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Compensatory and catch-up growth in cattle

Tiago Alves Corrêa Carvalho da Silva
B.Sc. Agronomic Engineering

A thesis submitted for the degree of Doctor of Philosophy at
The University of Queensland in 2017
School of Agriculture and Food Science

Abstract

Variation in nutrition leads to periods of restricted and accelerated changes in the liveweight of cattle, but less is known about the effect of level of nutrition and/or specific nutrients on skeletal growth. The endochondral ossification process at the epiphyseal growth plate drives longitudinal bone growth and by inference sets growth in skeletal muscle via a passive stretch mechanism. The physiological and morphological mechanism behind animal growth that drives adaptation of mammals during the transition from a low to high plane of nutrition remains a major biological question. Two experiments examined the effect of protein and energy on skeletal growth the perspective of the dimensional changes, trabecular bone remodelling (histology and bone biomarkers) and hormone. Data was then collated from these and other experiments conducted within this laboratory to develop a growth curve in liveweight-for-hip height of well-fed cattle and to identify deviations from this "normal" growth relationship.

In the first experiment (Chapter 4), the effects of low and high crude protein (CP) content diets during metabolizable energy (ME) restriction on subsequent re-alimentation in *Bos indicus* and *Bos taurus* cattle were evaluated. Three treatment diets were applied; a control diet (High CP-High ME) and two restricted pair-fed ME intake diets differing in CP content (Low CP–Low ME and High CP-Low ME) for 93 days followed by re-alimentation of all treatment groups offered *ad libitum* access to the High CP-High ME diet for 103 days. In the second experiment (Chapter 5), a 2 x 5 factorial design was used to determine the effect of supplementation (0, 1, 2.5, 5, 10 g protein meal/kg LW.day) of a low CP hay during the first dry season (169 days) and weaning weight [Early (118 kg) *vs.* Normal (183 kg)] on long-term (~2 years) growth in liveweight and the skeleton and reproduction of replacement heifers in northern Australia. After the first dry season all treatment groups were subjected to the same level of nutrition by grazing the same pasture together.

Across both experiments, higher plane of nutrition increased liveweight gain and skeletal elongation growth. Increases in ME and CP intake in cattle were positively associated with the height of proliferative and hypertrophic zones as well as with the diameter of terminal hypertrophic chondrocytes measured in growth plate biopsies of the tuber coxae. In addition, the diameter of terminal hypertrophic chondrocytes showed significant correlation with the broader measure of hip height gain in both experiments. Plasma bone-specific alkaline

phosphatase and pyridinoline appeared to be effective bone biomarkers of formation and resorption in growing cattle respectively.

Low ME intake severely reduced the plasma insulin and insulin-like growth factor 1 concentration in cattle, independent of CP intake. Cattle with the higher CP intake during ME restriction had higher concentration of triiodothyronine in the plasma and this was correlated with larger terminal hypertrophic chondrocytes at the tuber coxae growth plate as well as increased hip height gain. After nutritional restriction, cattle showed an increase in dry matter intake and liveweight gain for approximately 40 to 60 days after commencement of the re-alimentation phase followed by a subsequent decrease to values similar to unrestricted counterparts. Skeletal growth of previously restricted cattle was greater than unrestricted counterparts at the same age and was associated with increased proliferative and hypertrophic zones heights without differences in any measured plasma hormone concentration. Nutritional restriction at the early weaning weight did not cause permanent stunting, however it increased the time frame necessary to achieve reproductive target liveweights for satisfactory pregnancy rates (>80%) of replacement heifers in northern Australia.

In Chapter 7, a "normal" liveweight-for-hip height relationship model was generated for well-fed *Bos indicus* cattle using data from the two experiments in this thesis as well as results from 4 other studies by our reseach group and a groups of mature fistulated steers. A decrease from the normal liveweight-for-hip height relationship was observed during nutritional restriction. The difference between the actual liveweight of an animal and the expected liveweight based on its hip height was identified as the "Liveweight gap". A significant relationship was found between liveweight gain during compensatory growth and the Liveweight gap. The return to the normal liveweight-for-hip height relationship was associated with the decrease in dry matter intake during compensatory growth.

It is concluded that cattle can exhibit catch-up growth in skeletal growth and compensatory growth in liveweight after a period of nutritional restriction. The results support the concept that catch-up growth in the skeleton is caused by a delay in growth plate senescence during nutritional restriction and that compensatory growth in weight is caused by a deviation from the liveweight-for-hip height relationship probably related to the passive stretch mechanism of bone length on skeletal muscle.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Silva, TACC; Quigley, S; Kidd, LJ; McLennan, SR & Poppi, DP. 2014. 'Skeletal Growth, Liveweight Gain and Feed Intake of Bos indicus and Bos taurus Cattle Undergoing Compensatory Liveweight Gain' in *30th Biennial Conference of Australian Society of Animal Production*, Canberra-NSW, vol. 30, p. 289.

Silva, TACC; Kidd, LJ; Quigley, S; McLennan, Anderson, ST & SR; Poppi, DP. 2016. 'High protein content stimulates bone elongation on energy-restricted cattle' in *31th Biennial Conference of Australian Society of Animal Production*, Adelaide-SA, vol. 31, p. 87.

Silva, TACC; McCosker, K; Schatz, T; Quigley, S; McLennan & SR; Poppi, DP. 2016. 'Strategies for replacement heifers in Northern Australia: Weaning weight' in Northern Beef Research Update Conference, Rockhampton-QLD, p. 139.

