and bone resorption to allow for bone to be continually renewed (Jilka, 2003). Regulation of these cell types comes from growth factors located in the matrix.

Osteoblasts secrete the organic component of bone matrix called osteoid by secreting collagen and ground substance (Craig et al., 2016). Osteoblasts also have a regulatory function in the mineralisation of osteoid with hydroxyapatite (Hall, 2005, Craig et al., 2016). During the time of mineralisation 10-20% of osteoblasts along the bone-forming surface are surrounded by osteoid to become mature bone cells called osteocytes (Craig et al., 2016). Osteoblasts also activate osteoclastic bone resorption of the bone matrix by producing colony stimulating factor, RANK and interleukin-1 beta osteoclast activation factor to promote osteoclast maturation (Sinclair et al., 2014, Paternoster et al., 2010).

Osteocytes are the most abundant cell type in bone tissue and reside in small spaces within the mineralised matrix called lacunae (Marks Jr and Popoff, 1988). Processes

extend from the osteocytes through the mineralised matrix in canaliculi which connect to the lacunae (Hall, 2005). The canaliculi provide a network for osteocytes to facilitate cell to cell communication. Osteocytes play an important role in assessing mechanical loading by forming a mechanosensation network. Osteocyte processes detect changes in fluid flow shear forces which activates them to produce nitric oxide, prostaglandins, bone morphogenic proteins and Wnt proteins to modify the activity of osteoblasts and osteoclasts. Osteocyte apoptosis can be stimulated by both lack of mechanical stimulation and excessive mechanical loading to optimise bone architecture and mass in response to changes in mechanical demands (Sinclair et al., 2014). Osteocyte death promotes the secretion of RANKL resulting in osteoclastic resorption of unneeded or damaged bone.

Bone lining cells are quiescent flat-shaped osteoblasts present on endosteal bone surfaces where no new bone formation or resorption is occurring (Florencio-Silva et al., 2015). Bone lining cells play a functional role in creating a barrier between the extracellular fluid compartment of bone tissue and that of surrounding tissues (Marks Jr and Popoff, 1988). Lining cells can be activated by mechanical and biological signals to regress into metabolically active osteoblasts (Sinclair et al., 2014). In times of bone resorption the presence of parathyroid hormone (PTH) activates osteoblasts to allow for the coupling of bone resorption with formation at previously inactive sites (Canalis, 2013).

Also present on bone surfaces, are osteoclasts, found in shallow pits called Howship's lacunae (Craig et al., 2016). Osteoclasts are large multinucleated cells found in areas of bone resorption or bone removal (Canalis, 2013). Osteoclasts are derived from monocyte/macrophage lines making them rich in acid phosphatase and other acid hydrolases that are packaged into primary lysosymes. Actin-like filaments anchor the osteoclast to the bone to allow for resorption to take place (Craig et al., 2016). An acidic environment is created by the osteoclast, forming a sealed compartment that enhances the activity of the hydrolases that break down the organic component of the bone matrix (Sinclair et al., 2014). Bone matrix that has been broken down is then endocytosed by osteoclasts and further degraded by secondary lysosomes. Once the osteoclasts have completed its role in resorption, the osteoclasts detach from bone and undergo apoptosis (Craig et al., 2016).

1.2.4. Bone growth

The skeleton has evolved to provide maximum strength with minimal mass and this is achieved via bone modelling and remodelling (Sinclair et al., 2014). Bone is formed using one of two mechanisms, intramembranous ossification or endochondral ossification (Bonucci, 1992). Bones involved in locomotion are formed by both intramembranous and endochondral ossification allowing for simultaneous longitudinal and circumferential growth.

1.2.4.1. Intramembranous ossification

Intramembranous ossification occurs when the bone develops from a fibrous membrane seen in flat bones such as the skull, face and under the periosteum of long bones (Hall, 2005). Mesenchymal progenitor cells migrate during embryonic development to specific regions where they aggregate. As the cells condense, they form the membrane and template for bone development (Franz-Odendaal, 2011). Vascular sites then develop on the bone template to provide an increase in nutrient supply while mesenchymal cells become enlarged and rounder to enable differentiation into osteoprogenitor cells (Cashman and Ginty, 2003). Mesenchymal cells develop into osteoblasts that secrete osteoid, an unmineralised collagen-proteoglycan matrix that can bind calcium to create woven bone (Craig et al., 2016). Woven bone is later remodelled and replaced with lamellar bone (Cashman and Ginty, 2003). The osteoblasts become trapped and transform into osteocytes in lacunae (Hall, 2005). Mesenchymal cells on the bone surface form the periosteum. The periosteum comprises of two layers called the fibrous layer and the inner osteogenic cambium layer. Osteoprogenitor cells on the inner cambium layer differentiate into osteoblasts to secrete osteoid (Breeland and Menezes, 2020, Sinclair et al., 2014).

1.2.4.2. Endochondral ossification

Endochondral ossification begins in a similar fashion to intramembranous ossification with the migration and aggregation of mesenchymal cells. However, the mesenchymal cells then become chondroblasts to make cartilage matrix (Bonucci,

1992). The cartilage model provides the template for adult bone and expands with interstitial and appositional growth (Figure 1.2a) (Craig et al., 2016). Interstitial growth causes the increase in bone length whereas the increase in bone width is caused by new chondrocytes that differentiate from the chondrogenic layer of the perichondrium surrounding the cartilage mass.

Perichondral cells produce osteoblasts to form the bone tissue. The connective tissue surrounding the middle of the cartilage changes from perichondrium to periosteum as it becomes invaded with capillaries (Craig et al., 2016). Periosteal bone then forms around the cartilage model (Figure 1.2b).

As the chondrocytes enlarge, the matrix is compressed, and osteoblasts begin to synthesize alkaline phosphatase and surrounding surfaces begin to mineralise (Masrour Roudsari and Mahjoub, 2012). Chondrocytes then die off and the matrix breaks down causing the neighbouring lacunae to become interconnected. Blood vessels begin to enter the diaphyseal area and vascularise the cavity (Figure 1.2c). Osteoprogenitor cells arise from cells migrating from the periosteum that line the spicules and differentiate into osteoblasts. Osteoblasts then produce osteoid on the chondrocytes matrix scaffold to form endochondral bone and this becomes the primary ossification centre (Figure 1.2c).

Endochondral ossification continues as the chondrocytes continue to divide at each end of the growing bone (Craig et al., 2016). As bone continues to develop, secondary ossification centres are formed at one or both ends depending on the bone (Figure

1.2d) (Craig et al., 2016). These secondary ossification sites are known as epiphyses and remain separated from the primary ossification centres in the diaphysis and metaphysis by the physis.

Limited growth occurs in the articular epiphyseal complex, the epiphyseal side of the growth plate is capped by trabecular bone to prevent further growth at that site (Figure 1.2e). Proliferation of chondrocytes and therefore endochondral ossification can continue on the metaphyseal side of the growth plate until maturity (Figure 1.2e) (Craig et al., 2016).

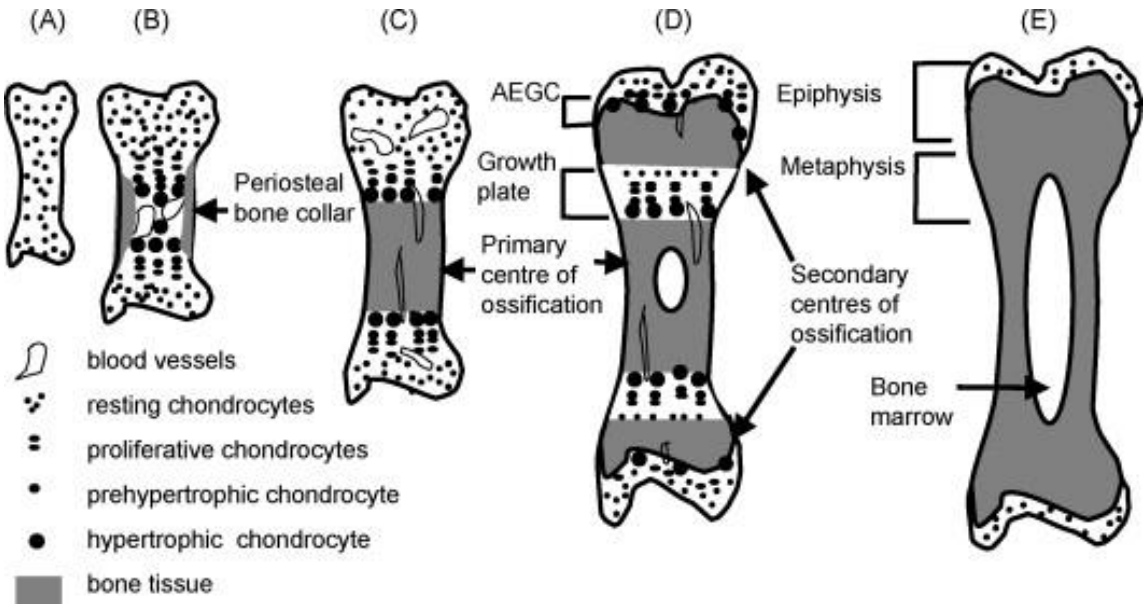

Figure 1.2 Diagram of long bone development showing position and stages of ossification from fetal growth to mature bone. Cartilaginous model develops during fetal development (a), followed by development of the periosteum (b). Primary ossification centre contains trabecular bone surrounded by cortical bone (c). The medullary cavity forms in the diaphysis and a secondary ossification centre develops at the epiphyses. After birth growth still occurs due to cartilage at the epiphyseal plates (d). Mature bone only has the remnants of the epiphysis with only thin lines presented (Mackie et al., 2008). Reprinted with permission from Elesvier.

During active growth, the physis can be organized in to three zones. The resting zone is anchored to the trabecular bone of the epiphysis (Craig et al., 2016). The next zone is the proliferative zone, where tightly packed columns of proliferating chondrocytes are present. The chondrocytes then enlarge, and the matrix prepares for mineralization in the hypertrophic zone (Nilsson et al., 2005). Within the hypertrophic zone there are 3 additional zones; zone of maturation, zone of degeneration and the zone of provisional calcification. The maturation zone prepares the matrix for mineralisation and chondrocyte growth so that matrix can then move into the zone of maturation in preparation for mineralisation and death of chondrocytes. The provisional calcification zone is where chondrocyte death occurs allowing calcium to be released and the matrix to be mineralised (Thabet, 2014, Craig et al., 2016).

The physis is responsible for longitudinal growth and controls the length of the bone (Akers and Denbow, 2013a). In times of inadequate feed intake or calcium deficiency, growth in the physis can be halted and a layer of bone can form and seal the physis (Craig et al., 2016). When growth resumes, the layer of bone is displaced which results in a growth arrest line (Craig et al. 2016). If the suspension in growth is significant enough, it may prevent bone from reaching peak bone mass. Histological data from animals with humeral fractures show growth arrest lines and often multiple growth arrest lines that suggest affected animals go through multiple periods of inadequate nutrition (Dittmer et al. 2016). However, little is known about the effects of inadequate nutrition on bone growth and strength in heifers.

1.2.5. Bone modelling and remodelling

With a larger body mass, bone must proportionally increase in size and strength to maintain strain on bone within physiological limits (i.e. the mechanostat theorem and Wolffs law) (Frost 1987). An increase in animal size creates a greater loading strain on bone via bodyweight and muscular forces, resulting in a larger bone strength (the importance of loading is further discussed in section 1.4.5). Maturation (closure of growth plates) occurs earliest in distal vs proximal bones and this subsequently alters the approach to increase bone strength. Mechanical strain occurs as a result of applied mechanical stress from muscle contraction, impact loading and gravitational forces which causes bone modelling and remodelling to occur (Hart et al., 2017). The nature and magnitude of bone response to changes in strain is dependent on the site examined and the stage of maturation/age of the animal. Within the site, strain environment, strain magnitude, strain rate, strain frequency, strain distribution, number of loading cycles and rest-recovery periods determine the bones response to mechanical loading (Hart et al., 2017).

1.2.5.1. Bone modelling

Bone modelling is the process in which bones are shaped or reshaped by the independent action of osteoblasts and osteoclasts. Bone modelling is determined by growth and mechanical loading to determine bone shape and size (Sinclair et al., 2014). Mature bone can also be modelled if there is a permanent change in strain on the bone and to provide an increase in periosteal size (Langdahl et al., 2016).

1.2.5.2. Bone remodelling

Microdamage is linear microcracks of 50 - 100 μm in length within the bone matrix as a result of repeated loading cycles. Microcracks occur from physiological habitual loading of bone and stimulates cellular responses to maintain and adjust the matrix to maintain resistance to bone strain (Sinclair et al., 2014). Bone remodelling occurs throughout an animal's life and relies on the sequential work of osteoclasts and osteoblasts. Remodelling is characterised by 4 phases: Osteoclasts are recruited (activation phase) to resorb bone (resorption phase). Once osteoclasts have finished removing bone, they undergo apoptosis and osteoblasts are recruited (reversal phase) to lay down new secondary bone which is subsequently mineralised (formation phase) (Cashman and Ginty, 2003, Langdahl et al., 2016). Although secondary bone is initially not as strong as primary bone (formed by bone modelling), it is stronger than the damaged matrix it replaces (Sinclair et al., 2014). Once peak bone mass has been achieved bone remodelling is balanced until later life when age related bone loss begins. Remodelling-based resorption is responsible for increases in endosteal size. Bone resorption only takes 10-20 days whereas bone formation takes 3-6 months (Cashman and Ginty, 2003). If bone resorption and formation is not balanced abnormalities can occur. Abnormalities in bone remodelling can result in a loss or gain in bone and are the basis of high and low bone mass syndromes. A common low bone mass syndrome is osteoporosis (reduced bone quality and quality). Excessive

remodelling can also result in bone destabilisation which increases strain on neighbouring bone regions (Langdahl et al., 2016).

Another important role of bone remodelling is for calcium homeostasis. During pregnancy and lactation, additional calcium can be taken from bone when insufficient calcium is present in the diet (Reinhardt et al., 1988). However, preserving bone strength whilst supplying the necessary amount of calcium can be difficult. If bone strength cannot be maintained, the animal may be at risk of bone fracture (Langdahl et al., 2016).

1.3. Modifiers of bone

1.3.1. Seasonality

Feed supply on New Zealand (NZ) farms is seasonal, where pasture availability changes dependent on the time of year. Season impacts the quality and quantity of feed available, requiring supplementation to meet animal demands for growth and production in times of poor or limited pasture growth e.g. winter (Rattray et al., 2007). In contrast, overseas systems do not rely as heavily on pasture availability, so growth as measured by live weight gain is more linear. New Zealand studies have shown that during winter (June-August) a growth check often occurs due to lower pasture quality and availability (Handcock et al., 2016). One potential indication that an animal has gone through a growth check is the presence of a growth arrest line. Growth arrest lines occur when an animal has undergone a significant and sustained period of nutritional deficit (Craig et al., 2016). Therefore, it is possible that animals affected by

a seasonal growth pattern may show signs of growth arrest. A constant growth rate would indicate that adequate pasture and supplementation occurred during winter and is considered a linear growth trajectory (grown to target). Handcock et al. (2021) reported that different growth trajectories (linear vs seasonal) did not affect height or length of the heifer at 12 months old. However, girth circumference (capacity) was reported to be greater in heifers grown to live weight target than heifers that grew in a seasonal pattern. The significant effect of girth was subsequently not present at 15 months of age, but heifers grown in a seasonal pattern were taller at 15 and 21 months of age. The difference in height is likely attributed to differences in an earlier pubertal age (38 days) achieved in heifers grown to target than heifers grown in a seasonal pattern so longitudinal bone growth ceases at an earlier age (Handcock et al., 2021).

1.3.2. Nutrition

Bone is dynamic and changes throughout life. Changes in bone can be a result of nutritional imbalances as these can disrupt bone growth, modelling, or remodelling. (Shamay et al., 2005). Insufficient dietary energy causes a decrease in plasma IGF-I concentrations and an increase in plasma GH concentration. Low IGF-I concentrations results in growth arrest (Brameld, 1997). The severity of the abnormality is dependent on the phase of skeletal development, age of the animal, and the length of time the animal is exposed to the factor . Due to their rapid rate of bone turnover and growth, younger animals are more susceptible to the effects of nutritional imbalances. Older animals are also affected by nutritional imbalances, but the severity compared to

growing animals is less significant (Craig et al., 2016). It can be difficult to pinpoint the exact cause of bone abnormalities as it is often a combination of nutritional imbalances over an animal's life and the effect of the imbalance can be diverse among different species (Craig et al., 2016). If imbalances are corrected, provided treatment occurs early and correctly, the effect on bone can be repaired. In the case of severe or prolonged deficiencies causing diseases such as osteoporosis, the effects may be permanent.

In immature animals, growth arrest lines are often observed in osteoporotic bones, which can indicate that the cause of osteoporosis is due to a sustained period of nutritional deficit. Bone resorption takes 2-3 weeks, whereas it takes 3 months to deposit the same amount of bone. Therefore, if bone resorption is increased due to metabolic signalling, bone formation would not be able to keep up and this results in bone deficits (osteoporosis) (Harada and Rodan, 2003). Fast-growing growth plates are most susceptible to nutritional imbalances (Craig et al., 2016).

The most common metabolic bone disorder in cattle is osteoporosis, which is characterised by a reduction in the quantity of bone with the quality remaining normal. Osteoporosis results in a weaker bone and increases the chance of fracture without trauma. In a farming scenario, this could be observed as a higher incidence of spontaneous fractures in the herd or flock. Other than an increase in fractures, osteoporosis can be hard to detect radiographically or at necropsy unless the loss of bone is at least 30-50% (Craig et al., 2016). Nutrient deficiencies such as calcium or

copper cause significant reduction of bone mass and can result in osteoporosis. In NZ copper deficiencies can occur in sheep and cattle due to the lack of copper in soils and poor supplementation (Grace et al., 2010).

Another common cause of osteoporosis in farm animals is starvation, as a result of drought, or overstocking, leading to a lack of pasture or lack of quality pasture. Younger animals are more prone to the effects of undernutrition due to the energy requirements for growth and development. Periods of nutrient deficiency will lead to growth arrest lines and serous atrophy of medullary adipose tissue, in addition to osteoporosis (Craig et al., 2016)

Early weaning is common in dairy calves when compared to beef calf systems. Dairy calves are often weaned at 2-3 months of age (80-100 kg) which is younger than beef calves that are weaned at 5-7 months of age (180-250 kg) (Back, 2017, Morris, 2017). The timing of early weaning means that when pasture quality is at its seasonal low point in later summer/autumn, dairy calves are grazing pasture only, whereas beef calves are still receiving more than 30% of their energy intake from milk (Roca Fraga, 2013), allowing beef calves to achieve greater growth rates through the consumption of a comparatively high energy milk diet.

Another potential factor in dairy calf nutrition is the use of milk replacer vs whole milk. There appears to be differences in responses to feeding milk replacer and whole milk, where calves fed the two milk types with the same dietary energy had differences in growth. Body composition of calves fed milk replacer was compared

with calves fed *ad libitum* whole milk (Shamay et al., 2005). Calves fed *ad libitum* milk were 16 kg heavier at the end of the rearing period compared to calves fed *ad libitum* milk replacer. Differences in weight could be attributed to differences in wither height, with calves fed *ad libitum* milk 2% taller at 60 days of age than calves fed milk replacer.

Ellenberger et al. (1989) reported steers fed an *ad libitum* diet had an average growth rate of 1.40 kg/day compared to steers on a restricted diet that had a reduced growth rate of 0.37 kg/day when grown from 240 to 307 kg. Steers fed the restricted diet had increased GH at 45.6 ng/mL compared to steers on *ad libitum* diet, which had a GH concentration of 23.4 ng/mL. However the lower IGF-I in steers on a restricted diet (108 ng/mL vs 167 ng/mL) than steers fed *ad libitum* contributed to a slower growth rate (Ellenberger et al., 1989). Therefore, differences in diet can affect regulation of growth hormones that regulate live weight gain and bone.

The effect of early nutrition in relation to bone development has been thoroughly researched in humans due to the high incidence of osteoporosis in later life. Bone mineral acquisition prior to puberty (peak bone mass) is crucial to prevent osteoporosis in later life and periods of severe nutrient deficiency can prevent peak bone mass from being achieved (Javaid and Cooper, 2002). A common example of severe nutrient deficiency in humans is adolescents with anorexia nervosa (AN), causing low body mass and disruptions in hormonal concentrations involved in bone regulation (Turner et al., 2001). Anorexia nervosa can also affect longitudinal bone growth where at times of anorexic illness, bone growth and maturation slowed.

However, once sufficient feeding occurred the bone maturing mechanism was resumed, and longitudinal bone growth is accelerated. The age of skeletal maturation would therefore be delayed (Pfeiffer et al., 1986). In contrast, the rate of bone mass accrual towards peak bone mass can be decreased and is likely a contributing factor to the increased occurrence of osteoporosis in affected individuals. Miller et al. (2005) found approximately 34.6% of adolescents with AN will end up with osteoporosis and 30% of adolescents with AN had a history of fractures.