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If I have seen further, it is by standing upon the shoulders of giants **(Isaac Newton)**

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
AA	Amino acids
ADF	Acid detergent fibre
AFRC	Agriculture and Food Research Council
AIC	Akaike information criterion
ALS	Acid-labile subunit
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BAP	Bone-specific alkaline phosphatase
BCFA	Branched-chain fatty acids
BCS	Body condition score
BFR	Bone formation rate
BMD	Bone mineral density
BMP	Bone morphogenetic proteins
BMU	Basic multicelular unit
BrdU	5-bromo-2'-deoxyuridine
BS	Bone surface
BV/TV	Bone volume
Ca	Calcium
CL	Corpus luteum
CLA	Conjugated Linoleic Acid
CP	Crude protein
Cr	Chromium
Cr-EDTA	Chromium-ethylenediamine tetraacetic acid
CSIRO	Commonwealth Scientific and Industrial Research Organization
CTX-1	C-terminal crosslinked telopeptide of type I collagen
Cy9	9th coccygeal vertebrae
d	Day
D1	Deiodinase type 1
D1KO	Deiodinase type 1 knockout
D2	Deiodinase type 2
D2KO	Deiodinase type 2 knockout

dL	Decilitre
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DOM	Digestible organic matter
DOMI	Digestible organic matter intake
DPD	Deoxypyridinoline
e.g.	Exempli gratia (for example)
ECM	Extra celular matrix
EDTA	2,2',2'',2'''-(Ethane-1,2-diyldinitrilo)tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	Eye muscle area
EMCP	Efficiency of MCP production
etc	Et cetera
FA	Fatty acid
fl	Femtoliters
FME	Fermentable metabolizable energy
FOM	Fermentable organic matter
g	Gravity
g	Gram
GH	Growth hormone
GHIH	Growth hormone-inhibiting hormone
GHRH	Growth hormone-realising hormone
h	Hours
HH	Hip height
HHG	Hip height gain
HPLC	High performance liquid chromatography
HW	Hip width
HWG	Hip width gain
HZ	Hypertrophic zone
HC	Hypertrophic chondrocyte
i.e.	That is
ICTP	Cross-linked carboxyterminal telopeptide of type I collagen

IGF-1	Insulin-like growth factor I
IGFBP	IGF binding proteins
IRMA	Immunoradiometric assay
K	Potassium
kg	Kilogram
KO	Knockout
L	Litre
LH	Luteinizing hormone
Ln	Natural logarithm
LSD	Least significant difference
LW	Liveweight
LWG	Liveweight gain
M-CSF	Monocyte-macrophage colony-stimulating factors
MAR	Mineral apposition rate
MCP	Microbial crude protein
ME	Metabolizable energy
min	Minutes
MJ	Mega joules
mL	Millilitre
mm	Millimetre
mmol	Millimole
MMP's	Matrix metalloproteinases
MP	Metabolizable protein
mRNA	Mensseger RNA
N	Nitrogen
NBF	Neutral buffered formalin
NDF	Neutral detergent fibre
NEFAs	Nonesterified fatty acids
NH_3N	Ammonia N
NPN	Non-protein N
NRC	National research council
OCN	Osteocalcin
OM	Organic matter
OMD	Organic matter digestibility

OMI	Organic matter intake
OPG	Osteoprotegerin
P	Phosphorus
P:E	Protein to energy
P=/>/<	Probability of significant difference between treatments
PCR	Polymerase chain reaction
PD	Purine derivatives
PFA	Paraformaldehyde
PTH	Parathyroid hormone
PTU	Propylthiouracil
PUFA	Polyunsaturated fatty acid
PUN	Plasma urea nitrogen
PYD	Pyridinoline
PZ	Proliferative zone
r	Correlation coefficient
R^2	Coefficient of determination
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligant
RDN	Rumen degradable nitrogen
RDP	Rumen degradable protein
RIA	Radioimmunoassays
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RSE	Residual standard error
rT3	Reverse T3
RZ	Resting zone
S	Sulphur
SD	Standard deviation
SEM	Standard error of the mean
T3	3,5,3’- triiodothyronine
T4	Thyroxin
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TH	Thyroid hormone

THR	Thyrotropin-releasing hormone
TR	Thyroid receptors
US	Urea-ammonium sulphate
VFA	Volatile fatty acid
μm	Micrometre

Chapter 1. General Introduction

Australia is currently the third largest exporter of beef in the world (USDA, 2017). The red meat industry employs around 200,000 people including on-farm production, processing and retail (RMAC, 2015). In 2015-2016 the gross value of Australian cattle production is estimated at A$13 billion (Australian Bureau of Statistics, 2017). The larger proportion of the Australian cattle herd is concentrated in Queensland (42% of 24.8 million head). Overall, the beef cattle production systems (e.g. property size, breeds and markets) in Australia differ greatly specially when comparing Northern and Southern Australia. However there is an overall reliance on grass fed animals. It is estimated that only 2.7 million cattle are grainfed which represents 39% of the adult cattle slaughtered (ALFA, 2015). The cattle industry have an important economic, social and cultural role in the Australian society.

Grazing ruminants possess a digestive chamber (i.e. reticulo-rumen) which allows them to utilize cellulolytic carbohydrates as an energy source (Van Soest, 1994). The position of the digestive chamber prior to the true stomach (i.e. abomasum) enables the use of microbial protein as the main source (from 60 to 85%) of the total absorbable protein in the small intestine (Storm and Ørskov, 1983). These characteristics allow human societies to utilize ruminants to produce several food (e.g. meat and milk) and non-food (e.g. wool, leather, traction power and transport) items without the necessity of feeding a human edible product (e.g. grains) and assume considerable social-economical relevance as domestic animals.

In tropical and sub-tropical environments forage growth and quality is largely determined by rainfall which occurs mostly in the wet season. The variation in forage growth and quality throughout the year affects the rate at which it can be harvested by animals. Contrary to popular belief, in tropical and subtropical areas the environmental factors associated with dry season (e.g. low temperatures and low water availability) tend to enhance the nutritional value of C4 pathway grasses (Wilson et al., 1976; Van Soest et al., 1978; Wilson, 1983). However due to the slower forage accumulation rates during this period, the pasture grazed in the dry season has a longer regrowth period during which there is a loss of nutritional value due to greater maturation stage. In general terms, forage available during the dry season in tropical grasslands has a low dry matter digestibility (DMD; <55%) and crude protein content (CP; <60 g CP/kg DM) and a high neutral detergent fibre (NDF) content (Leng, 1990; Boval and Dixon, 2012).

Boval and Dixon (2012) reported dietary CP and DMD values estimated by NIRS analysis of faecal samples of cattle grazing native pastures in northern Australia ranged from 30 to 150 g CP/kg DM and 44 to 68% DMD, during the dry and wet seasons respectively. The output of ruminant production in grazing systems is directly affected by the variation in pasture quality and quantity throughout the year. Winter et al. (1991) reviewed data on liveweight (LW) production of extensive grazing cattle in Australia and demonstrated that 48% of the literature reported gains over 0.6 kg/day during the wet season while 47% reported liveweight loss or maintenance during the dry season. In addition, Winter et al. (1991) stated that cumulative LW gain (CLW) of 100 kg/head/year is typical within the extensive grazing systems in northern Australia. However, in improved grazing systems in northern Australia CLW of 142 and 168 kg/head/year and productivity per area varying from 25 to 76 kg LW/ha.year have been recorded (Bowen et al., 2015; Bowen et al., 2016).

Animal productivity in tropical pastures can be enhanced by implementation of techniques such as grazing management, improving soil fertility and the use of improved pastures (Corsi et al., 2001). For instance, in intensive Brazilian tropical pastures Gimenes et al. (2011) reported LWG of 1.0, 0.8, 0.4 and 0.3 kg/day during summer (wet season), autumn, winter (dry season) and spring respectively. The values of LWG reported by Gimenes et al. (2011) are in agreement with the average LWG of 0.2 and 0.8 kg/day for non-supplemented cattle observed in a compilation of Brazilian experiments during the dry and wet season respectively (Dórea, 2010). The CLW and productivity per area observed by Gimenes et al. (2011) ranged from 150 to 130 kg/head/year and from 886 to 697 kg LW/ha.year depending upon the different grazing managements adopted. In a subtropical area of USA (Florida), CP content of bahia grass declined from 120 to 60 g CP/kg DM while *in vitro* organic matter digestibility (OMD) declined from 55 to 35% between May to September over three consecutive years (Williams et al., 2002). In addition, the LWG of grazing heifers in this experiment was approximately 0.8 and 0.2 kg/day in May-June (wet season) and September-August (end wet season) respectively (Williams et al., 2002).

Despite significant differences in forage species and composition, grazing and cattle management and cattle genotypes across cattle production systems in the tropics, the influence of seasonal rainfall on cattle liveweight gain is similar. This variation in nutritional plane as influenced by seasonal conditions and its effect on cattle physiology and productivity is a common challenge for grazing cattle throughout the tropics. This thesis will examine the growth, morphologic and physiologic

parameters of cattle during and after periods of nutrient restriction, with a particular emphasis on the way in which bone (skeletal growth) responds to such changes in nutrient supply.

Chapter 2. Literature review: Nutritional effect on cattle growth

2.1 Introduction

This review will describe the definitions of terms present in the literature that are currently used to describe animal growth following a period of nutritional restriction. Then the physiological and morphological mechanisms that control endochondral ossification and bone turnover as well as the nutritional effect on the endocrine regulation of these processes are reviewed. Finally, it will focus on reviewing the main factors cited by the literature as possible determinants of cattle performance following a period of nutritional restriction.

2.2 Nutritional restrictions in beef cattle production in Northern Australia

Approximately 97% of the total cattle herd in Australia is located in pasture-based grazing systems at any one time (ALFA, 2015). The variability in pasture quantity and quality throughout the year and how producers manage this impacts on the productivity and profitability of the cattle industry. Generally the year is divided into a wet season and a dry season. Wet season pastures are typically more digestible with higher CP and mineral content compared to dry season pastures which are of lower quality, resulting in lower growth and recovery of body condition of cattle in the dry season. Liveweight gain in the wet season following a dry season is often faster than expected and this phenomenon is called compensatory growth in the cattle industry. The use of protein meal supplements during the dry season is a well-developed tool to attenuate the nutritional CP deficit that occurs (Poppi and McLennan, 2010). The adoption of this practice is limited due to the high cost of supplements and the possibility of erosion of any benefits of supplementation due to compensatory growth that may occur in the following wet season. The 2010 Pastoral Industry Survey of the Northern Territory (NT) reported that only 20% and 3% of properties supplemented weaners under 100 kg and between 100-150 kg LW respectively throughout the dry season (Cowley et al., 2015). Weaner pellets, copra meal and cottonseed meal were the most commonly used supplements provided to weaners in the NT. The adoption of protein meals as a supplement is unlikely to increase until the development of new and more affordable protein sources (e.g. algae grown on property) become a reality (Costa, 2012). Thus nutritional restriction (i.e. CP, metabolizable energy (ME) and minerals), especially during the dry season and its effects on the following wet season, is a reality that needs to be researched in order to achieve higher biological and economical efficiency in these production systems.

2.3 Protein and energy balance in ruminant nutrition

The balance of dietary protein and energy in ruminant nutrition are well known to have significant impact on rumen metabolism and animal performance (Poppi and McLennan, 1995; Moore et al., 1999). Studies with other species have also demonstrated that the balance between these two nutrients play a role in controlling some endocrine parameters such as the somatotropic axis and thyroid hormones (Clemmons and Underwood, 1991; Ramos et al., 2000; Passos et al., 2001).