1.3.3. Puberty

In the heifer, puberty is the transition from a state of ovarian inactivity to a state of regular ovulations (Hickson et al., 2011). Puberty in the heifer occurs when 48-51% of mature body weight is attained, with age having a lesser effect (Handcock et al., 2021). Therefore, the age at which a heifer reaches puberty is heavily dependent on growth rate (Nelsen et al., 1982).

The onset of puberty is signalled from the central nervous system that stimulates the release of the gonadotrophin releasing hormone (GnRH) from the hypothalamus. Increases in GnRH stimulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Akers and Denbow, 2013b). Secretion of LH and FSH acts on the ovaries to stimulate secretion of oestrogen. Oestrogen plays a crucial role in bone regulation in females, by facilitating the draw of calcium from bone stores for embryonic skeletal development and lactation (Harada and Rodan, 2003). Early in puberty, the increase in sex and growth hormones

cause the elongation of long bones known as a growth spurt. However, in later stages of puberty acquisition, oestrogen promotes bone resorption and inhibits further appositional growth (Falahati-Nini et al., 2000). A reduction in osteoclastic function, inhibits bone resorption and promotes the closure of the physis (Riggs et al., 2002). Once the physis is sealed, endosteal and trabecular bone formation is increased along with an increase in mineralisation. Oestrogen also stimulates the uptake of calcium from the intestinal tract and retention of calcium in skeletal stores increasing bone density and strength (Riggs et al., 2002).

1.3.4. Transition from pregnancy to lactation

A dairy cow faces a unique set of challenges with regards to lactation and pregnancy. The normal lactation period for a dairy cow is 271 days, resulting in approximately 13 weeks between the cessation of lactation and parturition (LIC and DairyNZ, 2019). The cow is then bred again within 3 months of parturition (Horst et al., 2005).

The change from pregnant and nonlactating to nonpregnant and lactating is called the transition period. The transition period is the time of sudden calcium demand on the cow and can lead to hypocalcaemia if the cow is unable to supply the necessary calcium (Horst et al., 2005). Calcium demand, towards the end of lactation, for the maintenance of body tissues and fetal growth are easily met by dietary calcium (Reinhardt et al., 1988). However, the onset of lactation creates a large demand for calcium for colostrum production, this is in the range of 30-50 g/day compared to 10 g/day for milk production in late pregnancy (Horst et al., 2005). Hypocalcaemia

usually occurs in the first 12-24 hours after parturition, due to inadequate upregulation of PTH and significant loss of calcium in colostrum, which can be nine times the amount present in the plasma pool (Horst et al., 2005). Cows exhibiting signs of hypocalcaemia for more than a few hours often present with calcium concentrations below 0.5mmol/L (Grace et al., 2010).

In the first days of lactation the secretion of PTH is increased to reduce losses of calcium through urine, to increase bone Ca resorption, and increase the production of 1,25-dihydroxyvitamin D_3 in order to increase calcium absorption from the intestinal tract (Reinhardt et al., 1988). The secretion of PTH is triggered when calcium concentrations drop below 1.25 mmol/L in extracellular fluid (Ferneborg, 2010). Bone stores will then be utilised from 1 to 2 weeks after parturition. However, this could be earlier if calcium supplementation in the diet is insufficient (Reinhardt et al., 1988). If the cow is facing metabolic alkalosis due to high intake of potassium and/or sodium, the response of bone and kidneys to PTH can be inhibited, resulting in a decrease in bone resorption and production of 1,25-dihydroxyvitamin D_3 in response to hypocalcaemia (Horst et al., 2005). To prevent hypocalcaemia, farmers can use a low dietary cation anion difference (DCAD) to stimulate the cows metabolism to increase calcium utilisation and reduce milk fever (IVERSI, 1998).

Negative calcium balance usually occurs in the first 6-8 weeks of lactation with a loss of approximately 13% of calcium stored in bone. As feed intake increases, the intestinal tract increases in size due to cellular hypertrophy (Horst et al., 2005). The

increase in serum 1,25-dihydroxyvitamin D_3 concentration and large intestinal surface area allows the cow to absorb enough dietary calcium to meet the demands of lactation and replenish calcium lost in the early stages of lactation (Horst et al., 2005).

1.3.5. Loading

As an animal moves, mechanical forces are placed on bone from ground reaction forces and the contractile forces from muscles. The direction and size of the force placed on the bone determines maintenance and transformations in bone mass (Harada and Rodan, 2003). Transformations of bone are explained by Wolff's law and the mechanostat theory, where bone is deposited in sites where it is required and resorbed where it's not required (Elliott et al., 2016). The alignment of trabecular bone in the metaphysis and epiphysis is dependent on the direction of compressive forces from weight-bearing. The deformation of bone is termed "strain" and can be calculated as the change in length in relation to the original length (Elliott et al., 2016, Sinclair et al., 2014).

Under normal physiological conditions where bone is within a homeostatic range, a slow turnover of bone will occur, and osteoblast and osteoclast function will be balanced. A decrease in the mechanical stress placed on bone due to complete or partial immobilisation causes an increase in the resorption of bone and therefore decrease in bone strength and stiffness (Figure 1.3) (Harada and Rodan, 2003). An example of this was reported in Firth et al. (2012) where diaphyseal and epiphyseal bone parameters were examined in horses after withdrawal from training. After

training ceased a significant decrease in bone mineral density (BMD) was observed with no change in bone area in the diaphysis (Firth et al., 2012).

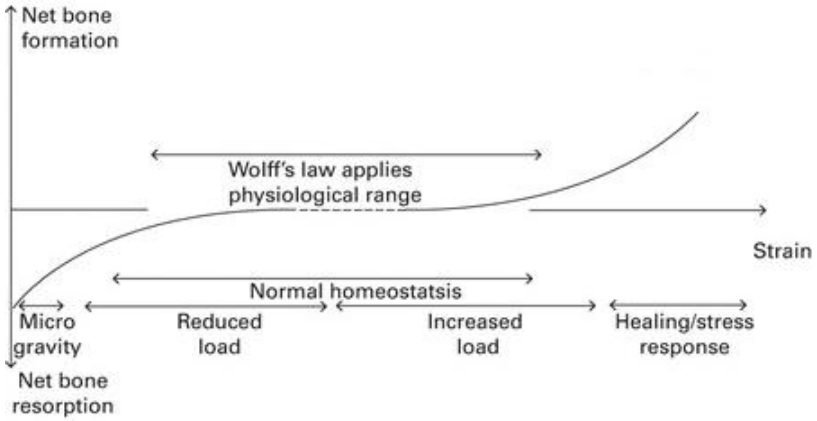

Figure 1.3: Graph showing bone homeostasis adapted from Elliott et al. (2016)

The ability of bone to withstand changes in tension and compression can be measured using Young's modulus. The value of Young's modulus for a material is dependent on the density of the material, and the width of the material tested. A smaller surface area to volume ratio would result in a higher modulus (Choi et al., 1990). The density of trabecular bone is lower than that of cortical bone, resulting in a lower tensile strength in trabecular bone compared to cortical bone (Rho et al., 1998). Choi et al (1990) reported that cortical bone was 19% stronger than trabecular bone, and that subchondral bone had a lower density (and Young's modulus) than trabecular and cortical bone. Changes in density also result from factors such as age and nutrition (Craig et al., 2016). Animals that have a reduction in bone density are likely to have a lower tensile strength of bone and a higher risk of fracture (Craig et al., 2016).

Mechanical loading of a bone is assessed by a mechanosensation network made up of osteocytes (Bonewald, 2006). Changes in fluid flow shear forces (strain) are detected by osteocyte processes triggering modification of osteoblast and osteoclast activity. Lack of mechanical loading, or excessive mechanical loading, stimulates osteoclastic resorption of excess bone or damaged bone (Bonewald, 2006). Loading within a mild to moderate range prevents osteocyte apoptosis, permitting bone formation (Craig et al., 2016).

The initial response to increasing loading strain is to increase bone size. However, if strain rates are high enough, an increase in bone density can be induced (Vainionpää, 2007). In young horses, significant increases in density at the mid diaphysis were only observed after the introduction of intensive exercise such as galloping (Firth et al., 2012, Rogers et al., 2012). In comparison, calves, which are not a cursorial species, do not have the same predisposition to run at high speed and are therefore unlikely to subject the distal limb to the high strain rates observed in galloping horses. Even when calves were run on a concrete path this failed to induce sufficient high strain rates to increase bone density, with the primary response being an alteration in bone circumference and focal response in morphology to the strain (deposition of bone on the dorsal surface) (Logan et al., 2019). In addition commercial beef farming systems with a large reliance on hill grazing does not provide sufficient strain to induce an increase in bone density (Bijen et al., 2020).

1.3.7. Copper

Copper is an important factor in many biochemical and physiological pathways including nerve, bone, immune and growth functions. To increase bone strength, collagen is cross-linked by the enzyme lysyl oxidase which requires copper (Cu) as a cofactor (Hill and Davidson, 1986). In cattle, Cu is stored in the liver, which contains 40-70% of total body Cu. In healthy cattle, Cu ranges between 100-1730 μmol/kg in fresh tissue. When dietary Cu intake is inadequate, liver stores are mobilised to maintain serum Cu concentrations (Grace et al., 2010). However, in New Zealand, 75% of pastures have inadequate copper to meet the dietary requirements of cattle and must be supplemented to prevent copper deficiency (Knowles and Grace, 2014).

Copper concentrations within the animal is affected by age, breed, individual variation, seasonal variation and endoparasites. Diet also plays an important role in copper concentrations where high supplementation of minerals such as molybdenum, sulphur, cadmium, iron and zinc result in reduced copper absorption and concentration (Grace et al., 2010, Parkinson et al., 2010). Inadequate copper concentrations may also occur during growth, pregnancy and lactation. In dairy heifers, a combination of growth and pregnancy results in a substantial demand for copper, which can deplete liver copper reserves and result in a low concentration of copper in serum. Copper loss from the body is approximately 1 mg Cu/kg LW gain during growth and approximately 2.1 mg Cu/ day during pregnancy (third trimester).

If copper from the diet is inadequate to meet the increased demand, then heifers may deplete any liver reserves and become copper deficient (Grace et al., 2010).

Copper deficiencies can be caused by insufficient supplementation, parasitism and/or genetic abnormalities (Grace et al., 2010). The effects of copper deficits are visible in calves with signs of stiffness, lameness, diarrhoea, and mild anaemia. The effects of subclinical copper deficiencies in adult cattle are less obvious with the effects being limited to subtle losses in production and possibly diarrhoea. However, in severe cases, enlarged joints and fractures can occur (Suttle et al., 1972).

Osteoporosis can occur when liver copper levels are less than 50-75 μmol/ kg fresh tissue (Suttle and Angus, 1976). However, visible signs of copper deficiency such as impaired growth and ataxia occur at a much lower copper concentration (20-30 μmol/kg and 5-35 μmol/kg of fresh tissue respectively). Therefore, cattle can develop osteoporosis from copper deficiency with no visible signs (Grace et al., 2010). Two types of copper deficiencies can occur, with primary deficiencies being caused by a lack of copper in the diet and secondary deficiencies occurring when copper in the diet is adequate, but the uptake of copper is impaired by increased levels of molybdenum or sulphate (Gay et al., 1987).

Deficiencies in copper affect the ability of lysyl oxidase to crosslink collagen molecules and therefore is associated with an increase in the fragility of bone, and reduced cartilage integrity (Hill and Davidson, 1986, Suttle et al., 1972). A lack of copper also results in a decrease in osteoblast activity affecting bone growth and mineralisation

(Suttle et al., 1972). Radiographic and histological lesions are observed in animals with copper deficiencies and can be found on the growth plate of the metacarpal and metatarsal with a widening of the physis and fragmentation of the metaphysis. Bones also showed a decrease in cortical thickness and a reduction in the trabeculation. Observations from calves with copper deficiencies have shown osteoporotic bones and physeal lesions (Suttle and Angus, 1978).

1.4. Measuring bone

1.4.1. Existing methods

The gold standard for measuring bone parameters in a non-invasive manner has been Dual-energy X-ray absorptiometry (DEXA). DEXA is desirable for its low radiation dose and robustness for movement and positional variation. However, DEXA creates a 2 dimensional result of a 3 dimensional bone model (Gasser, 1995). By doing so, bone measures are limited to bone mineral content, bone area and bone mineral density. However, bone density calculated in this manner is dependent on bone size and does not represent true volumetric density. DEXA does not distinguish between cortical and trabecular bone so descriptions of bone growth are limited (Choksi et al., 2018).

A method that allows for the quantification of mechanical bone strength is the three-point bending test. The three-point bending test is carried out by applying force to the centre of a bone with the ends fixed until it reaches breaking point and therefore the elastic modulus can be calculated (Walker et al., 2015).

1.4.2. pQCT

To understand bone growth in terms of bone mineral accrual and geometry a technique such as peripheral quantitative computed tomography (pQCT) can be used. PQCT uses 3-dimensional bone densitometry to produce measures of bone mineral status and geometry and allows trabecular and cortical bone to be distinguished (Stagi et al., 2016). Bone density calculated using pQCT is independent of bone size and provides a volumetric measure of bone density (Stagi et al., 2016). Along with assessing bone parameters, pQCT can provide information on surrounding muscle and therefore provides valuable information on the interrelationship of bone and muscle. The use of pQCT scanners is limited in the size of the gantry only allowing for scans of peripheral bones to be scanned in living patients. Therefore, scanning of proximal bones such as the humerus in cattle is limited to post-mortem. However, unlike DEXA, pQCT is sensitive to movement and positional variation. The combination of pQCT sensitivity to movement and long scanning times means that subjects should be immobilised, and animals anaesthetised which is not always practical when carrying out live animal scans.

1.4.3. Measures and SSI using pQCT

Periosteal circumference is defined as the outer circumference of the bone whilst endosteal circumference is the circumference of the inner surface of the bony tissue (Nahian and Chauhan, 2020). Cortical thickness is estimated by subtracting the endosteal circumference from the periosteal circumference (Figure 1.4). Total bone

area is calculated from the periosteal circumference and includes the bone marrow (Hasegawa et al., 2001).

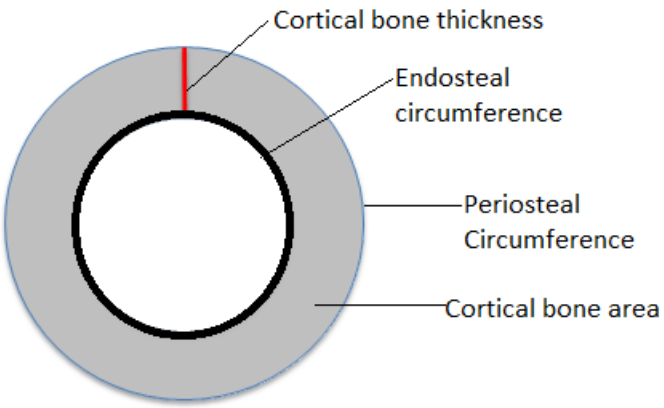

Figure 1.4. Cortical dimensions derived from pQCT scans.

The stress–strain index is a derived measure of bone strength using pQCT data and avoids the requirement for destructive testing using techniques such as the three-point bending test. The stress–strain index describes the ability of bone to withstand bending from lateral, dorso-palmar and torsional forces and is calculated by incorporating the index of material stiffness (bone mineral density) and bone geometry (cross-sectional moment of inertia) (Ferretti, 1995). The stress strain index is calculated as follows: $SSI=\Sigma i=l,n r_i^2 aCDND r_{max}$ where r_{max} is the distance of the voxel from the centre, CD is the apparent cortical bone density, ND is the normal cortical bone density, r_i is the pixel position from the centre and *a* is the area of the pixel (Hasegawa et al., 2001).

A combination of these measures allows for the quantification of bone parameters to investigate changes in size and morphology. The importance of being able to quantify bone morphology has been demonstrated for many bone disorders such as osteoporosis in elderly women and juvenile bone fractures (Stagi et al., 2016). Therefore, pQCT makes an ideal tool for measuring bone morphology and strength in the humerus of heifers affected by spontaneous humeral fractures.

1.5. Summary and implications

Failure of bone to reach adequate size and strength increases the risk of spontaneous and traumatic fractures. An example of this is humeral fractures in first lactation dairy heifers. Once humeral fractures begin to occur on a farm, it is too late for preventative measures to occur. The presence of growth arrest lines and osteoporotic bones indicate that there is a sensitive stage of growth where bones are affected. Bone is affected by changes in loading, nutrition, timing of puberty and the transition period. However, the timing most critical to bone development is not well described in cattle. Using a combination of pQCT and measures of stature provides valuable insight into how heifers grow and how bone responds in relation to growth. Therefore, the aim of this thesis was to investigate the relationship between live weight and bone morphology in the bovine forelimb to identify potential risk periods for spontaneous humeral fractures in first-lactation dairy heifers.

Therefore, the main objectives of the work presented in this thesis are:

- To evaluate whether computed tomography scans of the metacarpus can be used as a predictor for humeral bone structure.
    - Hypothesis- Computed tomography scans of the metacarpus can predict humeral bone structure.
- Describe the relationship of pQCT-derived measures of bone strength and morphology within two different bones, in the proximal and distal limb, with gross measurements of size and growth.
    - Hypothesis- Measures of bone strength using pQCT are highly correlated with gross measures of animal size.
- Examine the effect of sex and age on bone morphology post puberty.
    - Hypothesis- The onset of puberty causes the divergence in measures of bone morphology in bulls, steers and heifers.
- Describe proportional changes in stature with weight in Holstein Friesian, Jersey and Holstein-Friesian Jersey crossbred calves from birth to 15 months.
    - Hypothesis- Proportional changes in stature are affected by breed from birth to 15 months of age.
- Compare and contrast pQCT derived measures of bone morphology of bones collected from 2-year-old dairy heifers affected and unaffected by humeral fractures.
    - Hypothesis- pQCT derived measures of cortical bone thickness and stress strain index differs between heifers affected and unaffected by humeral fractures.

Chapter 2

Can bone measures of the bovine metacarpus predict humeral bone structure?

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2.1. Abstract

Peak bone mass in cattle is influenced by factors prior to puberty and insufficient peak bone mass prior to the first lactation predisposes heifers to the risk of spontaneous humeral fractures. At post-mortem examination, affected heifers present with osteoporosis (reduced periosteal circumference and reduced cortical wall thickness). Due to its location, there is no cost-effective way to scan the humerus in the live animal. However, the metacarpal bones (MC3/MC4) are straightforward to scan in a live anaesthetised animal. The aim of this experiment was to determine if the metacarpal could be used to predict bone mass and material properties of the humerus. The left humerus and metacarpal bones were obtained from 57 cattle aged six weeks to eight years. Bones were scanned at the mid-diaphysis using peripheral quantitative computed tomography (pQCT), to obtain measures of bone mass and material properties (strength). Strong positive relationships were observed (R^2=0.89 - 0.98, P<0.0001) for pQCT measurements of bone mass and strength. Thus, the metacarpus is a good predictor of bone content and material properties of the humerus across cattle of differing age and maturity.

2.2. Introduction

Spontaneous humeral fracture is an emerging condition affecting at least 4% of all dairy farms in New Zealand. It is predicted that approximately 5,000 heifers per year are lost to this syndrome, resulting in an economic loss of around $10 million to the New Zealand dairy industry (Dittmer et al., 2016). Currently there is no method to

identify heifers that are at risk of humeral fractures. At present, a herd can only be identified as being at risk of humeral fractures when multiple heifers have been affected, at which point there is limited opportunity to increase bone quality and quantity on a farm level. Post-mortem examination of humeral fractures has identified reduced cortical bone thickness in affected bones (Dittmer et al., 2016).

To understand why the humerus, in particular, appears to be most at risk of fracture, bone developmental stages need to be considered. This includes changes in bone measurements with growth and nutritional factors when peak bone mass occurs. Humeral growth is constant with growth in size of the animal, whereas bones in the distal limb, such as the third and fourth metacarpal (MC3/MC4), have a decrease in growth trajectory as the animal gets larger. Berg et al. (1978) compared the proportion of bone to total bone weight of carcasses reported in different studies and demonstrated that as the size of the carcass increased, the proportion of metacarpal weight to total bone weight decreased whereas, the proportion of bone in the shoulder (including the humerus) remained constant (Berg et al., 1978). This shows how the humerus is dynamic in response to growth, while the metacarpus is less so.

The majority of peak bone mass is accumulated at puberty. Just prior to puberty, there is a rapid increase in bone mass, due to rising secretions of growth hormone (GH), insulin-like growth factor 1 (IGF-I) and sex steroids (Glastre et al., 1990). The increase in circulatory GH and IGF-I concentrations promote bone turnover by stimulating osteoblast proliferation and differentiation (Green et al., 1985). Precursors for male

and female sex hormones, such as dehydroepiandrosterone, are also related to bone strength. Increases in muscularity at puberty is also thought to be a factor in increasing bone mass during puberty at approximately 12 months old (Meier et al., 2017, Gilsanz et al., 2011). However, growth continues in cattle until around 20-22 months, at which point it slows considerably (Handcock et al., 2018). Therefore, it could be expected that the bone dimensions continue to change based on age and growth until this point.