The supply of rumen degradable protein (RDP) is essential to maintain the microbial activity in the rumen. The RDP fraction is degraded in the rumen and converted into ammonia, amino acids and peptides. Satter and Slyter (1974) suggested that maximum microbial protein production is achieved when rumen ammonia concentration is greater or equal to 50 mg ammonia N/L. Limiting supply of RDP leads to decreased dry matter digestibility, microbial growth efficiency and volatile fatty acids concentration (Griswold et al., 2003). However, when dietary protein supply is in excess of requirement there is an additional energy cost to metabolize the excess protein and to synthesize and excrete urea. For dairy cows, it has been estimated that each gram of N in excess increases heat production by 4.1 to 7.6 kcal, and decreases retained energy and milk gross energy by 4.2 to 6.6 kcal and 52 to 68 kcal, respectively (Reed et al., 2017). Moreover, losses of protein or incomplete net transfer occurs in scenarios where CP content of the diet exceeds 210 g of CP/kg of digestible organic matter (Poppi and McLennan, 1995). The regulation of protein and energy balance in ruminant nutrition have a greater level of difficulty in grazing scenarios where forage composition is greatly affected by environmental conditions and management. Supplementation can be a tool to manipulate protein and energy balance supply in grazing animals.

The selection of source and type of supplements needs to be based on the forage composition and on the target requirement. In a meta-analysis of Brazilian experiments Detmann et al. (2014) described that the efficiency of nitrogen utilization is linearly associated with the dietary content of digestible organic matter (DOM) and relative production of microbial crude protein in the rumen. Moreover, the data presented shows a significant quadratic response on forage intake when increasing the CP:DOM relationship via supplementation. Maximum forage intake was observed at 288 g CP/ kg DOM. According to Illius and Jessop (1996) an imbalance of the protein to energy ratio are presumed to constrain intake because of the build-up of the excess metabolite. For instance, the acetate clearance in adipose tissue depends on glucose supply to balance the NADPH and ATP requirements for triglyceride formation with the supply of these from acetate catabolism. In a low protein to energy

scenario where there is a shortage of gluconeogenic precursors, the glucose deficiency would lead to a rise in blood acetate and result in a negative feedback causing intake reductions.

This section outlines the main nutritional outcomes of protein and energy relationship imbalance in ruminants. Further aspects of dietary protein and energy balance on the endocrine parameters are covered in depth in section 2.12 of this literature review.

2.4 Compensatory and catch-up growth

This section outlines some of the terms used to describe compensatory and catch-up growth which are often interchanged. From this, a new definition is proposed which is used throughout the thesis but this is done to avoid the confusion and loose use of terms which appear in the literature.

After an animal has gone through a period of nutritional restriction it has been commonly observed that they will grow faster than counterparts who were not restricted when both are put on a high plane of nutrition. For cattle in the tropics this is most commonly observed as faster growth during the wet season after a period of nutritional restriction during the previous dry season (Kidd and McLennan, 1998). The practical importance of this is that animals may be supplemented during the dry season to maintain a high growth rate, but the subsequent compensatory growth of the restricted cattle may erode any benefits of supplementation and pastoralists may have wasted money on expensive supplements. This is not a new observation. The animal's capacity to reach the full size of its counterparts after periods of suppression of growth was reported a century ago by Osborne and Mendel (1915). They stated that the growth curve of rats after the period of suppression were comparable with growing rat of the same size and sex. The term compensatory growth was introduced by Bohman (1955) to describe the higher growth when cattle grazed summer pasture after wintering on low quality hay. Tanner (1963) demonstrated examples where weight, length and skeletal maturation retarded in humans due to nutritional or illness disorders were overcome after correction of each restriction factor. He named the faster growth rate during the recovery phase as catch-up growth.

Ryan (1990) classified the different responses as complete, partial and no compensation according to the growth of recovering animals. Complete compensation is stated as animals that have increased growth rates maintained long enough to achieve the same weight for age as not restricted animals. Partial compensation occurs when animals increase growth rate but do not attain the same weight for

age as those not restricted. No compensation response defines animals which do not show a difference in growth rate during re-alimentation. Jobling (2010) proposed a new nomenclature to classify the different growth curves obtained after restoration of “optimum” conditions. He considered catch-up growth the convergence of growth trajectories of animals that have different growth histories. Compensatory growth was defined as the animal’s capacity to growth faster than unrestricted cohorts, after a period of reduced growth resulting from reduced food availability or some unfavourable environmental perturbation. During this phenomenon, growth is more rapid than that of size-matched (in terms of either mass or length) animals that have not experienced any growth depression. The nomenclature proposed by Jobling (2010) is more specific and completes the previous definitions proposed by Ryan (1990). However, this nomenclature is based on the growth path of tissues (i.e. muscle or bone) and does not take into account the physiological and behavioural responses observed during these phenomena. Another problem that arises from this terminology is that, in order to match animals at a given LW or size, the comparison is made between different points in time. Thus, it is necessary that the experimental animals be subjected to the same nutritional and environmental (or any other factor that may affect growth) conditions during the whole experimental period. This is possible under strict experimental conditions but unrealistic when analysing real-word scenarios as the conditions cannot be met under seasonal variation in the environment and pasture.

There is evidence in the literature that cattle can have higher LWG after a period of nutrient restriction compared with unrestricted counterparts when compared within the same LW range (Ryan et al., 1993a). In contrast there is limited evidence that cattle or other mammals can have higher rates of bone elongation after a period of nutrient restriction compared with size matched unrestricted counterparts. However in many situations, restricted animals are able to grow faster than unrestricted animals at the same age and reach full mature skeletal size and LW but at a later point in time (Lawrence and Pearce, 1964b). The terms compensatory and catch-up growth have been used synonymously on some occasions and this may contribute to some of the confusion surrounding this topic (Blum et al., 1985; Ryan, 1990; Yambayamba and Price, 1991b). Predominantly compensatory growth has been used to describe changes in LW (Fox, 1970; Hornick et al., 2000; Keogh et al., 2015) whereas catch-up growth has often been used to describe the changes related to bone growth (Boersma and Wit, 1997; Gafni et al., 2001). The original papers, which first utilized these terms, were both describing the faster growth rate of previously growth impaired individuals when compared to aged matched unrestricted counterparts (Bohman, 1955; Tanner, 1963). Tanner (1963) provides the first hypothesis to explain the process whereby a previously growth retarded animal can achieve the same mature size as an unrestricted counterpart; he also named the intersection of two different

growth path trajectories as catch-up. From this collation of studies, it is apparent that compensatory and catch-up growth are phenomena that share a common origin and the difference between the two relate primarily to the main variable used to assess growth.