Bones from cattle with spontaneous humeral fractures have osteoporosis (Dittmer et al., 2016). Deficits in nutrition may cause osteoporosis, and in farm animals in particular, nutrient deficiency associated with starvation is one of the most common causes of osteoporosis (Craig et al., 2016). Inadequate nutrition (total energy and protein requirement) on farm may occur due to drought or overstocking, leading to a lack of pasture, or lack of quality pasture, or both. Young animals are more prone to the effects of undernutrition due to the energy requirements of growth and development, for example in dairy calves when milk quality or quantity is limited. Cooper et al. (1997) reported that human infants with low birth weights had a reduced bone mineral content at an older age compared to bone mineral content in human infants of a normal birth weight. This could be a possible risk factor for humeral fractures, when calves fed restricted diets early in life could have lower bone mineral content and, therefore, be at risk of fracture.

Bone composition can be measured using peripheral quantitative computed tomography (pQCT), providing quantitative data on trabecular and cortical bone

material properties and distribution (Firth et al., 2011). In the anaesthetised animal, pQCT of the distal limb can be performed quickly and reliably. Scanning of distal limb, such as the metacarpus, can be achieved with pQCT in live cattle and in the humerus from animals submitted for post-mortem examination. To interpret any differences in bone composition seen on pQCT, an understanding of normal bovine bone structure and the effects of age is required.

Due to the location of the humerus, the ability to scan the bone in a cost-effective manner is difficult. Scanning of the humerus is only possible at post-mortem when the bone can be removed. This makes it difficult to predict heifers at risk of humeral fracture. It is possible that bones such as the metacarpus that are easily accessible in a live animal, could act as a proxy to predict humeral structure. Therefore, the aim of this study was to determine if pQCT measures of the metacarpal bone could be used to predict the structure of the humerus.

2.3. Materials and methods

The metacarpus and corresponding humerus were collected opportunistically from a total of 57 animals from three different studies. As well as bone scans and size, weight, age, and general breed type were recorded. The bones can be divided in to three cohorts. The first cohort (n=17) of bones were collected from a dairy-calf-nutrition study in which calves were fed either 5 L or 10 L of milk a day, and euthanized either at 6 weeks or 12 weeks of age. The second cohort (n=17) of bones was collected from a terminal study in which beef/dairy cross steers were sent to the abattoir at one year of

age. The third cohort (n=23) of bones were collected from animals of mixed age submitted for post-mortem examination for non-orthopaedic issues.

Metacarpal length was measured with a ruler, and determined as the distance between the lateral aspect of the lateral condyle of the MC4 and the proximal aspect of the lateral MC4. Humeral length was measured from the proximal end of the humeral head at the lateral aspect to the end of the trochlea at the distal end using a box ruler.

The mid-diaphysis of each bone was scanned using peripheral quantitative computed tomography (pQCT; XCT 2000, Stratec Medical) with the bone being scanned at 50% of the total bone length starting from the distal end with a voxels size of 0.3 mm^3. Voxels were assigned, within the manufactuers software, as either "trabecular" bone (≤710 mg/mL), or as "cortical" bone (>710 mg/mL). Data derived from the scan included measures of total bone content, cortical content, cortical density, total area, trabecular area, cortical area, cortical content, cortical density, cortical area, cortical thickness, periosteal circumference and endosteal circumference.

2.3.1. Statistical analysis

Data are presented as least-square means ± SEM by bone, study and parameter. Correlations were analysed between the metacarpus and humerus. A General Linear Model was used to generate LS means and regression coefficients for bone parameters. The model used the fixed effect of cohort. Where a cohort effect was significant ($P<0.05$), the interaction of cohort and bone was fitted and separate regression

equations were produced for each cohort by bone. All statistical analyses were conducted using the Statistical Analysis System software version 9.4 (SAS Institute Inc., Carey NC, USA).

2.4. Results

The age and weight range for the calf cohort was 11 to 90 days and 35.5 to 99.5 kg, respectively. All animals in the yearling cohort were approximately one year old and ranged from 303 kg to 392 kg. The mixed-age cohort ranged from two years to eight years of age, but live weights in this cohort were not routinely recorded.

There were significant differences among age cohorts for the all the remaining bone parameters listed in Table 2.1. These were associated with significant differences ($P<0.05$) between the calves and the older cohorts of yearlings and mixed-age cows. The yearlings and mixed-age cows had similar values for many of these parameters.

At the mid-diaphyseal site of the humerus there were significant differences for all parameters. In contrast to the metacarpus, the general pattern for the humerus was the presence of significant differences in parameters between all age cohorts.

Table 2.1 Composition of study population showing the calves, yearlings and mixed-age cohorts with their average age, weight with standard deviations and main production type for each cohort. Means of bone measures and standard error of mean by cohort and bone.

	Calves	Yearlings	Mixed-age	P value
Cohort				
n	17	17	24	
Mean age	61±6 days	1 Year	4.4±0.5 years	
Mean weight*	68.6±4.6 kg	345.8±5.1 kg	-	
Main production type	Dairy	Dairy/Beef	Dairy	
Metacarpal				
Bone length (mm)	174.8±2.4^{a}	207.9±2.8^{b}	206.0±2.4^{b}	<0.001
Total bone area (mm^2)	345.9±19.1 ^{a}	759.7±19.1 ^{b}	727.8±16.14 ^{b}	<0.001
Total bone content (mg/mm)	254.4 ± 15.3 ^{a}	679.0 ± 15.3 ^{b}	695.9±13.1 ^{b}	<0.001
Periosteal circumference (mm)	65.7±1.4 ^{a}	97.6±1.4 ^{b}	95.4±1.2 ^{b}	<0.001
Endosteal circumference (mm)	43.7±1.9^{a}	55.2±1.9^{b}	50.4±1.6^{b}	<0.001
Cortical thickness (mm)	3.5±0.2^{a}	6.8±0.2^{b}	7.2±0.1^{b}	<0.001
Cortical bone area (mm^2)	193.5±10.4 ^{a}	513.0±10.4 ^{b}	518.8±8.9 ^{b}	<0.001
Cortical bone content (mg/mm)	223.4±15.9 ^{a}	646.2±15.9 ^{b}	663.9±13.7 ^{b}	<0.001
Cortical bone density (mg/cm^3)	1152.6±11.3^{a}	1257.0±11.3^{b}	1278.9±9.7^{b}	<0.001
Stress strain index (mm^3)	1060.0±113.9^{a}	4536.0±134.7^{b}	4445.9±115.8^{b}	<0.001
Humerus				
Bone length (mm)	211.6±2.8^{a}	240.3±2.8^{b}	265.5±2.4^{c}	<0.001
Total bone area (mm^2)	634.9±42.3 ^{a}	1522.7±42.3 ^{b}	1808.5±36.4 ^{c}	<0.001
Total bone content (mg/mm)	386.3±30.0 ^{a}	1266.5±30.0 ^{b}	1429.0±25.8 ^{c}	<0.001
Periosteal circumference (mm)	88.7±2.1 ^{a}	138.2±2.1 ^{b}	150.5±1.8 ^{c}	<0.001
Endosteal circumference (mm)	65.24±2.24 ^{a}	84.2±2.24^{b}	98.4±2.0 ^{c}	<0.001
Cortical thickness (mm)	3.71±0.18 ^{a}	8.6±0.18 ^{b}	8.3±0.16 ^{b}	<0.001
Cortical bone area (mm^2)	288.65±22.23 ^{a}	953.8±22.23 ^{b}	1030.5±19.1 ^{c}	<0.001
Cortical bone content (mg/mm)	339.01±29.20 ^{a}	1202.8±27.6^{b}	1351.3±23.6 ^{c}	<0.001
Cortical bone density (mg/cm^3)	1174.19±6.67^{a}	1262.5±6.67^{b}	1310.3±5.73^{c}	<0.001
Stress strain index (mm^3)	836.3±476.5^{a}	12878.7±476.5^{b}	15121.6±409.7^{c}	<0.001

*Mixed age cows at the time of post-mortem were not routinely weighed.

Strong correlations were observed between the mid-diaphyseal measurements of the humerus and metacarpal (R^2=0.66-0.97, P<0.001) for periosteal circumference, total bone mineral content, cortical bone content, cortical bone density, total area and cortical area, cortical bone mineral content, cortical bone area, cortical thickness and stress strain index (an index based on the summative weighting of voxel BMD and the square of its location relative to the central axis) (SSI; Table 2.2.). A weak correlation (R^2=0.46; P<0.001) existed for endosteal circumference.

Table 2.2 Correlations between bone parameters in the metacarpal with the same measure in the humerus in the calf, yearling and mixed age cohorts.

	Correlation	P- value
Total bone area	0.89	<0.001
Total bone content	0.96	<0.001
Periosteal circumference	0.92	<0.001
Endosteal circumference	0.46	<0.001
Cortical bone thickness	0.91	<0.001
Cortical bone area	0.96	<0.001
Cortical bone content	0.95	<0.001
Cortical bone density	0.66	<0.001
Stress Strain Index	0.97	<0.001

Several parameters, generally those associated with size measurements rather than material properties, had high R^2 values, indicating that the metacarpus could be a suitable predictor of the same parameter in the humerus (Table 2.3.). Despite the different intercept, there was no interaction (P>0.05) of metacarpal parameters with cohort, so that the relative differences between the bones for these parameters were consistent among ages.

Table 2.3 Regression equations for prediction of the same bone parameter in the humerus where there was no study interaction in dairy cattle in the calf yearling and mixed-age cohorts.

	Intercept Calves	Intercept Yearlings	Intercept Mixed-age	Coefficient all studies	R^2
Total area	237.6	650.2	972.6	1.15	0.92
Total bone content	135.3	596.5	742.3	1.0	0.95
Periosteal circumference	26.6	45.9	60.3	1.0	0.94
Endosteal Circumference	43.4	56.62	73.2	0.5	0.75
Cortical bone thickness	2.30	5.88	5.42	0.4	0.91
Cortical bone area	93.9	580.4	509.5	1.0	0.95
Cortical bone content	150.2	656.7	790.2	0.9	0.95
Cortical bone density	1229.8	1323.1	1371.9	-0.1	0.82
Stress strain index	-974.2	-1356.6	1169.0	3.1	0.98

An interaction of cohort and bone was observed for cortical and subcortical bone mineral density and trabecular bone mineral density (Table 2.4.). This resulted in the generation of different regression coefficients for each age group. The yearling cohort had the least change in the humerus relative to the metacarpus compared to the mixed-age and calf cohorts, as shown by the low coefficient for all four traits. The mixed-age cohort had the largest difference between the two bones, which is represented by large coefficients in the regression equation.

Table 2.4 Regression equations where there was a study effect for prediction of the humeral cortical and subcortical density and trabecular bone density in dairy cattle in the calf yearling and mixed-age cohorts.

	Calves		Yearlings		Mixed age		
	Intercept	Coefficient	Intercept	Coefficient	Intercept	Coefficient	R^2
Cortical/subcortical density	1509.1	486.0	1189.6	-0.20	0.6	0.03	0.88
Trabecular bone density	79.2	29.4	77.0	0.02	0.6	0.09	0.34

2.5. Discussion

Simple correlations comparing the mid-diaphysis of the metacarpal and humeral bones demonstrated that the metacarpal is a good predictor of humeral bone structure in cattle. The relationships were strongest with the cortical bone and total bone area parameters.

With increasing age there was an increase in periosteal circumference, but this was not associated with the same relative increase in cortical wall thickness, and the rate of increase in cortical wall thickness was lower in the humerus than in the metacarpus. This is likely to be associated with bending strain associated with the different anatomical locations of the bones (proximal versus distal) and the relatively greater increases in bending strength obtained by increasing bone circumference and cortical wall thickness (van Der Meulen et al., 1993). This observation was supported by the stress strain index, where the SSI of the bone increased with increased mean periosteal circumference between the yearling and mixed-age cohorts, despite no corresponding increase in cortical thickness.

The SSI was approximately three times higher in the humerus than in the metacarpus in the yearling and mixed age cohorts. Bone is dynamic and changes in response to increases in loading by increasing bone strength (the mechanostat theory). A larger SSI in the humerus, and hence greater resistance to bending strain, implies that the mechanical forces that act on the humerus are greater than those acting on the metacarpus, especially in older cattle. This may explain why reduced cortical

thickness and periosteal circumference predispose dairy cattle to spontaneous humeral fractures, but not fractures of distal limb bones such as the metacarpus.

Mean bone parameters of the metacarpus in the yearling cohort and mixed-age cohort were not significantly different. However, in the humerus, the mixed-age cohort had significantly higher bone parameters compared to yearlings. Development in the metacarpus, is, therefore likely to be completed by one year of age, while the humerus continues to develop. This is in agreement with Pomeroy (1978) who suggested that the development of upper limbs such as the humerus occurs later compared with the metacarpus. The continuing development of the humerus after one year of age shows how dynamic the humerus can be in response to body growth and increases in muscular forces placed on the bone.

2.6. Conclusions

To conclude, pQCT scans of the metacarpus allow for predictions of humeral structure in cattle, possibly enabling the detection of animals at risk of humeral fractures and providing an opportunity for preventative measures to be taken.

Chapter 3

Bone morphology and strength in the mid-diaphysis of the humerus and metacarpus in dairy calves prior to weaning.

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Foreword to Chapter 3

The previous chapter (Chapter 2), was used to validate the use of pQCT derived measures to asess bone morphology in the metacarpus and humerus. Results demonstrated that there was a strong relationship between the metacarpus and humerus. Therefore, it is possible that pQCT measures of the metacarpus could be used to predict the same parameter in the humerus. Results indicated that there was differences in bone maturity between the distal (metacarpus) and proximal (humerus) regions of the forelimb. The next step of this research was to relate observed bone paramters with measures of stature (Chapter 3). Measures of live weight and stature (wither height, girth, wither-rump length, and leg length) were examined against pQCT-derived parameters in the metacarpus and humerus. The pQCT methodology was continued in Chapters 3, 4 and 6.

3.1. Abstract

Calf growth rate in relation to future milk production has been thoroughly studied, however, the observation of growth arrest lines in bones from heifers with humeral fractures has highlighted the need to understand bone growth in relation to calf growth. The aim of this study was to describe the relationship of peripheral quantitative computed tomography (pQCT)-derived measures of bone strength and morphology with gross measurements of size and growth in pre-weaning dairy calves. Liveweight, height, body-length, girth and leg length were measured at one, six and twelve weeks of age. At these intervals, the mid-diaphysis of the metacarpus was also scanned in the live animal using pQCT. At six and twelve weeks old, a subset of calves were euthanised and the humerus was collected and scanned at the mid-diaphysis using pQCT. Differences in growth patterns were observed between the metacarpus and humerus over time. Weight was the best predictor for measures of periosteal circumference and stress strain index (R^2= 0.49-0.58) in the metacarpus, and also the best predictor for measures of stress strain index at all ages in the humerus (R^2= 0.94). The strong relationship with weight and bone measures emphasises the need for adequate preweaning nutrition for future bone growth.

3.2. Introduction

The association of growth and future production in dairy cattle has been well described within the literature (Van De Stroet et al., 2016, Shamay et al., 2005). However, the effect of growth and environment on subsequent bone development in dairy heifers has often been overlooked. Humeral fractures in first lactation dairy heifers were first reported in 2008, and represent a significant economic and welfare issue for the New Zealand dairy industry, with an estimated within herd incidence of 4 – 25%, and approximately 5000 dairy replacements each year may be affected (Dittmer et al., 2016). A case-control study of humeral fractures showed a reduction in cortical thickness and osteoporosis. While the risk factors for humeral fracture are multifactorial, a major contributor is believed to be poor nutrition early in a heifer's life (Dittmer et al., 2016). However, there is currently no published literature to inform on the timing and severity of nutrition restriction required to have an effect.

Bone growth and development has been thoroughly described, particularly in relation to osteoporosis, in humans and animal (murine) models. Historically, particularly in human studies, data has been obtained using Dual-energy X-ray absorptiometry (DEXA) which had been considered the gold standard of non-invasive measurements. However, a limitation of DEXA is the inability to distinguish between changes in cortical and cancellous bone due to scanning a 3-dimensional model (bone) in 2-dimensions. Using DEXA, bone mass can be

measured, but changes in cancellous bone and more importantly cortical bone cannot be quantified (Choksi et al., 2018). In contrast, peripheral quantitative computative tomography (pQCT) provides a non-invasive technique of determining bone parameters, but is able to describe not just bone mass and density, but also measures of bone geometry, and thus calculation of precise measures of bone strength and resistance to fracture (Gasser, 1995).

There is limited published literature describing the effect of sex and other factors on bone growth and development in livestock, with much of the published literature focusing on horses (Davies et al., 1984, Firth et al., 2012). There is some limited data on bone growth and development in cattle, however, the majority of this has been undertaken using DEXA, preventing description of changes in bone geometry and microarchitecture (Logan et al., 2019). Therefore, the aim of this study was to describe the relationship of pQCT-derived measures of bone strength and morphology within two different bones, in the proximal and distal limb, with gross measurements of size and growth.

3.3. Materials and Methods

This report details a prospective observational study with serial and terminal sampling to ensure measurement of bone parameters at the mid diaphysis of two bones in the proximal and distal limb on three separate occasions during pre-weaning growth. Measures of frame size and weight were taken to allow for comparison of size and bone parameters.

3.3.1. Animals and management

The cohort consisted of 21 female Friesian-Jersey crossbred calves born at Massey University's Dairy 1 farm during the 2016 spring calving season. Calves were fed *ad libitum* colostrum from birth to three days old. At four days of age, calves were assigned into two feed groups and offered either 10 L (n=9) or 5 L (n=12) of milk per day, split over two feeds (am and pm). From birth, calves were housed in a shed, and at approximately three weeks old all calves were managed at pasture, excluding a subset of six calves from the 5 L cohort that remained in the shed with the intention to restrict exercise. The six calves that remained in the rearing shed were offered access to a balanced ration of meal (Sharpes Pellets, Commercial mix, 18% crude protein) at a rate that provided equivalent estimated daily Digestible Energy (DE) and nutrients equivalent to the calves with access to pasture.

3.3.2. Sampling and measurements

Calves were measured at birth and at time of pQCT scanning for live weight and the following stature measures: wither height, girth, leg length (from ground to elbow of the right forelimb) and wither-rump length (stature measures).

In cattle the third and fourth metacarpus are fused and are subsequently referred to as the metacarpus within the text. The metacarpus (MC3/MC4) was scanned at one (scan one), six (scan two) and twelve (scan three) weeks of age. The metacarpus of the right limb was scanned whilst calves were in lateral recumbency, and anaesthesia was induced with either 6 mg/kg followed by 0.3 mg/kg/min during anaesthesia of propofol (Fresofol 1% MCT/LCT) or 2 mg/kg followed by 0.2 mg/kg/min during anaesthesia of Alfaxalone (Alfaxan multidose 10mg/ml).

Of the 21 animals, eight were euthanised at the six-week scan (three from 5L restricted exercise, three from 5L unrestricted exercise and two from 10L) and eight animals at the twelve-week scan (three from 5L restricted exercise, two from 5 L unrestricted exercise and three from 10 L). One calf from the 10 L group was euthanised after the one-week scan and one calf from the 5 L non restricted exercise group after the six-week scan due to ill-thrift not related to the current study. This left three live calves from the 10 L group after the twelve-week scan that were returned to the herd for a future study. At euthanasia the humerus and metacarpus were dissected-out and cleaned of muscle mass, double wrapped in plastic and

stored frozen (-20°C freezer) until subsequent pQCT scanning. All procedures related to the management and use of the animals during the experiment were approved by the Massey University Animals Ethics Committee (MUAEC 16/65).

3.3.3. Sampling Techniques

All pQCT scanning was carried out using an XCT 2000 peripheral quantitative computed tomography machine (Stratec Medical). For each bone, a 2 mm slice at the mid diaphysis was obtained with a voxel size of 0.3 mm^3. In the metacarpus, the mid-diaphysis was defined as 50% of the total bone length using the lateral aspect of the lateral condyle of the fourth metacarpal (MC4) and the proximal aspect of the lateral MC4. In the humerus, the mid-diaphysis was defined as 50% of total bone length, measured from the most distal aspect of the trochlea, to the proximal end of the humeral head at the lateral aspect.

Within the manufacturers software, voxels >710 mg/cm^3were assigned as "cortical" bone. Data derived from the scan included measures of total bone content, cortical content, cortical density, total area, trabecular area, cortical area, cortical content, cortical density, cortical area, cortical thickness, periosteal circumference, endosteal circumference and stress strain index (SSI). The stress strain index is the ability to withstand bending from lateral, dorso-palmar and torsional forces and is calculated by incorporating the index of material stiffness (bone mineral density) and bone

geometry (cross-sectional moment of inertia) (Firth et al., 2011). These will be referred to as bone parameters in the statistical models.

3.3.4. Statistical analysis

Statistical analysis was conducted using SAS version 9.4 (SAS Institute Inc., Cary NC, USA). Bone parameters and stature measures were analysed using a general linear model that included the fixed effect of age at measurement. Rearing treatment (milk allowance and exercise) was tested in this model but was removed because it failed to reach significance ($P>0.05$) for any parameter considered. Similarly, change in bone parameters and stature measures were analysed using a general linear model that included the fixed effect of period (first to second scan versus second to third scan). Bone parameters were regressed on body weight on day of measurement to calculate the regression coefficient for body weight at each measurement age. Bone parameters were analysed using a multivariate regression with stepwise selection, which considered stature measures as predictors of each bone parameter within measurement event. The threshold for inclusion in the multivariate regression model was $P<0.05$.