The medical fraternity utilise two additional terms to describe the response of individuals that suffer from nutritional disorders. An individual is considered stunted when their height is below two standard deviation from median ('normal') height-for-age of the reference population (WHO, 1986). The term describes the effect of slower skeletal growth rather than a permanent effect on height and stunted individuals may achieve normal height for age if catch-up growth does occur. Wasting is described as a deficit in weight of soft tissue for a given individual compared to what would be expected for a person of the same height (i.e. weight-for-height), which can be a result of low LW or LW loss. The occurrence of stunting and wasting are commonly associated though the time necessary to recover from it differs greatly in response to restoration of adequate nourishment. For instance, Damen et al. (1994) showed that children with celiac disease recovered their weight-for-height within 3 months of adopting a gluten-free diet while it took 2 to 3 years to return to a normal height-for-age. Ashworth (1969) reported that children suffering from protein-calorie malnutrition (stunted and wasted) had accelerated rate of weight gain and intake at the beginning of a recovery period until they reached their normal weight-for-height. After patients reached normal weight-for-height, growth was still greater compared to children at the same age but similar to a children of that height and weight. These descriptions are very similar to the pattern observed in cattle following a period of nutritional restriction although, there is more information describing the pattern of LWG than skeletal growth following a period of nutritional restriction in cattle. In addition, descriptions such as wasting and stunting are not widely used in agriculture but similar concepts are used. A practical example of this is when a producer would rather select an animal with a bigger frame size but with a low body condition score (BCS) than an animal in similar LW but greater BCS to be finished in a feedlot. The assumption here is that the animal in a lower BCS will have a greater LWG compared to the animal with greater BCS as it attempts to return to a normal growth trajectory as quickly as possible, as described above for humans.

The relationship between height and LW in cattle undergoing nutrient restriction and compensatory growth has not been extensively investigated before. The difference between a low LW for a given height or a low height for a given age after a period of nutritional restriction in cattle may be one explanation for the differing interpretation of results regarding compensatory growth in cattle. In this thesis compensatory growth will be described as the process whereby an animal undergoes increased

LWG following a period of nutritional restriction until it reaches the normal weight-to-height relationship. During compensatory growth animals may or may not achieve higher LWG than size matched (i.e. LW or height) unrestricted counterparts. In this thesis catch-up growth will be described as the faster skeleton elongation rate exhibited when previously restricted animals are compared to unrestricted counterparts at the same chronological age. This is assumed to be the process whereby an animal increases its height-for-age relationship.

Another point of debate is related to what cohort of animals the restricted animals should be compared when considering whether growth was compensatory, catch-up or otherwise. The majority of experiments in this area are divided into two phases. During the first phase the control group usually receives *ad libitum* access to a high quality diet and the restricted group would receive a set proportion of the intake of the control group or would receive a similar diet, also *ad libitum,* but with a reduced amount of a specific nutrient (e.g. energy, protein, minerals or vitamins). During the second phase the previously restricted group is normally offered the same *ad libitum* diet as the control group for a given period of time, usually named as recovery, re-alimentation or compensatory period. Interpretation of results is based on a comparison of growth rates or cumulative growth exhibited during the recovery phase to that achieved by unrestricted cohorts. The usual critique to these comparisons is related to the size dependence nature of growth rates since growth is related to body size (Nicieza and Álvarez, 2009). This critique may or may not be valid depending upon what is considered to be compensatory growth in the first place.

This discussion has outlined the various methods by which compensatory and catch-up growth may be defined. For the reasons discussed previously, catch-up growth will refer to a previously restricted animal which exhibits faster bone elongation when compared to age matched unrestricted counterpart at the same given point in time; i.e. it will refer to skeletal growth. Compensatory growth will refer to faster growth rate (LWG) of any soft tissue in the body (i.e. muscle, fat, organs or whole body) when compared at the same given time with that of aged matched unrestricted counterparts; i.e. it will refer to LWG. This implies that animals follow a growth curve over their lifetime of a defined weight–to-height relationship and any deviation from this as a result of nutritional or environmental restriction will result in the animal gaining LW faster when conditions permit as it seeks to return to the normal weight–to-height relationship, whereupon it will follow the normal LW and height change. The intersection of two different growth trajectories of any variable (e.g. liveweight, height) will be considered complete catch-up of the variable measured. Therefore catch-up and compensatory growth

describe rates of bone and LW growth respectively whereas the term complete catch-up refers to previously restricted animals reaching the same body size or liveweight than unrestricted counterpart.

2.5 Regulation of body growth after a period of growth restriction

This section describes the main theories developed to explain the physiological mechanism that controls growth following a period of growth suppression, including the response of bone elongation within these models.

The first hypothesis to explain the mechanism whereby a previously restricted animal could grow faster than an unrestricted counterpart was proposed by Tanner (1963). This explanation involves a central nervous system mechanism, “sizostat”, which recognizes the actual body size and the difference between it and an age-matched target size. The difference, between actual and target size, would be determined by the concentration of a circulatory factor released by growing organs. The difference in concentration would be detected by the central nervous system, which in turn releases another stimulating factor and enhances growth rate accordingly. Tanner’s neuroendocrine hypothesis was challenged thirty-one years later by the delayed growth plate senescence hypothesis described by Baron et al. (1994). This new proposal was developed analysing rabbits which had one of their tibia growth plate sides slowed by glucocorticoid application (dexamethasone) over 4 weeks. Once the infusion treatment was over, the treated growth plate grew faster than the opposite control tibia side. Since the faster growth rate was only observed on the side that had been slowed by dexamethasone treatment, it demonstrated that the neuroendocrine hypothesis would not be able to explain this local effect.