3.4. Results

The calves had a mean birth weight of 35.2 ± 0.9 kg with a range of 29.0-42.5 kg. At the twelve-week scan, the age at which calves would have been weaned, they weighed on average 101.9 ± 2.3 kg. At the time of each pQCT scan, stature measures had increased significantly from the previous measure (Table 3.1., $P<0.05$).

At the mid-diaphysis site of the metacarpus there were significant increases with age at scanning ($P<0.05$) in all bone parameters measured with the exception of endosteal circumference, which did not change between scans (Table 3.1.). There were significant increases in most of the bone parameters of the humerus at six and twelve weeks of age ($P>0.05$), excluding cortical density. Mean bone measures were greater in the humerus than the metacarpus at each age, including stress strain index which at 12 weeks was 3398.9 ± 146.4 mm^3, which was approximately twice that calculated for the metacarpus (1431.4 ± 49.4 mm^3). The primary contributors to the increase in stress train index during growth differed between the two bones. In the metacarpus, between the six to twelve-week scan there was an 11% increase in periosteal circumference and 30% increase in cortical thickness, which resulted in a 49% increase in stress strain index. In contrast, in the humerus, a 22% increase in periosteal circumference in association with a similar 27% increase in cortical thickness, resulted in a 91% increase in the stress strain index.

Table 3.1 Least squares means and standard error of stature measures and pQCT bone measures of the humerus and metacarpus at 1, 6 and 12 weeks of age in dairy calves.

Age	1 week	6 weeks	12 weeks	P-value
Stature				
N	21	20	11	
Liveweight (kg)	36.1±1.6[a]	57.0±1.65[b]	102.0±2.3[c]	<.001
Age (days)	6.1±0.6[a]	38.9±0.6[b]	84.1±0.9[c]	<.001
Wither-rump length (cm)	87.0±1.3[a]	97.2±1.4[b]	118.2±1.9[c]	<.001
Height (cm)	75.0±0.8[a]	82.0±0.8[b]	94.6±1.1[c]	<.001
Girth (cm)	76.8±0.9[a]	89.8±0.9[b]	107.4±1.3[c]	<.001
Leg Length (cm)	48.6±0.7[a]	51.9±0.7[b]	57.8±1.0[c]	<.001
Metacarpus				
N	21	20	11	
Bone length(mm)	160.2±1.3[a]	168.9±1.4[b]	181.0±1.9[c]	<.001
Periosteal circumference (mm)	60.6±0.9[a]	63.5±1.0	70.6±1.4[c]	<.001
Endosteal circumference (mm)	43.6±1.1	43.6±1.1	45.5±1.5	0.546
Total bone area (mm^2)	293.3±9.3[a]	321.1±9.5[b]	397.5±13.4[c]	<.001
Total bone content (mg/mm)	181.2±4.7[a]	218.7±4.8[b]	297.5±6.8[c]	<.001
Cortical bone thickness (mm)	2.7±0.1[a]	3.1±0.1[b]	4.0±0.1[c]	<.001
Cortical bone area (mm^2)	140.5±3.7[a]	167.8±3.8[b]	231.1±5.4[c]	<.001
Cortical bone content (mg/mm)	151.1±4.2[a]	194.35±4.4[b]	269.6±6.0[c]	<.001
Cortical bone density (mg/cm^3)	1075.7±5.8[a]	1142.9±6.0[b]	1166.7±7.4[c]	<.001
Stress strain index (mm^3)	734.1±34.1[a]	962.35±34.9[b]	1431.4±49.4[c]	<.001
Humerus				
N	0	8	8	
Periosteal circumference (mm)	-	79.4±2.3[a]	97.0±2.3[b]	<.001
Endosteal circumference (mm)	-	58.9±2.3[a]	70.7±2.3[b]	0.003
Total bone area (mm^2)	-	504.4±32.16[a]	752.1±3.6[b]	<.001
Total bone content (mg/mm)	-	305.6±15.0[a]	466.6±15.0[b]	<.001
Cortical bone thickness (mm)	-	3.3±0.1[a]	4.2±0.1[b]	<.001
Cortical bone area (mm^2)	-	226.3±11.4[a]	350.7±11.4[b]	<.001
Cortical bone content (mg/mm)	-	264.4±12.7[a]	413.4±12.7[b]	<.001
Cortical bone density (mg/cm^3)	-	1169.8±5.3	1179.4±5.3	0.228
Stress strain index (mm^3)	-	1776.4±146.4[a]	3398.9±146.4[b]	<.001

[a, b, c] Means with different superscript within row at each age are significantly different ($P<0.05$).

At the mid-diaphysis site of the metacarpus, increases in total area and periosteal circumference were positively associated with bodyweight (Table 3.2.). In contrast, endosteal circumference at twelve weeks, was better explained by wither-rump length and height. In the regression model for cortical content in the metacarpus at six weeks, weight was the best stature measure to explain increases in content. However, at one and twelve weeks, girth slightly improved the fit of the regression model, compared to the use of liveweight.

When using a stepwise selection model, girth was a major predictor of total size and area, but there was only moderate improvement above using only weight in the model. Total content, cortical thickness, cortical area, cortical content, and stress strain index was positively associated with weight (R^2 =0.73-0.94), however, cortical thickness was also negatively associated with height. Cortical density was not significantly affected by any stature measure ($P<0.05$).

Table 3.2. Regression equations at each scan age of the metacarpal fitting with stepwise selection using weight, girth, birth weight, wither-rump length, height, and leg length as potential predictors. Regression equations for the mid-diaphyseal site of the humerus for scans at 6 and 12 weeks of age fitting with stepwise selection using weight, girth, birth weight, wither-rump length, height and leg length as potential predictors. Weight R^2 indicates the model fit when using only weight as a predictor for each bone measure at each scan. Missing value indicates that the parameter was not significant at the P<0.05 threshold.

Bone	**Scan**	**Intercept**	**Weight**	**Girth**	**Wither-rump Length**	**Height**	**Leg**	**Weight R^2**	**Step wise R^2**
METACARPUS									
Periosteal	1	31.7±6.9	0.8±0.2					0.49	0.49
circumference (mm)	2	42.9±5.1	0.4±0.1					0.50	0.50
	3	39.0±10.6	0.3±0.1					0.53	0.53
Endosteal	1	11.95±8.2	0.9±0.2					0.45	0.45
circumference (mm)	2	27.5±6.7	0.3±0.1					0.26	0.26
	3	-72.1±30.5			0.4±0.1	0.75±0.3		0.32	0.72
Total area (mm^2)	1	20.1±64.7	7.6±1.8					0.50	0.50
	2	119.0±50.2	3.6±0.9					0.50	0.50
	3	42.0±122.2	3.5±1.2					0.52	0.52
Total content	1	107.8±33.8	2.0±0.9					0.21	0.21
(mg/mm)	2	101.4±21.4	2.1±0.4					0.65	0.65
	3	NS						0.28	
Cortical thickness	1	NS						0.04	
(mm)	2	NS						0.15	
	3	NS						0.00	
Cortical area	1	82.5±27.4	1.6±0.8					0.20	0.20

(mm^2)	2	72.4±16.7	1.7±0.3			0.67	0.67
	3	NS				0.30	
Cortical Content	1	-10.4±73.0		2.1±1.0		0.20	0.21
(mg/mm)	2	84.5±20.0	1.9±0.3			0.65	0.65
	3	-82.9±182.7		3.3±1.7		0.27	0.32
Cortical Density	1	NS				0.00	
(mg/cm^3)	2	NS				0.01	
	3	NS					
Stress strain index	1	-33.5±191.7	21.3±5.3			0.47	0.47
(mm^3)	2	117.7±180.1	15.0±3.1			0.58	0.58
	3	-32.1±503.8	14.4±4.9			0.52	0.52
HUMERUS							
Periosteal circumference (mm)		-1.7±10.3		0.9±0.1		0.82	0.84
Endosteal circumference (mm)		20±15.7			1.0±0.1	0.62	0.68
Total bone area (mm^2)		-634.2±150.1		13.0 ±1.7		0.82	0.83
Total bone content (mg/mm)		126.6±21.3	3.4±0.3			0.92	0.92
Cortical bone thickness		6.1±1.8	0.03±0.01		-0.06±0.02	0.73	0.73
Cortical bone area (mm^2)		89.4±16.9	2.6±0.2			0.92	0.92
Cortical bone content (mg/mm)		21.2±17.3	1.2±0.2			0.68	0.68
Cortical bone density (mg/cm^3)		NS				0.00	
Stress strain index (mm^3)		-30.5±191.3	34.2±2.4			0.94	0.94

3.5. Discussion

The calves were reared on a commercial dairy farm using standard New Zealand farming practices. At twelve weeks of age, the average weight of 102 kg aligned with the industry standards of 90 kg at 8-12 weeks of age for a Holstein-Friesian-Jersey crossbred heifer calf (Back, 2017).

Differences in the pattern of growth between the metacarpus and humerus were observed that reflected the relative growth of the two bones during the study period and the differences in mechanical loading of the bones due to bodyweight and muscular strain. Endosteal circumference did not increase in the metacarpus, but periosteal circumference increased, demonstrating that cortical bone (>710 mg /cm^3) was being deposited on the outside of the bone resulting in increased cortical thickness, bone area/size and therefore, bone strength. However, in the humerus, endosteal circumference increased proportionally with periosteal circumference. This appositional growth is a highly efficient mechanism to increase bone strength (SSI) during growth, with minimal increases in bone mass, and thus only a marginal increase in the resources required for bone growth.

Mechanical loading affects bone structure in the form of changing its mass and architecture of bone to optimise (i.e. minimise) the effective strain rate. The different strains applied to the two bones during growth, and the tissue (structural) responses observed, reflected their anatomical position and relative growth potential. There was a consistent association of weight with bone parameter measures in the humerus,

which contrasted with the limited association (R^2) identified with bone parameter measurements in the metacarpus. The difference in associations of stature measures with bone can be explained by the anatomical location of the two bones (proximal vs distal) which generated very different patterns of strain, differing in both the magnitude and direction of the strain, placed on the two bones (Bijen et al., 2020, Bouza-Rodríguez and Miramontes-Sequeiros, 2014).

The greater SSI of the humerus mid-diaphysis implies that at this site, the humerus is subjected to greater force than the metacarpus. The shape of the humerus is derived by the direction and size of the muscle forces exerted on the bone as additional bone is placed on regions that have the largest bending strain (Raab-Cullen et al., 1994). Finite element analysis of the humerus indicates that first force (primary and majority of strain) on the humerus is torsion, attributed to combined force of the supraspinatus, infraspinatus, pectoralis and subscapularis muscles. The second force can be attributed to the triceps brachialis muscle. The third and final force group considered was force placed on the humerus attributed to body weight (Bouza-Rodríguez and Miramontes-Sequeiros, 2014). In contrast, the loading on the metacarpus in cattle is effectively loading under stance (compression), and low strain rate locomotion (i.e. walking) ((Bijen et al., 2020)). Girth was highly correlated with weight, so the slight improvement in model fit with the inclusion of girth may be due to girth providing an indication of frame size, and therefore an indirect measure of thoracic growth and muscle force.

During growth, in both the metacarpus and humerus, increases in strength (SSI) were obtained by increases in circumference, with only minimal changes in cortical bone density. Increases in bone strength is also likely to be a result of changes in bone microarchitecture as primary bone is replaced with lamellar bone tissue forming osteons (Craig et al., 2016). Bone mineralization is much slower so minimal changes in density would occur and the changes in architecture are unable to be detected at a pQCT level. Biologically, the first approach to increase bone strength is to increase bone size (circumference) and only after exposure to high strain rates (e.g. galloping) is there an increase in bone density. In young horses, significant increases in density at the mid diaphysis were only observed after the introduction of intensive exercise such as galloping (Firth et al., 2012, Rogers et al., 2012). In comparison, calves, which are not a cursorial species, do not have the same predisposition to run at high speed and are therefore unlikely to subject the distal limb to the high strain rates observed in galloping horses. Even when calves were run on a concrete path this failed to induce sufficient high strain rates to increase bone density, with the primary response being an alteration in bone circumference and focal response in morphology to the strain (deposition of bone on the dorsal surface) (Logan et al., 2019). The results from the current study agrees with Hiney et al. (2004) that, calves on a pasture based system will not reach the critical loading threshold to induce an increase in cortical density and no differences in cortical density between calves on restricted and unrestricted exercise would be expected.

The rate at which the humerus increased in strength (SSI) was higher than what is observed in the metacarpal bone, reflecting that the majority of bone growth in these animals was in the proximal rather than distal limb. This rapid and sustained growth in the humerus, in association with increases in periosteal circumference but not cortical thickness, may predispose the humerus to pre-pubertal growth perturbations due to malnutrition or illness. Pre-pubertal growth perturbations may affect the ability of the humerus reach peak bone mass and therefore increase the risk of humeral fracture in severe cases. This highlights the need for adequate growth prior to weaning and puberty, to not only allow animals to maximise production performance but to ensure peak bone mass can be achieved.

It is possible that the effect of pre-pubertal malnutrition (i.e. feed restriction) on bone development does not become obvious, or quantifiable, without histological examination, until later in life. The effect of diet, in this study, did not influence stature or any of the bone parameters so was not included within the model. It is possible that the lack of effect of diet on bone parameters was due to the low sample number of calves in each treatment group. However, it is likely that if an effect of diet was to occur, it would occur later in life as observed by Moallem et al. (2010). Therefore, the primary effect of diet (differing nutrition planes) may be alteration of the developmental pathway which may have been observed if scans had been repeated at an older age after the pre-weaning growth period. Support for this hypothesis can be derived from observations of the effect of early activity (including pre-weaning high

strain rate exercise) on bone development up to post-puberty in foals. The provision of additional canter exercise (30% higher workload than controls at pasture) did not have a significant quantifiable effect on bone size and strength until the horses were 12 months old. It is likely that changes were occurring to the developmental potential and microarchitecture of bone but changes in gross morphology of the bone were not observable until later in age (Firth et al., 2011). It is possible that a similar effect occurred in the calves in the current study, where developmental programming may have been occurring between the dietary treatments. If the effect of diet on weight becomes significant at a later age as proposed by Moallem et al. (2010), then the effect of diet may have become quantifiable using pQCT at a later age.

3.6. Conclusions

The current study demonstrated that weight, and to a lesser degree girth, are the main explanatory factors of bone development and strength in calves prior to weaning. The strategy to increase resistance to bending strain and torsion (SSI) differed between the humerus and the metacarpus. The rapid growth of the humerus during this pre-weaning period and the increases in periosteal circumference and cortical thickness, provides an explanation of why the humerus may be more susceptible to growth checks. Changes in cortical thickness in the metacarpus are likely to be attributed by factors not measured in the current study. The strong association of bone development with bodyweight indicates that in a practical setting bodyweight and average daily gain provide a good proxy measure for bone development and attainment of bone

mass in pre weaning dairy heifers. If the relationship between bone strength and weight is disrupted bone may not be able to reach adequate strength to withstand forces. Therefore, it can be concluded that satisfactory pre-weaning growth rates are required to maintain the relationship between weight and bone strength for future growth.

Chapter 4

The Effect of Sex and Age on Bone Morphology and Strength in the Metacarpus and Humerus in Beef-Cross-Dairy Cattle

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Foreword to Chapter 4

The previous chapter (Chapter 3) demonstrated that live weight was the main predictor of bone parameters such as SSI in the metacarpus and humerus in dairy calves. With an established relationship between live weight and bone in calves, the next step was to observe the relationship between live weight and bone in older animals. Therefore, in the following chapter (Chapter 4) the relationship between live weight and pQCT derived bone parameters was examined in bulls, steers and heifers aged between 8 and 24 months. In addition, the effect of sex on bone morphology was examined.

4.1. Abstract

In cattle, limited data have been reported about the relationship between live weight, bone size, and strength and how this relationship can be altered by factors such as sex and age. The aim of this study was to describe the relationship of peripheral quantitative computed tomography (pQCT)-derived parameters of bone strength and morphology with live weight, age and sex in beef-cross-dairy cattle. All animals were weighed the day before slaughter. The metacarpus and humerus were collected at slaughter and scanned at the mid-diaphysis using pQCT. Live weight was the primary explanatory variable for bone size and strength in all cohorts. However, the effect of age was significant, such that magnitude of response to liveweight was less in the 24-month-old cohort. Sex was significant within cohorts in that bulls had a shorter metacarpus than steers and heifers had a shorter metacarpus than steers at age of slaughter.

4.2. Introduction

As live weight increases, bone must increase in size and strength to maintain strain within physiological limits (Rubin and Lanyon, 1985). The application of this theory has been supported by research looking in to the relationship between bone and strain where increases in live weight or loading result in a rise in bone strength (Firth et al., 2011, Gibson et al., 2020). The nature and magnitude of the bone response is dependent on the region within the bone and the stage of maturation of the animal. The mid-diaphyseal site of long bones must resist the bending and torsion arising

from locomotion, so increases in live weight and therefore loading forces must result in an increase in strength (Firth et al., 2011). The primary response in long bones to increase "bone strength" is increasing bone size via appositional growth (Akers and Denbow, 2013a). During the bone remodelling process, bone is removed from the inner endosteal side and deposited on the outer periosteum to provide a larger cross-sectional area with a minimal increase in cortical bone thickness (Sinclair et al., 2014). However, the ability for bone to increase strength via appositional growth is limited once the animal attains puberty and there is a reduction in longitudinal growth when the growth plate is closed at bone maturity (Harada and Rodan, 2003).

An important cue for the initiation of bone maturity is the increase in oestrogen at puberty in both females and males (Singh et al., 2010). In cattle, the common definition of puberty is the onset of regular oestrous cycles for heifers, and the production of viable semen for bulls, both indicative of the animal developing the maturity to reproduce (Hickson et al., 2011, Gupta et al., 2016). For cattle, puberty is attained when animals reach approximately 48–51% of their mature body weight (Handcock et al., 2021). At the onset of puberty, oestrogen is secreted from the ovaries in heifers and the Leydig cells in the testes of bulls in low concentrations. In the early stages of puberty, the increase in sex and growth hormones causes elongation of long bones, colloquially referred to as a "growth spurt" (Singh et al., 2010). Distal portions of the limbs such as the metacarpus have limited longitudinal growth potential compared to proximal bones such as the humerus (Bartosiewicz, 1984). In late puberty, higher

concentrations of oestrogen inhibit bone resorption by reducing the number of osteoclasts produced and limiting osteocyte apoptosis (Harada and Rodan, 2003). A decrease in bone growth promotes the closure of the physis and allows for endosteal and trabecular bone formation. A lack of oestrogen, i.e., castration will prevent puberty acquisition and delay bone maturity resulting in a longer bone (Gilsanz et al., 1988, Purchas et al., 2002).

Inadequate bone growth prior to puberty and bone maturity can result in a smaller bone size and limited bone strength. A potential cause for reduced bone size is a period of malnutrition, where bone growth ceases and the physis is temporarily sealed. When bone growth resumes, the seal on the physis is displaced and is observed histologically and on radiographs as a growth arrest line (Harris line) (Craig et al., 2016).

Spontaneous humeral fractures have been reported in first calving dairy cows in New Zealand (Dittmer et al., 2016). It is estimated that 4% of dairy farms are affected by humeral fractures a year with an on-farm prevalence of 2–25% of replacement heifers resulting in a significant welfare issue. Due to the location of the humerus, the ability to examine the bone is restricted to post-mortem, however, bones such as the metacarpus are easily accessible and have been shown to be a good predictor of bone size and strength in the humerus (Gibson et al., 2019). Humeri from heifers with humeral fractures often have a reduced periosteal circumference and bone volume to total bone volume ratio (BV/TV). Growth arrest lines were also present indicating a

period of malnutrition, suggesting this may predispose affected animals to fracture (Dittmer et al., 2016). This highlights the importance of understanding bone growth prior to puberty. Therefore, the aim of this study was to examine the effect of sex and age on the relationship between live weight and pQCT derived measures of bone mass and strength in the metacarpus and humerus of cattle.

4.3. Materials and Methods

4.3.1. Animals

The experiment included three cohorts of beef-cross-dairy breed calves reared at pasture on three commercial farms in the Manawatu region of New Zealand. The first cohort were Hereford-sired, male calves born to Friesian and Friesian–Jersey crossbred dairy cows. Cohort two consisted of Stabilizer-sired calves born to 2-year-old, Friesian–Jersey crossbred heifers , and cohort three consisted of Hereford-sired, steers born to Friesian and Friesian–Jersey crossbred dairy cows.

Each cohort included a control group (steers slaughtered between 8–12 months of age) and a comparison group. At slaughter, measures of bodyweight, stature and, bone mass and architecture, were subsequently compared between the young steers and the comparison group of young heifers, young bulls or older steers at farms 1–3, respectively. The calves were purchased and moved to their respective farm at approximately 100 kg live weight, after having been artificially reared using commercial calf-rearing systems that involved weaning off milk at around 70 kg, and feeding high protein meal to approximately 100 kg live weight (around 3 months of

age) (Muir et al., 2002). Cattle were grazed under commercial conditions and managed with the aim of achieving high growth rates (target 1 kg/d). The predominant forage on offer was perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture. To achieve target growth rates during periods of low pasture growth, forage crops (plantain and chicory) were used to supplement the base pasture diet. Within cohort, all animals were grazed together as a single mob.