Bone elongation rate is a function of the rate of chondrocyte proliferation, terminal size of hypertrophic chondrocyte and rate of cartilage matrix secretion (Hunziker, 1994). In rats, reduction in both chondrocyte proliferation and hypertrophic expansion is related with reduced rates of bone elongation. Evidence suggests that factors which slow bone growth also slow growth plate senescence. Growth plate senescence is defined as the progressive decrease in bone growth rate which is accompanied by the structural changes observed at the growth plate level [e.g. decrease in size of resting (RZ), proliferative (PZ) and hypertrophic zones (HZ)] during this process. In this sense, senescence seems to be a factor of growth itself with a possible cell-cycle counting mechanism regulated by resting chondrocytes. Thus, increased growth rate after removal of restricting factors could be explained by the presence of a less mature growth plate when compared to age matched

counterparts (Gafni et al., 2001). The delayed growth plate senescence theory suggests a mechanism acting locally at the growth plate although it has long been observed that animals tend to maintain proportional growth (i.e. allometric growth) (Huxley and Teissier, 1936). The control of growth of distinct organs to maintain a relationship that fits its body has recently been more clarified. Lui et al. (2010) have identified a selection of genes that are down-regulated in a similar way across a number of different tissues including lungs, kidney and liver of juvenile rats during growth deceleration with increasing age. A similar response was also observed when a tryptophan-deficient diet was offered to newborn mice (Forcinito et al., 2011). The authors noticed that mice fed the restricted diet delayed the genetic program, of the previously described organs, when compared to same age cohorts. This suggests that this genetic mechanism acts in a negative feedback loop where growth deceleration is controlled by a multi-organ genetic program, which down-regulates a large set of growth-promoting genes. However it is unknown if this program also regulates other organs and if it exists at different local levels of control.

2.6 Catch-up growth

The term catch-up growth was used by Tanner (1963) to describe the process whereby humans could accelerate growth rates after a period of malnutrition or illness compared to unrestricted counterparts at the same chronological age. The main variable utilized in this work to describe growth was height (bone elongation) of individuals and since then the term catch-up growth has been mostly adopted for this purpose (Baron et al., 1994; Gafni et al., 2001; Cornelia et al., 2003; Emons et al., 2005; Marino et al., 2008; Pando et al., 2014; Gat-Yablonski and Phillip, 2015). As described above, some authors have used this term synonymously with compensatory growth (Yambayamba and Price, 1991a) while others describe it as a different phenomenon (Jobling, 2010). In this thesis the term will be utilized to describe a faster rate of bone growth following a period of bone growth restriction when compared to unrestricted age-matched counterparts. The review of catch-up growth presented below is structured on the methodology used to impose the growth restriction.

2.6.1. *Non-nutritional catch-up growth*

The observation that catch-up growth could be a phenomenon intrinsic to and regulated locally by the growth plate was first discussed by Baron et al. (1994). They observed that infusing dexamethasone into specific growth plates of rabbits decreased bone growth rates, and growth of these treated growth plates increased over untreated growth plates of the same animal after the dexamethasone treatment stopped. These observations led to the conclusion that the neuroendocrine

hypothesis proposed by Tanner (1963) was not able to explain the individual changes observed in these growth plates. Systemic dexamethasone treatment of New Zealand white rabbits for 5 weeks reduced liveweight, total femur length, the rate of proliferation of chondrocytes and height of the PZ and HZ when compared to non-treated controls (Gafni et al., 2001). Two days after dexamethasone treatment was stopped growth plates of treated rabbits showed higher growth, higher proliferation rate and higher PZ and HZs than control animals after two days of interruption of the treatment. Liveweight also showed some degree of recovery but remained less than control animals while there were no differences in femur length by the end of the experiment.

Marino et al. (2008) induced hypothyroidism in newborn rats by including propylthiouracil (PTU) in the drinking water for 8 weeks. The treatment led to a reduction in concentration of T4 and IGF-1 in plasma and reduced LW, tibia length, and heart, liver, and kidney mass. In addition, hypothyroid rats had a slower rate of chondrocyte proliferation, less proliferative chondrocytes per column and reduced overall growth plate height compared to the control rats. The authors reported a possible reduction in feed intake and these effects could be a combination of thyroid hormone deficiency as well as malnutrition. Once the treatment was discontinued, growth rates of hypothyroid rats was similar to that of control rats 5 weeks earlier. Increased bone growth during catch-up growth was associated with increased abundance of chondroadherin, osteoprotegerin, secreted frizzled-related protein 4 and nuclear protein-1 mRNA to levels similar to control rats at a younger age. Despite the increase in bone elongation after stopping PTU treatment, catch-up was not complete. At the end of the experimental period hypothyroid rats remained lighter with shorter tibia and tails than control rats indicative of incomplete catch-up. The authors suggested a possible mismatch between the rate of proliferation in the resting zone with proliferation in the PZ. In this scenario, if a greater inhibitive effect occurs in the PZ than in the resting zone, it would result in less growth leading to incomplete catch-up (i.e. permanent stunting). There is no doubt that the skeleton can increase growth rates after a period of growth inhibition compared to age-matched unrestricted cohorts. Maintenance of increased growth rates in order to achieve complete catch-up seems to be dependent on how growth restriction affects the different zones of the growth plate. Nevertheless, some of the experimental evidence such as Gafni et al. (2001) and Marino et al. (2008) are difficult to differentiate from the nutritional effect due to the increase in food intake after the interruption of drugs treatment. On the other hand, Baron et al. (1994) provides solid evidence that at least part of the control of long bone growth occurs locally. The possibility of a local and systemic mechanism occurring simultaneously is high, though it has not been fully demonstrated.