4.3.1.1. Cohort One (Steers (n = 14) Versus Bulls (n = 19)

Cohort one consisted of Hereford-sired, male calves born to Friesian and Friesian–Jersey crossbred dairy cows. Fourteen randomly selected calves had been castrated before 4 weeks of age, whilst the remaining 19 were left entire. At 11 months of age the calves were processed at a commercial abattoir. All procedures related to the management and use of these animals during the experiment were approved by the Massey University Animals Ethics Committee (MUAEC 18/120).

4.3.1.2. Cohort Two (Steers (n = 12) Versus Heifers (n = 12))

The initial group consisted of 140 Stabilizer-sired calves born to 2-year-old, Friesian–Jersey crossbred heifers. At 11 months of age, the 30 heaviest heifers and 30 heaviest steers were identified, and 12 of each sex were randomly selected from these subsets for processing at a commercial abattoir. All procedures related to the management and use of the animals during the experiment were approved by the Massey University Animals Ethics Committee (MUAEC 18/120).

4.3.1.3. Cohort Three (Steers Aged 8–12 Months (n = 60) or 24 Months (n = 20))

A cohort of 80 Hereford-sired weaner steers from predominantly Friesian–Jersey crossbred dams were purchased from a single commercial calf rearer at approximately 100 kg (3 months of age). Calves were managed as a single group and randomly assigned at 8 months of age to one of four slaughter ages, being 8, 10, 12 or 24 months of age with each treatment group balanced for live weight at 8 months of age (Pike et al., 2019). Cattle were processed at a commercial abattoir. All procedures related to the management and use of these animals during the experiment were approved by the Massey University Animals Ethics Committee (MUAEC 17/73).

4.3.2. Sample Collection

Unfasted live weight was recorded on farm the day before slaughter. Cattle underwent commercial slaughter and standard dressing procedures. The left metacarpus was collected at time of slaughter and the humerus was collected the following day at the boning-out stage. Both the humerus and metacarpus were double wrapped in plastic film and stored at −20 °C until scanning.

4.3.3. Scanning

The pQCT scanning was carried out using the same protocol previously reported by Gibson et al. (Gibson et al., 2020). Briefly, pQCT scanning was carried out using an XCT 2000 peripheral quantitative computed tomography machine (Stratec Medical, Pforzheim, Germany). For each bone, a 2 mm slice at the mid diaphysis was obtained with a voxel size of 0.3 mm. In the metacarpus, the mid-diaphysis was defined as 50%

of the total bone length using the lateral aspect of the lateral condyle of the MC4 and the proximal aspect of the lateral MC4. In the humerus, the mid-diaphysis was defined as 50% of total bone length, measured from the proximal end of the humeral head at the lateral aspect, to the end of the trochlea at the distal end.

Data derived from the scan included measures of total bone content, cortical (>710 mg/mL CaHA) and subcortical content, cortical and subcortical density, trabecular content, trabecular density, total area, trabecular area, cortical and subcortical area, cortical content, cortical density, cortical area, cortical thickness, periosteal circumference, endosteal circumference and stress–strain index (SSI). The stress–strain index is a derived measure of bone strength using pQCT data and avoids the requirement for destructive testing using techniques such as the three-point bending test. The stress–strain index described the ability of bone to withstand bending from lateral, dorso-palmar and torsional forces and is calculated by incorporating the index of material stiffness (bone mineral density) and bone geometry (cross-sectional moment of inertia) (Ferretti, 1995). The pQCT variables will be referred to as bone parameters in the statistical models.

4.3.4. Statistical Analysis

Statistical analysis was conducted using the Statistical Analysis System software version 9.4 (SAS institute Inc., Carey, NC, USA). All analyses were conducted using general linear models. The models for live weight and bone length included the fixed effect group (control vs. comparison).

4.3.4.1. Sex Effect

Bone parameters were compared between groups for each cohort. Models included the fixed effect of group (control vs. comparison) and the covariate of live weight. The interaction of group and live weight was considered but removed if not significant ($p > 0.05$).

4.3.4.2. Age Effect

Live weight was compared between groups for cohort three. Models included the fixed effect of group (8–12 months vs. 24 months) and the covariate of bone parameter. The interaction of group and bone parameter was considered but removed if not significant ($p > 0.05$).

4.4. Results

4.4.1. Effect of Sex/Cohort One and Two

There was limited effect of sex on bone parameters between bulls and control steers from cohort one in both the metacarpus and humerus when liveweight was included in the model (Table 4.1). Sex had a significant effect on metacarpus length. Most bone parameters had a moderate association with live weight (R^2 = 0.28–0.65) with the exception of endosteal circumference in the metacarpus and endosteal circumference and cortical bone density in the humerus which had limited variation which was reflected in the lower R^2 values for model fit.

In the humerus, cortical bone thickness was greater ($p < 0.001$) in steers than bulls (8.4 vs. 8.3 mm, respectively). There was a weight by sex interaction for cortical bone

thickness (p = 0.045), such that cortical bone thickness increased with live weight at a rate of 0.01 mm/kg in steers and 0.02 mm/kg in bulls.

Table 4.1. Least squared means and standard error for bone parameters in the metacarpus and humerus with the effect of sex in bulls and steers at 11 months of age from cohort one where there was no interaction of weight and sex.

				p-Value		
	Bull	**Steer**	**Live Weight Coefficient**	**Live Weight**	**Sex**	**R^2**
Live weight (kg)	305.9 ± 7.1	303.0 ± 6.9			0.773	0.00
Metacarpus						
n	16	17				
Bone length (mm)	205.8 ± 1.4	211.3 ± 1.3	0.21 ± 0.03	<0.001	0.008	0.59
Periosteal Circumference (mm)	98.3 ± 0.7	97.9 ± 0.7	0.10 ± 0.02	<0.001	0.661	0.50
Endosteal circumference (mm)	61.2 ± 1.0	61.3 ± 0.9	0.05 ± 0.02	0.055	0.897	0.12
Total bone area (mm^2)	770.5 ± 11.2	763.0 ± 10.8	1.5 ± 0.3	<0.001	0.634	0.50
Total bone content (mg/mm)	607.6 ± 8.0	600.7 ± 7.8	1.5 ± 0.2	<0.001	0.573	0.64
Cortical bone thickness (mm)	5.9 ± 0.1	5.8 ± 0.1	0.008 ± 0.002	0.002	0.457	0.28
Cortical bone area (mm^2)	471.4 ± 6.9	462.4 ± 6.7	1.1 ± 0.2	<0.001	0.355	0.56
Cortical bone density (mg/cm^3)	1206 ± 3	1213 ± 3	0.3 ± 0.1	<0.001	0.083	0.43
Cortical bone content (mg/mm)	568.6 ± 8.2	560.9 ± 8.0	1.4 ± 0.2	<0.001	0.505	0.61
Stress–strain index (mm^3)	4205 ± 84	4111 ± 81	13.6 ± 2.1	<0.001	0.426	0.59
Humerus						
n	16	17				
Bone length (mm)	233.5 ± 1.7	235.1 ± 1.6	0.14 ± 0.04	0.002	0.497	0.28
Periosteal Circumference (mm)	132.8 ± 1.2	133.3 ± 1.2	0.18 ± 0.03	<0.001	0.784	0.52
Endosteal circumference (mm)	80.6 ± 1.4	80.4 ± 1.4	0.10 ± 0.03	0.012	0.930	0.19
Total bone area (mm^2)	1407 ± 26	1417 ± 25	3.7 ± 0.7	0.001	0.804	0.51
Total bone content (mg/mm)	1171 ± 18	1184 ± 17	3.3 ± 0.4	<0.001	0.604	0.65
Cortical bone area (mm^2)	888.5 ± 14.1	899.2 ± 13.6	2.5 ± 0.4	<0.001	0.589	0.62
Cortical bone density (mg/cm^3)	1247 ± 4	1243 ± 4	−0.1 ± 0.1	0.315	0.436	0.05
Cortical bone content (mg/mm)	1108 ± 16	1118 ± 16	3.0 ± 0.4	<0.001	0.672	0.64
Stress–strain index (mm^3)	11,352 ± 268	11,503 ± 2560	46.4 ± 6.8	<0.001	0.688	0.61

In cohort two heifers had a shorter bone length and lower stress–strain index in the metacarpus than steers at the same live weight (Table 4.2.). Similarly, heifers had lower total bone content, cortical bone area and stress–strain index in the humerus than steers of the same live weight. Live weight had a significant effect for most parameters in both bones with the exceptions being cortical bone thickness in the metacarpal and humerus, and endosteal circumference in the humerus. Most bone parameters had a moderate association with live weight (R^2 = 0.28–0.62). There was limited variation of cortical bone thickness in the metacarpus and bone length and endosteal circumference in the humerus, which was reflected in the lower R^2 values for model fit.

Table 4.2. Least square means and standard error of bone parameters adjusted for live weight of heifers and steers at 11 months of age from cohort two where there was no interaction of weight and sex.

				p-Value		
	Heifer	**Steer**	**Live Weight Coefficient**	**Weight**	**Sex**	**R^2**
Live weight (kg)	314.3 ± 5.2	323.4 ± 5.2			0.228	0.07
Metacarpus						
n	12	12				
Bone length (mm)	198.5 ± 1.9	204.3 ± 1.9	0.1 ± 0.1	0.140	0.046	0.30
Periosteal Circumference (mm)	91.6 ± 1.0	94.7 ± 1.0	0.16 ± 0.04	0.001	0.054	0.53
Endosteal circumference (mm)	54.2 ± 1.4	57.3 ± 1.4	0.1 ± 0.1	0.015	0.145	0.37
Total bone area (mm^2)	670.1 ± 15.6	714.9 ± 15.6	2.3 ± 0.6	0.001	0.059	0.52
Total bone content (mg/mm)	556.3 ± 9.2	582.9 ± 9.2	1.2 ± 0.4	0.005	0.058	0.46
Cortical bone thickness (mm)	6.0 ± 0.1	6.0 ± 0.1	0.001 ± 0.005	0.817	0.999	0.00
Cortical bone area (mm^2)	433.4 ± 7.5	451.6 ± 7.5	1.0 ± 0.3	0.003	0.105	0.47
Cortical bone density (mg/cm^3)	1210 ± 4	1213 ± 4	−0.5 ± 0.2	0.005	0.681	0.32
Cortical bone content (mg/mm)	524 ± 9	548 ± 9	1.0 ± 0.4	0.012	0.080	0.41
Stress–strain index (mm^3)	3529 ± 102	3854 ± 102	14.5 ± 4.1	0.002	0.038	0.52
Humerus						
n	12	12				
Bone length (mm)	233.2 ± 2.5	235.3 ± 2.6	0.1 ± 0.1	0.279	0.584	0.10
Periosteal Circumference (mm)	125.9 ± 1.4	128.8 ± 1.5	0.2 ± 0.1	0.004	0.178	0.46
Endosteal circumference (mm)	75.4 ± 1.9	75.2 ± 2.0	0.1 ± 0.1	0.195	0.950	0.09
Total bone area (mm^2)	1265 ± 28	1322 ± 30	3.8 ± 1.2	0.004	0.192	0.45
Total bone content (mg/mm)	1059 ± 19	1149 ± 20	2.8 ± 0.8	0.002	0.005	0.62
Cortical bone thickness (mm)	8.0 ± 0.2	8.5 ± 0.2	0.01 ± 0.01	0.073	0.072	0.34
Cortical bone area (mm^2)	808.0 ± 16.2	869.3 ± 16.9	2.5 ± 0.7	0.001	0.019	0.60
Cortical bone density (mg/cm^3)	1245 ± 5	1253 ± 6	−0.6 ± 0.2	0.013	0.300	0.27
Cortical bone content (mg/mm)	1004 ± 18	1089 ± 19	2.7 ± 0.7	0.002	0.005	0.61
Stress–strain index (mm^3)	9447 ± 270	10,564 ± 283	39.6 ± 11.0	0.002	0.011	0.59

4.4.2. Effect of Age/Cohort Three

There was an effect of age on the relationship of live weight with all pQCT measured bone parameters. Live weight was greater at the same bone parameter value for the older animals. There was no relationship of live weight with cortical density in the metacarpus and humerus and also endosteal circumference in the humerus (Table 4.3.). All bone parameters had a strong association with live weight ($R^2 = 0.90–0.97$) in both the metacarpus and humerus.

In the metacarpus, there was a significant interaction of bone parameter and group in total bone area, total bone content, cortical bone thickness, cortical bone area, cortical bone content and the stress–strain index. Therefore, younger animals had a larger gain in live weight per unit of bone parameter (Table 4.4.).

Table 4.3. Live weight adjusted for each bone parameter in the metacarpus and humerus of control steers (8–12 months) and 24-month-old steers from cohort three for which there was no interaction of bone parameter and group. Values are the least squared mean (± standard error) live weight for each age group adjusted to the overall mean value of the bone parameter identified in that row.

				p-Value		
	8–12 Months Live Weight	**24 Months Live Weight**	**Bone Parameter Coefficient**	**Bone Parameter**	**Group**	**R^2**
Metacarpus						
Live weight (kg)	301.1 ± 5.7	580.1 ± 9.2			<0.001	0.90
n	57	22				
Bone length (mm)	305.5 ± 5.8	568.2 ± 10.0	1.0 ± 0.4	0.016	<0.001	0.90
Periosteal Circumference (mm)	328.4 ± 6.4	509.5 ± 13.5	5.1 ± 0.8	<0.001	<0.001	0.93
Endosteal circumference (mm)	304.4 ± 5.8	571.6 ± 9.8	2.0 ± 0.9	0.034	<0.001	0.90
Cortical bone density (mg/cm^3)	298.4 ± 6.2	587.1 ± 11.1	−0.4 ± 0.4	0.273	<0.001	0.90
Humerus						
n	17	18				
Live weight (kg)	345.8 ± 6.4	568.8 ± 6.2			<0.001	0.95
Bone length (mm)	372.7 ± 7.4	543.3 ± 7.1	2.1 ± 0.4	<0.001	<0.001	0.97
Periosteal Circumference (mm)	374.9 ± 9.4	541.3 ± 9.1	2.2 ± 0.6	<0.001	<0.001	0.97
Endosteal circumference (mm)	356.7 ± 8.3	558.4 ± 8.0	1.1 ± 0.6	0.059	<0.001	0.95
Total bone area (mm^2)	374.9 ± 9.1	541.3 ± 8.7	0.09 ± 0.02	<0.001	<0.001	0.97
Total bone content (mg/mm)	379.7 ± 9.5	536.7 ± 9.1	0.15 ± 0.03	<0.001	<0.001	0.97
Cortical bone thickness (mm)	351.7 ± 7.2	563.2 ± 7.0	11.5 ± 7.0	<0.110	<0.001	0.95
Cortical bone area (mm^2)	377.4 ± 9.1	538.9 ± 8.7	0.19 ± 0.04	<0.001	<0.001	0.97
Cortical bone density (mg/cm^3)	344.3 ± 6.6	570.2 ± 6.4	−0.3 ± 0.3	0.377	<0.001	0.95
Cortical bone content (mg/mm)	377.8 ± 9.5	538.6 ± 9.1	0.14 ± 0.04	<0.001	<0.001	0.97
Stress–strain index (mm^3)	371.1 ± 8.7	544.9 ± 8.3	0.007 ± 0.002	<0.001	<0.001	0.96

Table 4.4. Least squared means and standard error for live weight adjusted for each bone parameter in the metacarpus of control steers (8–12 months) and 24-month-old steers from cohort three. Regression coefficient for each group is presented with the interaction of bone parameter and group being significant

			Coefficient		*p*-Values			
	8–12 Months Live Weight	**24 Months Live Weight**	**8–12 Months**	**24 Months**	**Bone Parameter**	**Group**	**Bone Parameter x Group Interaction**	**R^2**
Total bone area (mm^2)	334.9 ± 7.1	544.3 ± 21.9	0.4	0.2	<0.001	<0.001	0.042	0.93
Total bone content (mg/mm)	336.7 ± 0.4	515.1 ± 24.8	0.7	0.3	<0.001	<0.001	<0.001	0.96
Cortical thickness (mm)	341.5 ± 7.5	541.2 ± 29.7	67.9	25.3	<0.001	0.004	0.048	0.94
Cortical bone area (mm^2)	365.0 ± 7.0	516.6 ± 24.4	0.9	0.3	<0.001	<0.001	<0.001	0.96
Cortical content (mg/mm)	368.3 ± 7.5	513.1 ± 25.3	0.7	0.3	<0.001	<0.001	<0.001	0.96
Stress–strain index (mm^3)	351.2 ± 7.9	542.0 ± 22.1	0.06	0.02	<0.001	<0.001	<0.001	0.94

4.5. Discussion

The results in the present study agree with an earlier observation from a younger cohort of cattle that identified live weight as the major explanatory variable for measures of bone mass and strength in the metacarpus and humerus (Gibson et al., 2020). In addition, in the current study, it was demonstrated that the relationship of live weight with measures of bone mass and strength was modified by age and sex.

Bone minimises strain such as that generated by increased bodyweight by changing content and geometry (size) (Frost, 1987, Rubin and Lanyon, 1985). However, as the animal reaches mature height and longitudinal bone growth ceases the expectation is there will be a decrease in the magnitude of the bone response to increasing live weight. This was observed in the age interaction in the metacarpus with the 24-month-old cohort demonstrating a two to three-fold reduction in the bone response for each

kg increase in liveweight. Cattle are not cursorial animals and thus most locomotion is at low velocities (strain rate) (Logan et al., 2019). Within commercial farming systems even management on hill properties did not appear to provide sufficient strain to induce an increase in bone strength (Bijen et al., 2020). In contrast, bone parameters in the humerus did not have an age interaction, which implies that the humerus was still capable of growth at 24 months in these cattle. This finding agrees with Berg et al. (1978) where the pattern of bone maturity described by where bones in the distal limb in the cow, such as the metacarpus, mature earlier than bones in proximal limb, such as the humerus. Differences in bone response to increases in liveweight is also a result of differences in the anatomical location of the bone and therefore differences in the direction and magnitude of the mechanical forces. The larger stress–strain index in the humerus demonstrates that at the mid-diaphysis, the humerus is subjected to a much larger force than in the metacarpus. The metacarpus undergoes compressional forces from stance resulting in a low strain rate (Bijen et al., 2020). Whereas in the humerus, torsional forces are placed on bone via surrounding muscles during locomotion (Bouza-Rodríguez and Miramontes-Sequeiros, 2014).

Steers had a longer metacarpus than bulls and heifers but had similar humeral lengths. Therefore, bone maturation (closure of the physis) in the distal limb of heifers and bulls had begun, but not in the steers. A likely cause for this difference in bone maturity is that the hormonal cue for the cessation of longitudinal bone growth, via puberty acquisition, was not occurring for steers. During puberty, increases in

circulatory concentration of both oestrogen (in heifers and bulls) and testosterone (in bulls) are associated with physeal closure (Harada and Rodan, 2003). However, each sex hormone has a differing subsequent effect on bone mass as the animal ages. At the onset of puberty oestrogen inhibits further appositional growth thus effectively restricting the periosteal circumference to that achieved at the time of puberty (Singh et al., 2010). Conversely, testosterone has a limited effect on bone resorption and does not inhibit increases in periosteal circumference (Falahati-Nini et al., 2000, Kirmani et al., 2011). The effect of testosterone results in a larger bone size in males and a later bone maturity than in the female (Bartosiewicz, 1984).

The presence of high concentrations of testosterone in bulls promotes increases in muscularity compared to heifers with low concentrations of testosterone (Choi et al., 2002). Increased muscle mass is associated with increased bending strain on the long bones and therefore increases in bone mass (Frost, 1987). Muscle and bone form the bone-muscle unit to provide a signalling pathway between each other. Skeletal muscle secretes growth factors such as insulin like growth factor (IGF-I) which stimulates bone growth (Tagliaferri et al., 2015). Therefore, increases in muscularity, due to increased circulatory concentrations of testosterone in bulls, should promote increases in the cross-sectional size of bone by appositional growth to prepare for a greater bending strain. The lack of testes in steers prevents testosterone from being produced but, the genetics for a large mature size will create a larger frame size than a heifer without the muscularity of a bull to promote the larger cross-sectional area in long

bones (Berg et al., 1979, Choi et al., 2002). The greater periosteal circumference and endosteal circumference contributed to the greater total bone content observed in the steers than the heifers and reflects the effect of oestrogen inhibiting appositional growth in heifers. The lack of difference in periosteal circumference, endosteal circumference and total bone content between bulls and steers indicates that appositional bone growth in both the metacarpus and humerus was still occurring at 11 months of age possibly due to a lack of a pubertal spike in circulatory testosterone and oestrogen. Therefore, to quantify differences between steers and bulls, due to the presence and absence of testosterone, the animals would need to be older and post-pubertal.

With an estimated mature weight of 770 kg, bulls in the current study would have been approximately 39.7% of mature weight, therefore, it is likely that bulls of this age were pre-pubertal (CSIRO, 2007). Therefore, longitudinal growth of the long bones, particularly the humerus, would continue after the age and weight at which the steers and bulls were slaughtered. As bulls reached puberty it would be expected that the increase in oestrogen will slow bone growth and mineralisation will increase. The effect of this would then be the divergence of the bone parameters, and the primary mechanisms used to increase resistance to bending strain, between the bulls and steers. This post-pubertal divergence in bone parameters between steers and bulls has been reported for the femur in cattle (Purchas et al., 2002). Post puberty, bulls had shorter femurs than steers of the same live weight. However, the weight of the femur

remained the same, possibly due to greater total bone density in the femur of the bulls. However, using live weight as a predictor for puberty does not precisely identify when puberty attainment occurred. The use of hormone assay would have been an effective way to identify puberty status but was unable to be undertaken due to the opportunistic nature of the bone collection from another study.

Increases in SSI in both the metacarpus and humerus were achieved by increasing the cross-sectional area of the mid-diaphysis, which reflects a common mammalian response to growth and loading in the long bones of the limb (Firth et al., 2011). In first lactation dairy heifers, heifers may present with humeral fractures, which are associated with reduced periosteal circumference and reduced BV/TV compared with control heifers (Dittmer et al., 2016). The data from the present study indicate that at 24 months the humerus was still experiencing appositional and longitudinal growth and is sensitive to changes in live weight. Therefore, in cattle, appositional growth of the humerus may be sensitive to periods of reduced nutrition between puberty and first lactation. Industry growth data provide evidence that many dairy heifers may encounter a "growth check"(reduced live weight gain) at this time (Handcock et al., 2019b). The resultant reduction in appositional growth of the humerus (and hence reduced bone mass and resistance to bending), due to the "growth check", may predispose dairy heifers to humeral fractures due to an inability to cope with mobilisation of bone tissue from the endosteal surface to supply calcium during the first lactation.

4.6. Conclusions

Live weight was the major explanatory variable of bone size and strength, with age and sex modifying the relationship. Differences in bone maturity meant that the rate of response in the metacarpus at 24 months to increases in live weight were less than that observed in 8–12 months. However, there was no interaction of age and bone response in the humerus, indicating it was still capable of growth.

The differences in growth capacity of the metacarpus and humerus was reflected in the sex-associated differences between groups, with the presence of oestrogen inhibiting longitudinal growth in the metacarpus and lack of sex hormones in the steers delaying inhibition of longitudinal growth. Heifers were characterised by shorter and smaller long bones in the metacarpus. However, the effect of puberty on the humerus had not yet occurred with minimal signs of bone maturation, indicating, that proximal bone growth was still occurring at 11 months.

Increased SSI with live weight and age occurred by increasing the cross-sectional area in the mid diaphysis rather than increasing bone density. At 24 months the humerus was still highly responsive to changes in live weight and thus any "growth check" would inhibit the ability to provide adequate cross-sectional area. Failure to attain optimal cross-sectional area at the time of peak lactation and associated increase in bone mobilisation could predispose heifers to humerus fracture.

Chapter 5

Live weight and bone growth from birth to 15 months of age in pure-bred and cross-bred Jersey and Friesian heifers.

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Gibson, M., Rogers, C., Hickson, R., Dittmer, K., & Back, P. 2021. Live weight and bone growth from birth to 15 months of age in pure-bred and cross-bred Jersey and Friesian heifers. New Zealand Journal of Animal Science and Production.

Foreword Chapter 5

The previous chapter (Chapter 4) demonstrated that the relationship between live weight and pQCT derived bone parameters continues after weaning. However, the relationship between live weight and bone can be altered by the effect of sex and castration. Bone maturity in the metacarpus was also confirmed to occur at approximately 12 months of age whereas the humerus continues to develop. The next step in this research was to examine changes in stature to relate with what has been observed so far in bone. Therefore, in the following chapter (Chapter 5), the pattern of stature growth in relation to live weight in dairy heifers from birth to 15 months of age was examined. In addition, the effect of breed on stature parameters was also examined.

5.1. Abstract

Live weight and live weight gain of dairy heifers has been described in New Zealand and overseas, however, the change in stature and pattern of proximal and distal bone growth has, to date, received little attention. This study describes the proportional changes in stature with live weight, in a cohort of commercial dairy heifers (Holstein Friesian, Jersey, and Holstein-Friesian Jersey) from birth to 15 months of age. Measurements of height, length, girth, and distal and proximal forelimb length (stature) revealed that at the same live weight, breed had a significant effect ($P<0.05$), however, the magnitude of the differences among breeds was consistently less than 1 cm, or 5%. As heifer live weight increased, the contribution of the distal limb to increases in height decreased, which is consistent with knowledge that physeal closure in cattle occurs earlier in distal limb bones, such as the metacarpus than in proximal limb bones, such as the humerus. In addition, the understanding of proximal and distal growth in cattle identifies when heifers are most susceptible to growth checks.

5.2. Introduction

In dairy heifers, the improvement in genetics for milk production has caused an increase in the demands of calcium from bone stores. If insufficient peak bone mass is attained prior to first lactation, a cohort of heifers may present with spontaneous humeral fractures due to osteoporosis (presenting with reduced circumference of the bone and reduced bone wall thickness) (Dittmer et al., 2016). It has been estimated that

affected farmers in New Zealand could be losing 2% to 3% of heifer replacements due to humeral fractures (J. Hunnam, unpublished data).

New Zealand dairy cows are predominantly Holstein-Friesian (33.1%), Holstein-Friesian- Jersey crossbred (48.5%), and Jersey (8.6%) (LIC and DairyNZ, 2019)Holstein-Friesians (HF) are known to have a larger mature weight than purebred Jersey and Jersey-Friesian crossbreds, resulting in a faster growth rate. Jersey calves are born smaller than Friesian calves and remain smaller at maturity (Handcock et al., 2019b). Animals with heavier mature weight tend to mature later than those with smaller mature weight, however, a larger size and faster growth rate requires higher dietary energy intake making them more susceptible to disruptions in growth from feed shortages (Rattray et al., 2007).

Growth in New Zealand dairy heifers is typically not linear due to seasonal variation and a heavy reliance on pasture quality. Times of poor pasture quality or quantity such as mid-summer with low rainfall and mid-winter with low temperatures results in slowed growth rates compared to spring (Handcock et al., 2019b). To understand how the heifer grows at a whole-body level research has been undertaken to look at changes in stature up until 21 months of age (Handcock et al., 2021). This describes the idea of the relationship between height, length, and girth but does not show the proportion of height change that comes from leg (metacarpus and radius) vs thoracic growth (humerus and scapula). It is known that mature height is achieved before

thoracic growth is completed however, the timing of this growth pattern is not fully described (Guilbert and Gregory, 1952). To gain a better understanding of key risk periods for humeral growth, the timing of proportional changes in stature and therefore bone growth needs to be investigated. Therefore, the aim of this study was to describe proportional changes in stature with weight in Holstein Friesian, Jersey, and Holstein-Friesian Jersey crossbred calves from birth to 15 months of age when puberty is likely to have been achieved.

5.3. Materials and methods

This experiment was conducted with the approval of Massey University Animals Ethics Committee (MUAEC 19/81).

5.3.1. Animals and management

The cohort consisted of 57 Friesian, Jersey, and Friesian-Jersey crossbred singleton heifer calves born at Massey University's Dairy 1 farm during the 2019 spring calving season (As described in Table 1). Dam parity was recorded for each calf as heifer or cow. Calves were housed for the first 3 weeks of life and at pasture thereafter. Calves were reared under commercial management, that involved ad libitum colostrum until three days of age, then 7 L/day of milk split over two feeds for approximately 6 weeks, then calves were reduced to 5L of milk fed once daily. Ad libitum access to a proprietary calf meal was provided until 5 months of age (SealsWinslow 20% muesli followed by SealsWinslow 18% pellets; SealesWinslow, Whanganui). Calves were

weaned at approximately 100 kg live weight, which occurred at 71-95 days of age. Once they exited the calf shed, calves were managed in a single group, except around weaning when calves fed milk were grazed separately to weaned calves, and for when the biggest 52 calves were grazed separately to smaller calves. At 185-109 days of age calves were moved to a grazier in Kimbolton, Manawatu and were grazed under commercial management, which involved drafting into up to three grazing herds, based on live weight to achieve target industry growth rates.

Stature measures were compared among breed groups where pedigree information was used to classify heifers in to one of 5 breed groups (Table 5.1.) (Handcock et al., 2019a).

Table 5.1. Breed group and composition (Friesian (F), Friesian-cross (FX), Friesian-cross-Jersey (FJ), Jersey-cross (JX) and Jersey (J)) for heifers with number of heifers per breed group.

Breed group	Breed composition	N
F	F ≥ 14/16	14
FX	10/16 ≤ F ≤ 13/16	9
FJ	F < 10/16 and J < 10/16	6
JX	10/16 ≤ J ≤ 13/16	12
J	J ≥ 14/16	16
Total		57

5.3.2. Sampling and measurements

Calves were measured at birth and then fortnightly until weaning. After weaning, calves were measured monthly until 15 months of age, except for at 7-8 months of age, when a nationwide COVID-19 lockdown resulted in two missed measurements.

Measurements included live weight, wither height, girth, wither-rump length (wither to tuber ischii), leg length (from ground to point of olecranon), toe length (from ground to the lateral aspect of the metacarpophalangeal joint), knee height (from ground to the lateral aspect of the antebrachiocarpal joint) and the cannon circumference (circumference of the metacarpus bone mid-length). All leg measurements were made on the left forelimb. MC3 length was equal to knee height minus toe height, thoracic length was equal to wither height minus leg length and radius height was equal to leg length minus knee height.

5.3.3. Statistical analysis

Statistical analysis was conducted using SAS version 9.4 (SAS Institute Inc., Cary NC, USA).

A third-order Legendre polynomial was fitted to age using random regression to obtain an average curve for live weight for the population and for each heifer. For this, live weight at 0, 30, 61, 91, 122, 152, 183, 213, 243, 274, 304, 335, 365, 395, 426, 456 days of age was estimated for each heifer.

A third-order Legendre polynomial was fitted to stature data using random regression on live weight to obtain an average curve for each stature parameter for the population and for each cow. From this, stature parameters at 50, 100, 150, 200, 250, 300 and 350 kg live weight were estimated for each heifer.

Stature parameters were analysed using general linear models. Each stature measurement was analysed separately at each live weight (50-350 kg, in 50 kg increments). The effect of dam parity (primiparous or multiparous) was tested but was not significant so was removed.

5.4. Results

The polynomial for live weight based on age was a good predictor of actual live weight (R2=0.996), and live weight increased with age (Figure 5.1.).

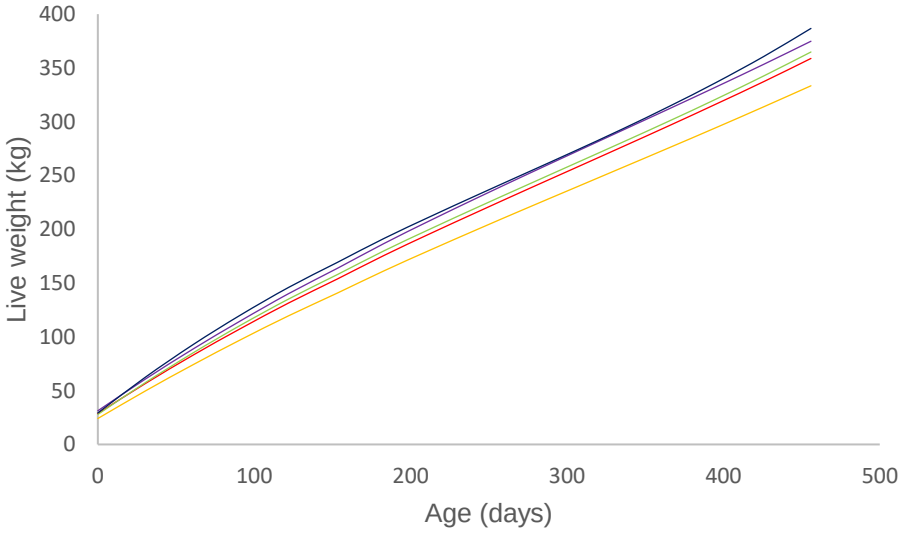

Figure 5.1. Live weight (kg) vs age (days) in Friesian (F) heifers (black), Friesian-cross (FX) heifers (purple), Friesian-cross-Jersey (FJ) heifers (Green), Jersey-cross (JX) heifers (red) and Jersey (J) (Yellow) heifers from birth to 15 months of age as defined in table 5.1.

Breed had a significant effect on many of the stature measures, however, the magnitude of the differences among breeds was consistently less than 1 cm, or 5% (Figure 5.2.). The greatest rate of increase in all stature measures occurred between 50 kg and 100 kg live weight.

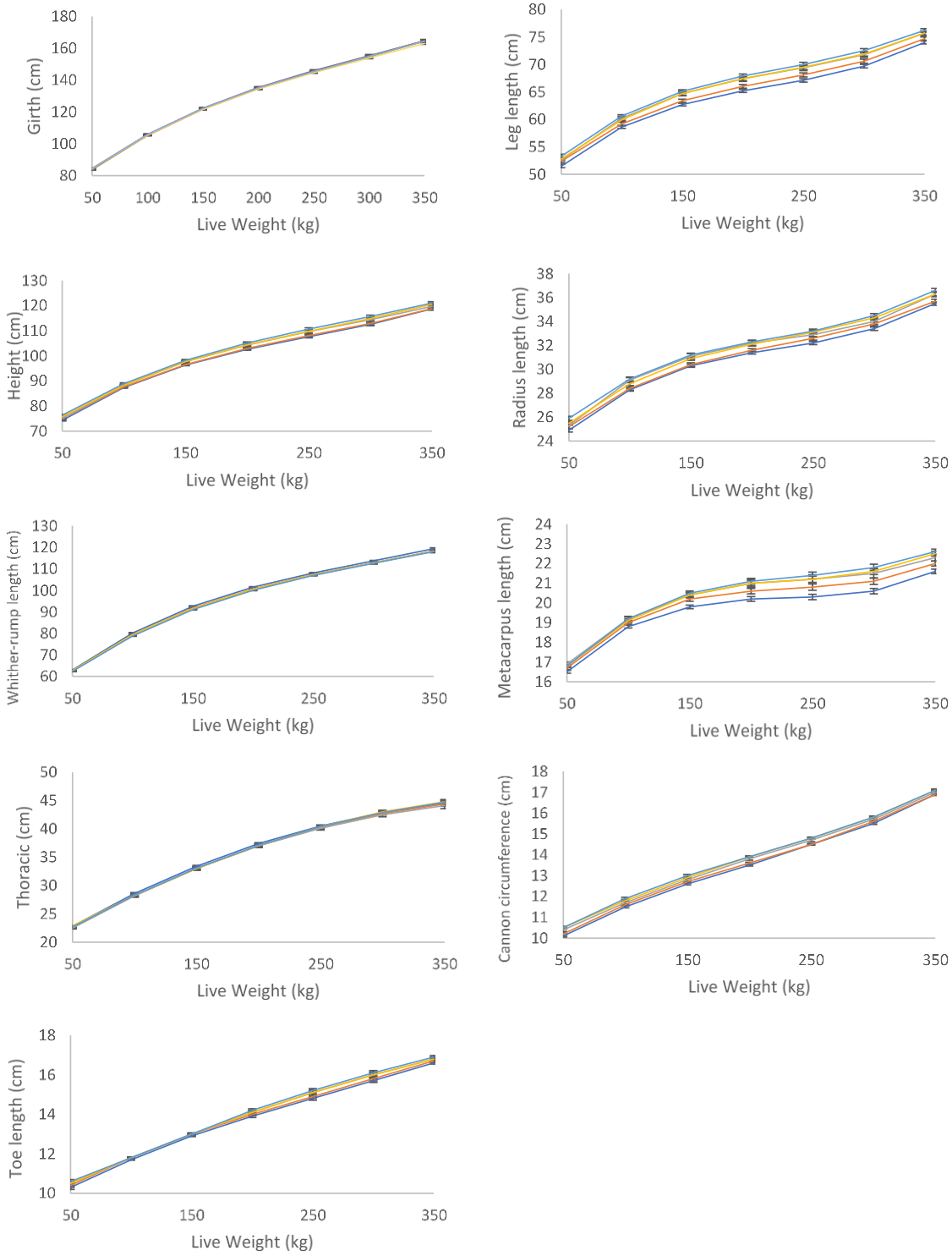

Figure 5.2. Stature (height, girth, thoracic length, and wither rump length) and leg (leg length, radius height, MC3 length, toe length and cannon circumference) measures in Friesian (F) heifers (black), Friesian-cross (FX) heifers (purple), Friesian-cross-Jersey (FJ) heifers (Green), Jersey-cross (JX) heifers (red) and Jersey (J) (Yellow) heifers from birth to 15 months of age as defined in table 5.1. with standard error bars.

As live weight increased, the proportion of Radius, MC3 and Toe length in the total height decreased whilst the proportion of thoracic length increased (Fig.3). At 50 kg, radius height made up the largest proportion of height, however, at 100 kg the proportion of thoracic length to total height had increased to be equal to that of radius, and thoracic height made up the largest proportion of overall height from 150 kg onwards. There was very little variation among animals as illustrated by the standard errors. For example, at 50 kg the toe length ranged from 13.3-14.3% of height and at 350 kg the MC3 length ranged from 17.9-19.4% of height.

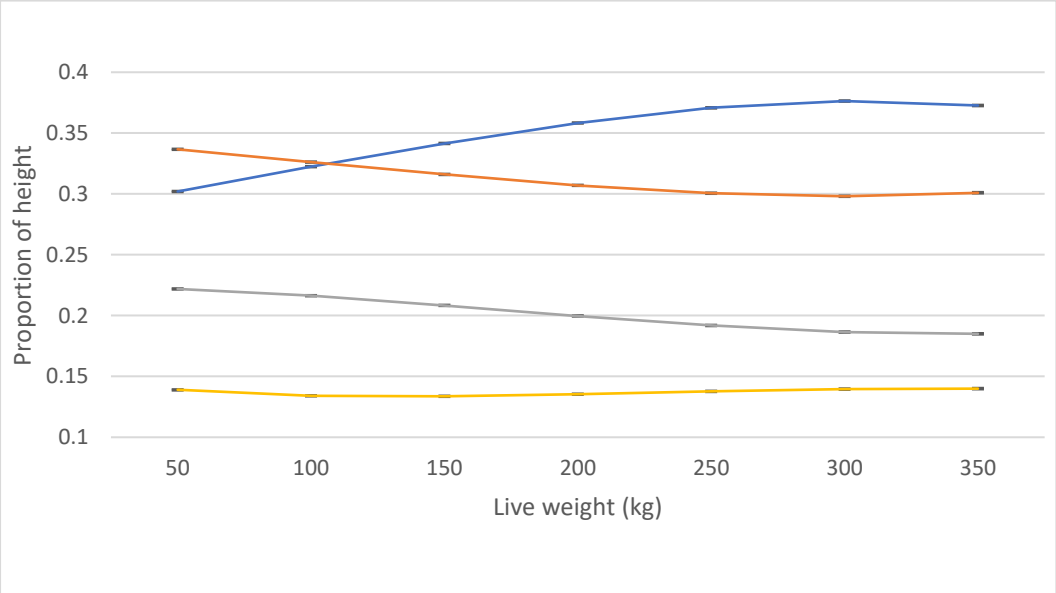

Figure 5.3. Proportion of Thoracic length (Blue), Radius height (Orange), MC3 length (Grey) and Toe length (Yellow) in relation to height of dairy calves from birth to 15 months of age with standard error bars.

5.5. Discussion

Feed supply on New Zealand farms is seasonal, where pasture availability changes dependent on the time of year (Rattray et al., 2007). Dairy industry data has indicated that during winter (June-August) a growth check in live weight often occurs, due to lower pasture quality and availability (Handcock et al., 2016). In the current study, live weight gain in heifers was linear, and live weight at 15 months was 333-387 kg for the different breed groups. This corresponds to approximately 78% of mature live weight, well above the industry target of 60% of mature live weight at 15 months. Therefore, the heifers in this experiment were well grown by New Zealand standards.

A linear growth pattern is likely to promote continuous appositional and longitudinal bone growth and mineralisation to achieve peak bone mass. Increases in animal size cause a greater strain on bone, requiring an increase in bone strength to maintain strain within physiological limits (Rubin and Lanyon, 1985). Given the relationship of bone strength with live weight, live weight, rather than age, was used as the predictor for stature measurements (Gibson et al., 2019, Gibson et al., 2021). This allowed comparison of how the body changed as the animal grew.

In the current experiment, increases in leg measures and height slowed at approximately 200 kg, whilst increases in capacity (i.e. girth) remained constant. Early in a heifer's life, increases in live weight are mainly comprised of growth in long bones (linear skeletal growth) causing increases in height (Guilbert & Gregory 1952). However, at puberty longitudinal bone growth ceases due to increasing oestrogen

secretion promoting physeal closure (Harada and Rodan, 2003). At 7 months of age, 80% of mature height is achieved but only 35-45% of mature weight has been attained. Longitudinal growth after 7 months of age slows as 90% of mature height is achieved by one year of age so contributions to live weight gain are the result of increases of capacity (Hammack and Gill, 2009).