2.6.2. *Nutritional catch-up growth*

Nutritional restriction is considered the leading cause of growth failure in children (Gat-Yablonski and Phillip, 2015). In 2015, UNICEF, World Health Organization (WHO) and World Bank (WB) estimated that 156 million children (23.2% of the population of children younger than 5 years) younger than 5 years old were stunted (height below minus two standard deviations from the median height or age of the reference population) (WHO, 1986; UNICEF-WHO-WB, 2016). In order to understand the effect of nutrition on bone elongation and catch-up growth, a series of studies have been made by imposing a 10 day restriction on growing rodents to a 60% food intake relative to a control group followed by an *ad libitum* phase (Gat-Yablonski, 2004; Even-Zohar et al., 2008; Gat-Yablonski et al., 2008; Pando et al., 2012; Pando et al., 2014).

In summary these studies have reported that during feed restriction there is a significant reduction in LWG and bone elongation. The height and number of chondrocytes within proliferative and hypertrophic phases are reduced. These were accompanied by a reduction in IGF-1R, measured as western immunoblot, at the growth plate as well as a decrease in the concentration of circulating IGF-1. In addition, during the energy restriction phase there was a decrease in HIF1α and miR-140-3p as well as an increase in SIRT1 and SIRT6 miRNA expression. These findings are important because they suggest a link (Figure 2-1) whereby nutrition affects epigenetic mechanisms that are known to regulate aging as well as cell proliferation, senescence and apoptosis via several transcription factors that govern metabolism and endocrine signaling (Gat-Yablonski and Phillip, 2015).

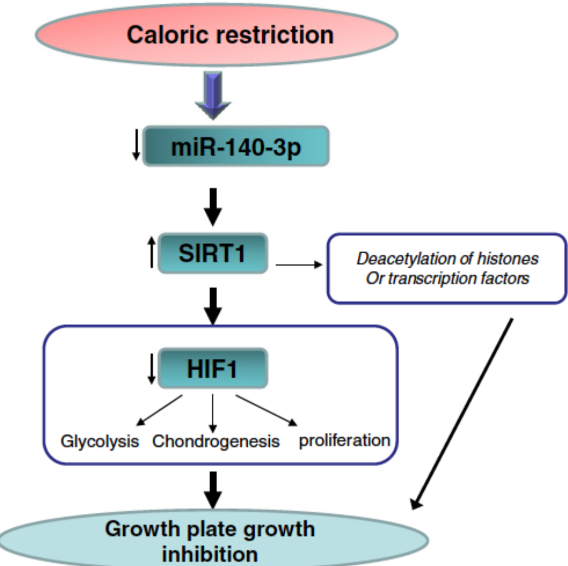


Figure 2-1 A suggested model of the effect of calorie restriction on growth plate. Reproduced from Pando et al. (2012)

As well as the changes at the growth plate level, it was also found that rats restricted to 60% of food intake of control group (described above) had reductions in trabecular bone volume and number and increase in trabecular separation. The mechanical analysis showed that bones of restricted rats were more susceptible to fractures than unrestricted counterparts. Upon re-alimentation during catch-up growth these process were rapidly (1 day) reverted and a substantial increase in bone elongation at the growth plate and trabecular bone formation was observed. Interestingly, at the first day of re-alimentation the load to fracture bone in the group undergoing catch-up growth was lower than when they were undergoing nutrient restriction which indicates a transitory period where individuals undergoing catch-up growth may be more susceptible to fractures than when they were nutrient restricted. After 26 days of re-alimentation, there were no differences in trabecular bone parameters (i.e. architectural and mechanical) between rats undergoing catch-up growth and their unrestricted counterparts, although catch-up was never complete in any of these studies with LW and humerus length always less in restricted rats compared to unrestricted counterparts at the end of the 10 or 26-day recovery period.

2.7 Bone and muscle development

The previous sections have described the cellular and hormonal changes that accompany bone growth and the effect of nutrition on these parameters. The next sections describe the allometric changes in LW and skeletal dimensions that occur under normal and perturbed growth and development.

Yayha and Millward (1994) studied the relationship of muscle and bone growth in rats and found that the muscle weight/bone length relationship was reduced by 41% at the end of 21 days of protein restriction when compared to an unrestricted control group. Upon re-alimentation, the muscle weight to bone length relationship was restored within 10 days and was not different to the control group. The authors concluded that muscle growth is a function of bone elongation and a permissive dietary substrate supply. Passive stretching is believed to be the mechanism by which bone elongation affects the length and therefore weight of skeletal-muscle (Holly et al., 1980; Young and Sykes, 1987; Goldspink et al., 1995). Passive stretch on skeletal-muscle leads to an increase in number of sarcomeres (Williams and Goldspink, 1973) and cross-sectional area of myofibres (Coutinho et al., 2004). In addition, it has been shown that muscles subjected to stretch stimulus had an increase in muscle weight, which was accompanied with a more significant increase in the rate of protein synthesis compared to protein breakdown (Goldspink, 1977). Interestingly, Brown et al. (1990), when studying the effect of stretch stimulus in nutritionally restricted chickens of different ages, observed