In the current experiment, proportional growth in relation to height in the distal parts of the leg such as the metacarpus and toe length decreased, likely a result of cessation of growth in the distal limb. At birth, metacarpal length is approximately 90% of mature length, so longitudinal growth of the bone is limited (Guilbert and Gregory, 1952). Proximal limbs such as the radius and humerus continue to grow at 15 months of age especially at the proximal growth plate in the humerus and distal plate in the radius (Bartosiewicz, 1984). The outlined growth pattern agrees with published descriptions of the pattern of growth in cattle where bones in the distal limb mature earlier than bones in the proximal limb (Bartosiewicz 1984; Berg et al. 1978). The difference in bone maturation also reflects differences in the size and direction of the forces placed on areas of the limb. There is a strong relationship between animal size and bone, where bone adapts to increases in animal size and loading by increasing strength (Gibson et al. 2020; Rubin & Lanyon 1985). Distal sections of the limb such as the metacarpus undergo compression forces from stance requiring a smaller bone strength compared to the humerus (Berg et al. 1978). In the humerus, torsional forces

from the surrounding muscles act on bone resulting in a much stronger bone than the metacarpus (Bouza-Rodríguez & Miramontes-Sequeiros 2014).

Jersey calves were consistently lighter than Friesian calves of the same age. At the same live weight, there were minimal effects of breed on stature measures demonstrating an optimal stature shape. However, a lighter mature weight in Jersey calves results in an earlier age at puberty (Hickson et al. 2011). The cessation of longitudinal growth with puberty occurs at a lesser live weight in Jerseys and results in divergence of metacarpus length among breeds, as breed groups with lower mature live weight are likely to have reached puberty at a lighter live weight. The beginning of this divergence in stature measures is observed in the current study where measures in the radius, MC3 length and height diverge between Friesian and Jersey heifers at approximately 150 kg. Divergence of breed fits with previous observations by Bijen et al. (2020) where breed affected the relationship between bone size and live weight in older animals.

With proximal limb development still occurring at 15 of months age, bones within the proximal limb such as the humerus will be susceptible to changes in growth trajectory. Proximal limb development in the current study is consistent with previous research in steers where growth in the humerus was still occurring at 24 months of age (Gibson et al. 2021). The effect of the first winter on live weight gain reported by Handcock et al. (2016), shows how times of poor quality and/or lack of pasture can change the

trajectory of longitudinal bone growth in the form of an altered wither height. Therefore, if the humerus is continuing to grow during the second winter, it is likely that if adequate pasture quality and quantity is not supplied then growth of the humerus may be affected. Heifers with humeral fractures often have growth arrest lines indicating a period of poor nutrition prior to the fracture occurring which possibly coincides with their second winter.

5.6. Conclusions

Contributions of live weight gain from height and length slowed at approximately 200-250 kg whilst capacity measures such as girth continued to increase, reflecting the known growth pattern of cattle where 90% of height is achieved in the first year. Therefore, growth in the metacarpus is restricted after 12 months whereas growth in the humerus continues. This raises the possibility that growth restriction after 15 months of age could influence the development of the humerus without affecting the metacarpus.

Minimal effects of breed on stature at the same live weight is likely the result of there being an ideal body composition at a given live weight. However, the lighter mature size and lighter weight at puberty with greater Jersey genetics is likely to result in the divergence of stature measures after this point. Increases in height will slow earlier in more-Jersey breed groups than for breed groups with more Friesian genetics due to differences in mature size and age.

Chapter 6

The mid-diaphysis is a poor predictor of humeral fracture risk indicating that predisposing factors are recent.

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Foreword Chapter 6

The previous chapters (Chapters 2-5) outlined what normal cattle bone growth looks like and the relationship between bone and stature. Age was an important contributor to bone maturity with bones in the distal limb such as the metacarpus maturing at an earlier age than proximal bones such as the humerus. With an understanding of what occurs during normal cattle bone growth, the next step was to compare bone morphology of heifers affected and unaffected by spontaneous humeral fractures. In addition, the effect of copper was examined to see if it was a predisposing factor in humeral fracture.

6.1. Abstract

The incidence of spontaneous humeral fractures in first lactation dairy heifers in New Zealand has emphasised the need to understand thoracic limb bone growth of dairy heifers. Previous research has indicated that a predisposing factor to spontaneous humeral fracture is nutrition. In addition, it has been hypothesised that liver copper concentration affects bone strength and may be a potential factor associated with humeral fracture risk. The aim of this study was to compare bone morphology in the mid-diaphysis of the metacarpus and humerus of heifers affected and unaffected by spontaneous humeral fractures and determine the effect of copper status at death on bone morphology. The metacarpus and humerus was collected from heifers affected and unaffected by humeral fractures and scanned using peripheral quantitative computed tomography (pQCT). The mid diaphysis of the humerus of the affected group had reduced cortical bone mineral density and a trend for reduced cortical content and total bone content which contributed to a reduced stress-strain index. The trend for reduced bone length in affected humeri provides additional support for the hypothesis of inhibited humeral growth. Heifers with low copper liver concentrations had reduced humerus length and reduced cortical bone mineral density. These data support the hypothesis that the developmental window for humeral fracture is recent and possibly associated with periods of inadequate nutrition.

6.2. Introduction

Spontaneous humeral fracture in first-lactation dairy heifers is an established condition affecting at least 4% of all dairy farms in New Zealand. It is predicted that

approximately 5,000 heifers per year are lost to this syndrome, resulting in significant economic loss to the New Zealand dairy industry (J. Hunnam, unpublished data).

Humeral fractures in cattle tend to be spiral fractures that originate from the proximal end of the diaphysis (Rakestraw, 1996). In cattle with normal cortical bone thickness, humeral fractures are rare (5% of all fractures in cattle and 18% of all long bone fractures) due to the protection provided by the biceps brachii, brachiocephalicus, and brachialis muscles that surround the humerus caudally, laterally and cranially (Rakestraw, 1996). Post-mortem examination of spontaneous humeral fractures have shown an association with lower cortical bone thickness in affected bones contributing to a decrease in bone strength (measured by stress strain index) (Dittmer et al., 2016). Growth arrest lines have also been observed in the humeri from heifers with spontaneous humeral fractures indicating a period of malnutrition at some point in the heifer's life (Dittmer et al., 2016). The combination of weaker bones and sudden draw of calcium due to the onset of lactation is likely to contribute to an increased risk of spontaneous fractures in the humerus. Additionally, copper deficiency, which is common in New Zealand cattle, is known to affect bone strength and can cause osteoporosis in severely deficient animals (Dittmer et al., 2016).

Identification of heifers at risk of humeral fracture is difficult due to the location of the humerus. The ability to examine the bone is restricted to post-mortem examination. Therefore, a herd can only be identified as being at risk of having a higher number of

animals with humeral fractures when multiple heifers have been affected, at which time there is limited opportunity to increase bone strength. However, the mid-diaphysis of the metacarpus is easily accessible, and has been shown to have bone parameters that are highly correlated with those of the humerus (Gibson et al., 2019). Peripheral quantitative computed tomography (pQCT) allows for quantification of bone morphology in both the metacarpus and humerus. However, due to the large size of the humeral head and small size of the gantry hole in the scanner, scanning is restricted to the mid-diaphysis. Currently there is minimal CT data on bone morphology in the humerus in cattle, however the incidence of spontaneous humeral fractures in first lactation dairy heifers has prompted the need to examine the humerus. Therefore, the aim of the study was to compare bone morphology in the mid-diaphysis of the metacarpus and humerus of heifers affected and unaffected by spontaneous humeral fractures and determine the effect of copper status at death on bone morphology.

6.3. Materials and Methods

6.3.1. Sample collection

6.3.1.1. Control group.

A collection of 29 metacarpals and 30 humeri (29 sets) were obtained from 2-year-old dairy cattle that died from non-bone related issues. Bones were collected from a casualty cow collection service (Wallace Corporation, Feilding) that was visited for

sample collection twice a week during October 2020. Additional bones were collected from Massey University's Post-mortem facility. Any cows with an ear tag indicating they were born in 2018 and an udder consistent with lactating at the time of death were selected. When possible (n= 16), a liver sample was collected and analysed for copper concentration at New Zealand Veterinary Pathology (IDEXX) Ltd (Palmerston North). Copper was categorised as normal (>95 μmol/kg), low (30-95 μmol/kg) and very low (<30 μmol/kg). A liver copper concentration of below 30 μmol/kg has been associated with impaired animal growth in cattle (Grace et al., 2010).

6.3.1.2. Affected group.

A collection of 86 metacarpals and 93 contralateral humeri (83 sets) from dairy cattle that had suffered humeral fractures were collected, through the help of veterinarians and farmers around New Zealand, during the 2019/2020 dairy season. Bones were collected by veterinarians and farmers following researcher requests via social media and email. Bones were sent disarticulated via courier and stored at -20 ℃ until scanning. Liver samples were also collected for 69 of the cases and analysed for copper concentration at New Zealand Veterinary Pathology (IDEXX) Ltd (Palmerston North). Copper was categorised as normal (>95 μmol/kg), low (30-95 μmol/kg) and very low (<30 μmol/kg). A liver copper concentration of below 30 μmol/kg has been associated with impaired animal growth in cattle (Grace et al., 2010).

6.3.2. Scanning

pQCT scanning was carried out using the same protocol previously reported by Gibson et al., 2021 and Gibson et al., 2020. Briefly, pQCT scanning was carried out using an XCT 2000 peripheral quantitative computed tomography machine (Stratec Medical, Pforzheim, Germany). For each bone, a 2 mm slice at the mid diaphysis was obtained with a voxel size of 0.3 mm. In the metacarpus, the mid-diaphysis was defined as 50% of the total bone length using the lateral aspect of the lateral condyle of the MC4 and the proximal aspect of the lateral MC4. In the humerus, the mid-diaphysis was defined as 50% of total bone length, measured from the proximal end of the humeral head at the lateral aspect, to the end of the trochlea at the distal end. Within the manufacturer's software, voxels > 710 mg/cm^3 CaHA were assigned as "cortical" bone. Data derived from the scan included measures of total bone content, (>710 mg/cm^3), cortical density, total area, cortical area, cortical content, cortical density, cortical area, cortical thickness, periosteal circumference, endosteal circumference and stress–strain index (SSI). The stress–strain index is a derived measure of bone strength using pQCT data and avoids the requirement for destructive testing using techniques such as the three-point bending test. The stress–strain index describes the ability of bone to withstand bending from lateral, dorso-palmar and torsional forces and is calculated by incorporating the index of material stiffness (bone mineral density) and bone geometry (cross-sectional moment of inertia) (Ferretti,

1995). The pQCT variables will be referred to as bone parameters in the statistical models.

6.3.3. Statistical analysis

Statistical analysis was conducted using the Statistical Analysis System software version 9.4 (SAS institute Inc., Carey, NC, USA). All analyses were conducted using general linear models.

Data are presented as least-square means and standard error for each heifer group and bone parameter. Bone parameters were compared between control and affected heifers using a general linear model that included the fixed effect of group.

In order to adjust for expected mature weight, bone parameters in the humerus were also compared between groups after adjusting for periosteal circumference of the metacarpus. These models included the fixed effect of group and the covariate of periosteal circumference in the metacarpus (as a predictor of mature size). The interaction of periosteal circumference in the metacarpus and group was considered but not significant so was removed (p>0.05).

Similarly, the effect of copper was tested using a general linear model that included the fixed effect of copper status (normal, low or very low) and the covariate of periosteal circumference in the metacarpus. The fixed effect of group was tested but not significant so was removed.

6.4. Results

In the metacarpus, endosteal circumference was greater in the affected heifers than the control heifers (Table 6.1. Affected heifers had lower cortical bone density (0.9% difference) in both the metacarpus and humerus than control heifers.

Table 6.1. Least squared means and standard error of bone parameters of control and affected heifers in the metacarpus and humerus.

Bone parameter	Unaffected	Affected	P-value
Metacarpus			
n=	29	86	
Bone length (mm)	206.9±1.3	209.3±0.8	0.114
Periosteal circumference (mm)	92.4±1.0	93.9±0.6	0.180
Endosteal circumference (mm)	46.3±1.0	48.7±0.6	0.041
Total bone area (mm^2)	681.4±14.7	704.5±8.5	0.178
Total bone content (mg/mm)	677.9±11.7	677.8±6.8	0.993
Cortical bone thickness (mm)	7.3±0.1	7.2±0.1	0.184
Cortical bone area (mm^2)	508.8±9.0	513.1±5.3	0.681
Cortical bone density (mg/cm^3)	1279.4±2.5	1267.6±1.5	<0.001
Cortical bone content (mg/mm)	651.0±11.4	650.3±6.6	0.955
Stress strain index (mm^3)	4031.1±126.4	4207.3±73.4	0.231
Humerus			
n=	30	93	
Bone length (mm)	258.3±1.9	257.1±1.1	0.575
Periosteal circumference (mm)	143.5±1.6	144.1±0.9	0.753
Endosteal circumference (mm)	93.6±1.5	94.8±0.9	0.501
Total bone area (mm^2)	1644.6±37.5	1657.4±21.3	0.767
Total bone content (mg/mm)	1285.6±26.2	1265.6±14.9	0.508
Cortical bone thickness (mm)	7.9±0.1	7.8±0.1	0.507
Cortical bone area (mm^2)	939.8±20.2	936.4±11.5	0.883
Cortical bone density (mg/cm^3)	1292.6±3.7	1280.4±2.1	0.005
Cortical bone content (mg/mm)	1214.6±25.3	1198.3±14.4	0.578
Stress strain index (mm^3)	13335.9±416.1	13261.3±236.3	0.876

Adjusting humeri measurements with metacarpus periosteal circumference, as a proxy for bodyweight revealed a pattern of moderate inhibition of longitudinal bone growth and reduced deposition of mineral content in affected heifers. Affected heifers presented with significantly reduced cortical bone density ($p<0.05$) and a strong trend for reduced total bone content ($p=0.057$) (Table 6.2). The lack of significant difference in cortical area indicates that the trend observed in lower cortical bone content was the result of a reduced cortical bone mineral density in affected heifers. The resultant effect of the reduced cortical bone mineral density and trend in lower total bone content was a trend for lower SSI observed in affected heifers.

Table 6.2 Least squared means and standard error of bone parameters in the humerus using periosteal circumference of metacarpus as a predictor between control and affected heifers.

Bone parameter	Control	Affected	Coefficient	*p*-Value		R^2
				Metacarpus Periosteal circumference	Group	
n=	29	83				
Bone length (mm)	259.7±1.6	256.5±1	1.2±0.2	<0.001	0.094	0.35
Periosteal circumference (mm)	144.7±0.9	143.5±0.5	1.3±0.1	<0.001	0.258	0.67
Endosteal circumference (mm)	94.9±1.2	94.3±0.7	1.0±0.1	<0.001	0.660	0.44
Total bone area (mm^2)	1673.2±21	1643.5±12.4	31.0±2.1	<0.001	0.228	0.67
Total bone content (mg/mm)	1297.1±17.6	1257.7±10.3	19.7±1.7	<0.001	0.057	0.54
Cortical bone thickness (mm)	7.9±0.1	7.8±0.1	0.05±0.01	<0.001	0.495	0.12
Cortical bone area (mm^2)	948.7±13.8	930.8±8.1	14.9±1.4	<0.001	0.267	0.53
Cortical bone density (mg/cm^3)	1291.2±3.7	1280.0±2.2	-1.0±0.4	0.008	0.010	0.13
Cortical bone content (mg/mm)	1224.4±17.8	1191±10.5	18.2±1.7	<0.001	0.110	0.50
Stress strain index (mm^3)	13605.4±240.4	13090±141.6	342.5±23.6	<0.001	0.068	0.66

Adjusting humeri measurements with metacarpus periosteal circumference, as a proxy for bodyweight revealed heifers in the very low liver copper concentration group had reduced humeral bone length (inhibited longitudinal growth) and low cortical bone density (Table 6.3). The lower cortical bone density was not associated with lower cortical area or content, which may explain the lack of difference identified in SSI.

Table 6.3. Least square means and standard error for bone parameters in the humerus using periosteal circumference in the metacarpus as a predictor for animals with normal, low and very low liver copper concentration.

Bone parameter	Normal	Low	Very Low	Coefficient	*p*-Value		R^2
					Metacarpus Periosteal circumference	Copper	
n=	33	31	21				
Bone length (mm)	260.2±1.6^{b}	256.9±1.6^{ab}	251.8±2.2^{a}	1.3±0.2	<0.001	0.013	0.44
Periosteal circumference (mm)	144.3±1.0	142.8±1.0	145.6±1.4	1.3±0.1	<0.001	0.218	0.65
Endosteal circumference (mm)	95.6±1.1	94.5±1.1	97.6±1.6	0.9±0.1	<0.001	0.286	0.42
Total bone area (mm^2)	1663.2±22.7	1627.3±22.3	1694.8±31.7	29.0±2.7	<0.001	0.205	0.65
Total bone content (mg/mm)	1267.2±18.0	1242.1±17.7	1257.8±25.1	20.4±2.1	<0.001	0.607	0.58
Cortical bone thickness (mm)	7.8±0.1	7.7±0.1	7.6±0.2	0.06±0.01	<0.001	0.815	0.19
Cortical bone area (mm^2)	930.6±14.1	913.8±13.8	932.4±19.7	15.1±1.6	<0.001	0.625	0.55
Cortical bone density (mg/cm^3)	1284.7±3.7^{b}	1285.7±3.6^{b}	1270.5±5.2^{a}	-0.4±0.4	0.307	0.045	0.11
Cortical bone content (mg/mm)	1195.4±18.0	1174.1±17.7	1184.4±25.1	19.0±2.1	<0.001	0.702	0.54
Stress strain index (mm^3)	13178.5±253.2	12946.1±248.7	13463.6±353.7	333.2±29.6	<0.001	0.484	0.65

6.5. Discussion

Previous research outlined the strong relationship between pQCT measures in the mid-diaphysis of the metacarpus and humerus (Gibson et al., 2019). Therefore, the basis of this work was to identify whether heifers at risk of humeral fractures could be identified using pQCT measures at the mid-diaphysis in the metacarpus. Whilst the mid-diaphysis was shown to not be a useful model for identifying at risk heifers, the current study provided indications for the temporal pattern of nutritional challenge that is associated with humeral fractures. Longitudinal bone growth occurs at the metaphysis making it a dynamic site that can be influenced by recent changes in growth. As longitudinal bone growth occurs, the affected bone produced in the metaphysis is pushed towards the diaphysis. Most longitudinal bone growth occurs in the first year of a heifer's life and because of this, changes to bone morphology observed in the diaphysis would be the result of an event that occurred in the first year of life. Humeral fractures originate in the metaphysis and thus it is an important site for quantification of differences in bone parameters. The lack of effect in the mid-diaphysis coupled with observations of growth arrest lines in affected bones reported by Dittmer et al. (2016) suggests that risk factors causing heifers to be susceptible to humeral fractures must be recent. The idea that the risk factor is recent aligns with observations by Handcock et al. (2019b), where the slowest growth (live weight gain) in a heifer occurs between 20 and 22 months of age as a result of changes in seasonal nutrition. Growth arrest lines occur due to the retardation of longitudinal bone growth

resulting in the temporary sealing of the physis with bone, which are often caused by malnutrition (Craig et al., 2016). If the deficit in nutrition is corrected, physeal growth will resume and the seal of the physis will be displaced into the metaphysis, resulting in what is termed a growth arrest line. However, if a growth arrest occurs early in a heifer's life, it is likely to be removed as the bone is remodelled during appositional and longitudinal growth. Therefore, if this proposed timing is correct the presence of growth arrest lines and lack of difference in the mid-diaphysis indicates that examination of the metaphysis may show reduced cortical bone density and a lower stress-strain index.

Bone size and strength has been shown to be strongly correlated with live weight (Gibson et al., 2020, Gibson et al., 2021). Due to the opportunistic nature of bone acquisition in both the control and affected groups live weights were not able to be consistently or reliably obtained. Therefore, bone parameters could not be adjusted for live weight to give an indication of bone size and strength in relation to animal size. However, previous research has found that bone parameters in the metacarpus can be used as a proxy for humeral bone parameters (Gibson et al., 2019). The metacarpus has minimal changes after one year of age and provides a gross measure of the size potential of the animal. Therefore, adjusting the humeral bone parameters to a common metacarpus parameter provided a mechanism to adjust for liveweight. With the humerus significantly shorter in length in affected heifers, and the tendency for a reduced stress-strain index and cortical bone density, the current results are

consistent with observations in human adolescents affected by anorexia nervosa (AN). Severe nutrient deficiency in AN results in low body mass and disruption to hormones involved in bone regulation (Turner et al., 2001). Longitudinal bone growth can be reduced during periods of AN. When sufficient feeding occurs, longitudinal bone growth resumes but at an accelerated rate. However, the effect on bone mass accrual can be more permanent resulting in an increased incidence of osteoporosis and fracture risk (Miller et al., 2005). The results in the current study and observations of AN emphasizes the potential importance of adequate nutrition for optimal bone mass accrual. Bone density will be further reduced at the onset of lactation in dairy cows as a result of calcium being lost from the matrix. Therefore, the onset of lactation is likely to be the tipping point for many heifers that experience spontaneous humeral fractures.

To provide a possible method for field collection of data the pQCT voxel size was set at 0.3 mm, which in a field setting provides a suitable trade-off between ability to detect differences and the time of image and data acquisition. A smaller voxel size would have provided greater resolution and reduced the error associated with the voxel sharing effect (Tjong et al., 2012). However, it was important test the ability of pQCT derived values at a setting that would have been used in a field setting to accurately test the possibility of using metacarpus values to predict humerus data and risk of fracture. Whilst pQCT provides a tool to quantify bone properties and spatial distribution it is likely that the resolution used with the pQCT (0.3 mm voxel size) was

insufficient to detect subtle changes (Schmidt et al., 2003). Therefore, to detect small changes between the control and affected animals, histology or microCT are required. The current results were limited to the diaphysis due to the limited size of the gantry of the pQCT machine and the large size of the humeral head and consequently the location of the metaphysis.

Although the relationship between bone parameters and copper was not different between affected and control heifers, low liver copper status was associated with reduced bone length and cortical bone density in the humerus. The reduced cortical bone density indicates that the lack of copper required for collagen cross-linking in bone has reduced the binding of calcium in the matrix. Copper deficiencies also causes a reduction in osteoblastic and osteoclastic activity, resulting in a decrease in osteoid production (Suttle et al., 1972). This supports the hypothesis of chronic inadequacy of copper within the diet (Nojiri et al., 2011). Examination of the metaphysis may provide more detail on the effect of recent copper status on bone development, as the metaphysis is the site reported to be most affected by copper deficiencies (Suttle et al., 1972). Another limitation is the use of copper status at the time of death as this does not provide an indication of whether the animal had previously had a copper deficiency and recovered and if so, how long the animal had been deficient or the severity of the deficit, both of which may impact the bone (Grace et al., 2010). The lack of difference in the relationship between bone parameters and copper concentration between affected and control heifers could also mean that copper concentration may

not be the primary contributing factor for humeral fractures. The opportunistic nature of the data collection for controls limited collection of liver copper status details. The low number of liver copper records for controls may have prevented us from identifying the relative influence copper status had in contributing to the risk of humeral fracture. The comparatively low number of control heifers in this sample reduced the power of the study. With the current number of samples, there was sufficient power to detect a 10% reduction in the stress strain index in the mid-diaphysis of the humerus. Without correction for body weight, to detect the 0.5% difference observed (Table 6.1) based on unadjusted means, approximately 29,000 control and affected heifers would be required. In contrast, when adjusting humeral bone parameters for periosteal circumference of the metacarpus, there was sufficient power to detect a 6% reduction in the stress-strain index. Based on the material collected in this study there was a 3.7% decrease in stress strain index which would have required only 198 control and affected heifers. The difficulty with sampling control heifers at the age of humeral fracture is the limited culling of non-diseased stock at this age. The lack of controls was a limitation, despite significant efforts being made to obtain suitable control samples.

The original aim of this research was to compare bone morphology in the mid-diaphysis of the metacarpus and humerus of both control and heifers affected by spontaneous humeral fractures. If there was a significant difference in morphology between control and affected heifers, future research would aim to establish a field

trial to see if the relationship of mid-diaphyseal measures in the metacarpus and humerus could be used to identify at risk heifers in a relatable manner in a field scenario. However, the lack of difference in the metacarpus and humerus relationship in control and affected heifers means the current method will not work. Therefore, further research into the metaphysis of the metacarpus and humerus may provide valuable insight into more recent changes in bone and allow for quantification of fracture risk in heifers.

6.6. Conclusions

Humeral fractures in dairy heifers originate in the distal metaphysis. The mid-diaphysis was a poor predictor of humeral fracture risk in this study. The limited differences at the mid-diaphysis highlighted that the contributing factor for fracture is recent and may be affecting bone propagation at the metaphysis but had limited effect on the more mature bone at the mid-diaphyseal site. Temporally, this would relate to the second winter which has the potential for restricted feed supply due to seasonal pasture growth. Growth arrest lines observed in case-control studies support this hypothesis. Further research should investigate differences in the metaphysis between affected and control heifers, and possibly use a variety of techniques to help elucidate the influence restricted feed supply may have on the development of bone within the metaphysis. Management of dairy heifers in the months prior to calving should also be examined to identify periods of growth restriction that may occur during the second winter or during the transition into the milking herd.

Chapter 7

General discussion.

7.1 General introduction

Studies of heifer growth in the dairy industry have largely been limited to the setting of liveweight targets to achieve reproductive and lactational outcomes. The incidence of spontaneous humeral fractures in first lactation dairy heifers has highlighted a lack of knowledge about bone growth in dairy heifers. Whilst live weight is recorded and reported, stature and skeletal growth has been largely overlooked in young cattle. To understand why the humerus is fracturing and not other bones, it is important to know when the humerus is growing and how that correlates with different stages of heifer rearing. Humeral fractures of this nature appear to be a New Zealand specific problem with no reports of cases overseas in industry or peer reviewed literature.

Within the first year of life, 90% of mature height of a heifer is achieved but only 50-60% of mature weight (Hammack and Gill, 2009). Thus, a greater proportion of increases in girth and therefore, humeral size, would be expected after one year of age. Given the seasonal pattern of heifer growth in New Zealand, it is expected that bone growth would reflect this non-linear pattern of growth as described by live weight gain. The presence of growth arrest lines, in bones collected from heifers affected by humeral fractures (Dittmer et al., 2016), suggests that normal bone growth and development has been disrupted. This may lead to inadequate bone mass to meet the demands for calcium at the time of first lactation.

The general aim of this thesis was to investigate the relationship between live weight and bone morphology in the bovine forelimb to identify potential risk periods for spontaneous humeral fractures in first-lactation dairy heifers.

7.1.1 Live weight is an ideal descriptor of bone morphology and strength.

Throughout the experimental chapters presented in the current thesis, measures of bone morphology have been shown to be highly correlated with live weight. Increases in liveweight are associated with an increase in bone size and strength in both the metacarpus and humerus. Demonstration of this relationship is in agreement with the mechanostat theory where bone increases in size and strength to maintain strain within physiological limits (Rubin and Lanyon, 1985). However, the relationship between bone and live weight changes with bone location, maturity status and sex of the animal (Chapters 2 and 4). Under New Zealand farming conditions, the load placed on long bones in cattle is insufficient to induce an increase in bone density. Cattle are not cursorial like horses so may not reach the same strain to induce an increase in bone density (Logan et al., 2019). Therefore, increases in resistance to bending by long bones in cattle is largely affected by bone size and to a lesser degree changes in cortical bone thickness (Chapters 2, 3, 4 and 6).

Differences in the stress strain index between the metacarpus and the much stronger humerus at all ages reflect the difference in loading and function of each bone (Chapters 2,3,4 and 6). In addition, the humerus also has a stronger relationship with

live weight than the metacarpus as shown by a higher R^2 for stress-strain index in Chapters 3 and 4. The main force placed on the metacarpus is compressional forces from standing and low velocity locomotion, with no muscular torsion, resulting in a low strain rate (Berg et al., 1978). In contrast, the humerus undergoes torsional forces placed on it by the biceps brachii, brachiocephalicus and brachialis muscles which provides the bone with its distinctive shape and requirement for increased resistance to bending (Rakestraw, 1996, Bouza-Rodríguez and Miramontes-Sequeiros, 2014). Therefore, increases or changes in live weight and as a result, muscularity, will have a greater effect on bone strength in the humerus than the metacarpus.

In the metacarpus, bone maturity occurred around one year of age, with only minor changes in the morphology of the bones examined after one year of age. The effect of sex is linked to mature size where bulls have a greater mature size than heifers. A greater mature size and increased muscularity will affect the size of bone. In Chapter 4 when both the metacarpus and humerus were adjusted for live weight, steers had a greater bone strength (SSI) and size than heifers, which reflects a greater genetic potential for mature size. Whilst there could not be a direct comparison between bulls and heifers in Chapter 4 due to differences in breed and farm between mobs, the lack of difference between bulls and steers emphasises the potential sex effect caused by testosterone between males and females. In contrast the significant differences between heifers and steers emphasises the effect of mature size and oestrogen on physeal closure.

7.1.2 Distal regions of the body have an earlier maturity than proximal regions.

The effect of puberty on bone creates a regional effect where bone maturity is promoted but does not occur in all bones at the same time. Distal regions of limbs have an earlier maturity than proximal regions (Berg et al., 1978) where the proportion of total bone weight in the distal limb decreased as live weight increased. However, in the study by Berg et al. (1978), when the proximal limb (humerus and scapula) was examined, the proportion of limb weight to total bone weight remained constant. The metacarpus is approximately 90% of its mature length at birth showing its limited growth potential (Guilbert and Gregory, 1952). Results from Chapters 2, 4 and 5 show that measures of metacarpal size and morphology had little change beyond a year of age whilst changes in bone size and morphology in the humerus and girth continued. The constant growth of the humerus also reaffirms the need for a larger and stronger humerus as the animal grows and limited requirement for increases in bone size and strength in the metacarpus. Therefore, it makes biological sense to have a later maturity in the humerus than in the metacarpus.

7.1.3 Description of stature

Changes in bone size and strength are proportional to changes in stature (Rubin and Lanyon, 1985). Liveweight trajectories have indicated that winter is a risk period for restricted live weight gain in heifers during their first and second year of life, when they are likely to achieve suboptimal growth rates (Handcock et al., 2017). With the majority of height achieved in the first year, increases in live weight in the second

winter are the result of an increase in girth (Chapter 5) (Hammack and Gill, 2009). Increases in girth, results in an increase in muscularity and therefore greater strain on the humerus resulting in a demand for a larger and stronger bone. Therefore, humeral growth must continue during the second winter, meaning it could be sensitive to potential deficits in pasture quantity and quality during this period.

The lack of breed effect on stature measures when adjusted for liveweight indicates that there may be an ideal body composition at a given weight (Chapter 5). Although stature was only measured until 15 months age, divergence between breeds was starting to occur. Differences in mature weights between Friesian and Jersey heifers result in different ages of maturity. Jersey heifers will have a lighter mature weight as cows, so are younger at maturity than Friesian cattle (Handcock et al., 2019b). Differences in maturity are also reflected in the observation of differences in height between breeds as distal limb bones cease longitudinal growth at a lighter weight in Jersey cattle and diverge from the still growing Friesian cattle, followed by a similar effect in changes of girth.

7.1.4 Lack of difference in control and fractured bones

Early research reported that there was a significant difference in cortical bone thickness and stress-strain index in the mid-diaphysis of bones from heifers with humeral fractures and unaffected heifers (Dittmer et al., 2016). However, there was no difference observed in bones analysed in Chapter 6. Early research looked at a small

subset of bones whilst current research looked at bones from a larger population. By using a larger population, there was more heterogeneity in both fractured and unaffected bones likely due to modifying factors such as live weight, breed, diet and farm (chapter 6). However, when metacarpal length was used as a mechanism to adjust for live weight, the cortical bone density was lower in the bones of affected heifers and there was a tendency for the humerus to be shorter and have a lower stress-strain index than control bones. The results of Chapter 6 and observations of anorexia nervosa in humans emphasises the importance of nutrition in early life to prevent the increased incidence of osteoporosis and fracture risk. In cattle, the presence of osteoporosis coupled with the onset of lactation where calcium stores are mobilised may create a potential tipping point to cause spontaneous humeral fracture.

A low liver copper status was associated with a reduced bone length and cortical bone density in the humerus when using the periosteal circumference of the metacarpus as a proxy for live weight. The reduced cortical bone density suggests that collagen cross-linking may be impaired as this can lead to decreased binding of calcium to the collagen matrix thus reducing bone density. Copper deficiency also causes a reduction in osteoblastic and osteoclastic activity resulting in decreased osteoid production (Suttle et al., 1972). Observations of reduced cortical bone density and copper deficiency supports the hypothesis that chronic inadequacy within the diet increases the risk of bone fracture.

The similarities between bones from fractured and control heifers at the mid-diaphysis, coupled with the site of origin of the fracture being in the metaphysis, indicates the metaphysis a potential site for differences between fractured and control heifers. If there are differences in the metaphysis it would indicate that the inhibition of bone quality and increased risk of fracture is a relatively recent event. Potential timings of this risk period may align with the seasonal variation of pasture supply that occurs in New Zealand.

7.1.5 Seasonality of NZ dairy systems

Not only is there a seasonal effect of winter influencing bone development but other variations in heifer management can also affect growth. In winter, heifers are also in the latter stages of pregnancy, so nutrient partitioning to the fetus will be at its greatest (Nicol and Brookes, 2007). If inadequate nutrition is supplied to the heifer, then energy stores will be used and possibly result in a growth check. Poor nutrition can also affect a heifer during the transition from pregnancy to lactation. As the heifer reaches parturition, increases in calcium are required for both the calf and the beginning of milk production (Horst et al., 2005). If insufficient calcium is provided in the diet, calcium bone stores may be mobilised resulting in a decrease in bone mass and resistance to bending (Reinhardt et al., 1988). Therefore, future research is required to ascertain the importance of feed management for bone strength during this period to enable the development of feeding guidelines for farmers.

Another important event in many heifer's lives just prior to calving may be the movement back to the home farm from a grazier. At this time heifer nutrition changes during the adjustment from the grazier to the home farm which can potentially result in a check in growth rates. Graziers are often contracted to grow heifers to a target liveweight gain so adequate nutrition is supplied. When heifers are moved back to the home farm, the transition may involve changes in diet composition and feed availability as well as changes in grazing herd and social hierarchy from mixing with older cows (Neisen et al., 2009), which may affect their access to feed (Bica et al., 2019).

7.1.6 Identifying at risk heifers.

Although a potential risk period has been highlighted in the current research, a method to detect heifers at risk of humeral fractures has not been achieved. The tight relationship between measures of the metacarpus and humerus means that the metacarpus may still provide good predictor of humeral bone structure (Chapter 2). However, the lack of difference in mid-diaphyseal bone morphology in the fractured and unaffected groups means that the use of the metacarpus to predict whether a heifer is at risk of fracture is not possible at this stage. Further work is necessary to establish the bone parameters that differ between fractured and control heifers, with focus around the metaphysis. Therefore, there is still currently no way to identify at risk herds until multiple fractures have occurred on farm.

7.2 Limitations

Stature measures were only measured up to 15 months of age in the thesis. Although this provided valuable insight into what occurs in the first winter and at puberty, subsequent growth cannot be assessed. The findings of this thesis have demonstrated that the period of greatest risk for environmental contributors to humeral fractures may have occurred after 15 months of age. However, when planning collection of the growth data with what is known about the potential recent change in bone that contributes to humeral fractures and the times frame for this prospective data collection was outside that of this thesis. Just prior to 15 months, differentiation in growth due to breed began to be seen where Jersey breed heifers were closer to their mature size than Friesian heifers. To provide information on how mature liveweight affects the timing of bone maturity, stature measures need to be recorded until a mature weight is reached at approximately 4-5 years of age (Arango et al., 2002); under commercial conditions can be logistically difficult to capture. In addition, data on the changes observed during the second winter of a heifer's life would also allow for quantification of increases in girth and, therefore, changes in humeral size. In this thesis, the heifers measured were all achieving above or near target liveweight, and consideration of measuring growth-restricted heifers may show greater impacts on stature. Although this research provides valuable information of what can be assumed to be ideal heifer growth conditions, it does not give an indication to what changes in stature would look like for a heifer affected by seasonal restrictions in feed availability.

There was large variance in pQCT-derived bone measures within both the control and fractured groups, indicating that within the industry there is significant variation in the phenotype of what is considered a "normal" dairy heifer at approximately 2 years old. Unaffected heifers were selected from those that had died of other causes and been sent to a disposal service, so it is possible that some of these animals had conditions which may have influenced the bone measurements obtained.

The results of the current work highlight that in future there is a need for information regarding live weight of the animal which will allow for adjustment of bone parameters for animal size. By doing so, any differences in bone morphology and strength between fractured and unaffected heifers can be more clearly described. However, with what is known about spontaneous humeral fractures and the likelihood that causes for fracture are multifactorial a large cohort of animals will be required to provide adequate power (as stated in Chapter 6) to quantify the impact each factor has on the risk of fracture.

Although measures of the mid-diaphysis provides valuable information on bone size and strength, the current status of the bone cannot be fully described with mid-diaphyseal data alone. The metaphysis is an active site in the bone where changes in growth and morphology are first observed. However, the size of the humeral head and small gantry on the pQCT machine meant that data on the metaphysis located in the humeral head could not be collected. In addition, the shape of the physis in the

humeral head makes identification of a common reference point difficult, and consequently limits the repeatability of the measurements. Therefore, recent changes in the humerus could not be quantified. It is known that the spiral fracture of the humerus originates in the metaphysis and this location is subjected to a large amount of force making it an important area to be subsequently quantified.

7.3 Future research

The general aim of this thesis was to investigate the relationship between live weight and bone morphology in the bovine forelimb to identify potential risk periods for spontaneous humeral fractures in first-lactation dairy heifers. This thesis described the association of cattle growth with bone development and the potential timing when the humerus is most likely to be affected. However, new questions have arisen from these results that justify further research.

- With only subtle differences in bone length and cortical bone density, future research should explore the differences in bone morphology in the metaphysis between affected and unaffected heifers. However, to do so, the current pQCT protocol would need to be improved. A possible improvement would be the use of a spiral CT that can accommodate a larger bone size. Although pQCT allows for the quantification of bone morphology and strength, it does not detect changes at a micro level due to the large voxel size. Micro CT shows changes in bone architecture and the current state of bone, based on signs of

modelling and remodelling to show bones response to factors such as lactation. In terms of heifers that have recently calved, it would be expected that there would be some remodelling to accommodate the onset of lactation, but some may also be due to the diet of the heifer, such as from copper deficiency. The use of histology would allow for bone tissue to be analysed in greater detail. Changes in bone histology could indicate if bone is trying to increase strength by resorbing bone from the endosteal side and placing it on the outer periosteum (appositional growth), to provide a larger bone and therefore greater bone strength. Although this would be observed in pQCT as an increase in endosteal circumference, histology would indicate signs of bone remodelling. Additional information regarding the mineral status of the bone and architecture can be described providing valuable information in conjunction with CT results. Therefore, future research should include bone analysis at a histological level.

- Research into bone growth and development with animals of known previous growth history would provide valuable information to factors that affect heifer growth and development. The first step would be to follow a herd of heifers from birth to mature live weight and measure stature at regular intervals with the addition of bone measures via serial slaughter to correlate with observations in stature. By recording bone parameters regularly, any changes in bone can be correlated with changes in live weight gain and events such as

puberty or change in farm. In addition, the age at which bone matures can be identified. Based on the potential importance of the second winter of life, emphasis should be placed on understanding a heifer's history in the 6 months prior to calving. Once an understanding of "normal" is obtained, factors of diet and farming practices can be manipulated at times when humeral growth is at its greatest.

Cohorts of animals should be divided up to make different treatment groups. Each treatment will have a restricted diet for one of the six months prior to calving. In addition, the length of time that the diet is restricted could also be altered. Records of live weight and measures of bone morphology such as size and content can then be taken to see how bone is affected and potential growth arrest lines identified. In particular, bone measures in the metaphysis should be used to look at recent changes in bone. Once the period that bone is most affected by nutrient deficit is identified, different levels of nutrient deficit can be tested to quantify the severity of deficit required to induce a significant reduction in bone strength. Information can then be used to look at how variations in growth from changes in diet and lack of nutrition change bone response to increases in live weight.

- The current bone scanning method using pQCT relies on post-mortem extraction of the humerus to be able to scan the bone. To continue with this

method and acquire adequate data requires a large number of heifers to be sacrificed. However, if a different method were used that would allow for live scans, such as quantitative ultrasound (QUS), repeated bone scans over a heifer's life could occur. By doing live scans the number of heifers required would be reduced and animal wastage would decrease. Research would also be able to be carried out on multiple farms.

7.4 Conclusion

The results presented in this thesis provide evidence that bone size and strength are highly correlated with live weight. However, the relationship between bone and live weight can be modified by factors such as sex and age. For example, the growth capacity of the metacarpus and humerus were affected by sex. The role of oestrogen in inhibiting longitudinal bone growth was demonstrated by a delay in bone maturation in steers compared with heifers and bulls at the same age. The effect of age was evident in that the metacarpus had matured by 12 months of age whereas the humerus was still growing at 24 months of age.

Observations of distal vs proximal bone growth align with what was observed in stature measures where increases in height and length slowed at approximately 200-250 kg whilst measures in capacity such as girth continued to increase. Therefore, with differences in bone maturity, and stature growth in proximal and distal regions of the

limb, it is likely that the humerus is still growing with increases in live weight in the lead up to the time of fracture at 24-27 months of age.

The second winter of life has been highlighted as a period in need of further research, as it is a period of significant humeral growth that coincides with potential feed restriction. Therefore, further investigation should focus on the six months prior to fracture as the most likely time for risk factors.

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Appendices.

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Gibson, M, Rogers, C, Dittmer, K, Hickson, R, Pettigrew, E & Back, P 2019. Can bone measures of the bovine metacarpus predict humeral bone structure? New Zealand Journal of Animal Science and Production, 79, 8-12	
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