that the passive stretch stimulus had different effects on muscle depending upon the birds age. Younger animals (7-week-old) showed an increase in muscle mass whereas older animals (28-months-old) did not respond to the stimulus. However, the different age groups were also from different genotype of birds which could have influenced the results. In cattle, Lehnert et al. (2006) imposed a liveweight loss diet (-14% of initial liveweight of 205 kg) over 120 days and observed that by the end of the nutritional restriction period there was a severe reduction in cross-sectional area of myofibers type 2X (69% reduction), type 2A (64% reduction) and type 1 (48% reduction) when compared to fast growing control group (LWG > 0.6 kg/day). After 109 days of re-alimentation the differences in cross-sectional area observed at the end of the nutritionally restricted period were reduced but myofiber areas of previously restricted cattle were still smaller when compared to fast growing cattle. However, Yambayamba and Price (1991a) found no difference in cross-sectional area or proportion of fibre type of previously restricted cattle when cattle were compared at the same liveweight of unrestricted controls. These results indicate that muscle increases in cross-sectional area and length responding to stretch stimulus but this process may be affected by the degree of maturity of the animal as well as the nutritional level. The process whereby skeletal-muscle increases in size and weight after a period of nutritional restriction remains unclear. However, data from different studies that compared carcass composition of cattle following a period of nutritional restriction showed no difference when the comparisons are made at the same slaughter liveweight (Lawrence and Pearce, 1964b; Morgan, 1972; Yambayamba and Price, 1991b). This seems to indicate that during compensatory growth muscle fibres increase in cross-sectional area as well as length and are comparable to unrestricted animals of a similar body size.

Hooper (1978) suggested that bone elongation might be the “pacemaker” for muscle growth. The effect of bone elongation on muscle length is mainly derived from a stretch stimulus, since they are attached, and this concept was further supported by Holly et al. (1980) and Swatland (1982). This concept led McLennan and Poppi (2011) to suggest a new approach to exploit compensatory growth in cattle by stimulating bone elongation during periods of under-nutrition (e.g. in the dry season in northern Australia) potentially resulting in a larger frame size for the muscle to rapidly realiment towards normal allometry when the quality and quantity of nutrients is increased (e.g. in the wet season in northern Australia).

2.8 Factors affecting compensatory growth in ruminants

Several factors have been identified as potential sources of the variation often reported in relation to compensatory growth after a period of nutrient restriction cattle (Wilson and Osbourn, 1960; Ryan, 1990; Hornick et al., 2000) and these are discussed below.

2.8.1. *Age at restriction*

The degree of maturity at the start of food restriction is suggested in the literature as one of the factors that can affect the rate and extent of compensatory growth and final size of the animal (Coleman and Evans, 1986; Morgan, 1972). There is a consensus that restriction in early stages of development will be more difficult fully compensate from than restrictions at a more advanced age. However, there is no agreement about the specific age or stage of development at which a feed restriction will prohibit the ability to fully compensate.

Berge (1971) compiled data from 74 experiments with cattle and classified cattle on the basis of their age at which feed restriction commenced (younger or older than 6 months old) and on the severity of the feed restriction imposed (percentage of liveweight difference between restricted and control cattle). The results indicate that older calves demonstrated greater increase in LWG during recovery than younger calves. Beyond that, the data indicates that LWG during recovery also increases with increasing severity of the feed restriction imposed. On average cattle exposed to feed restriction at a young age required 14 to 18 months to compensate 70 to 80% of the LW of their unrestricted counterparts while cattle exposed to feed restriction at an older age only needed 4 to 7 months for 70 to 80% compensation. Tudor and O'Rourke (1980) restricted calves to liveweight maintenance (mean LWG 0.05 kg/day) from 4 to 200 days of age and were offered either a feedlot diet (lucerne chaff/sorghum grain (1:1)) or improved pastures until reaching slaughter liveweight. In addition to these, a control group was fed a high quality diet from day 4 to 200, which resulted in an average LWG of 0.7 kg/day. No compensatory growth was observed in the group offered the feedlot diet following restriction when compared to control cattle offered the same diet. Meanwhile previously restricted cattle grew faster (0.5 *vs* 0.36 kg/day) than unrestricted controls in grazing conditions. The carcass analysis of these cattle showed that weight of visceral components, muscles and bones were not affected by nutritional restriction at early life (Tudor et al., 1980). In addition, animals finished in pasture were taller (wither and pelvis height) than animals finished in the feedlot. However, the pasture fed animals took approximately 300 days more to achieve the liveweight necessary for slaughter.

The effect of age on compensatory response is an important point to be taken into account when defining nutritional strategies for weaners in northern Australia. Early weaning is a recommended strategy to decrease nutrient requirement of dams and improve re-conception rates (Tyler, 2012). An early-weaned calf within this production system is typically between 3-4 months of age and 100 to 150 kg. Thus, a better understanding of the relationship between age at restriction and degree of compensation is needed. Despite the apparent overall agreement that restrictions in early age will require more time to achieve complete catch-up in LW, no specific physiological mechanism exists to explain this response.

2.8.2. *Severity of restriction*

The degree of LWG reduction in comparison to unrestricted animals is the parameter used to describe the severity of restriction. It may vary from severe restriction (i.e. LW loss), moderate restriction (i.e LW maintenance) or mild restriction (i.e. reduced LWG) (Wilson and Osbourn, 1960).

Typically the greater the severity of the restriction the greater the rate of compensation during the recovery period. A linear increase in LWG was reported for yearling steers during the recovery period as the severity of the prior restriction increased (LWG of 0.50, 0.38 and 0.28 kg/day) (Lewis et al. 1990). Each additional 0.1 kg/day LWG during the restriction period reduced LWG during the recovery period by 0.08 kg/day resulting in similar LW at the end of the recovery period regardless of LWG during the restriction period. Tolla et al. (2003) reported a linear increase in recovery index (39, 42 and 60%) after a 103 day recovery period in steers restricted to lose 15, 20 or 25% of LW during a 91 day restriction period. Similarly, Kidd and McLennan (1998) demonstrated that cattle in northern Australia only demonstrated substantial increment in wet season LWG when LW loss during the dry season was greater than 10%. These results demonstrate that increasing the severity of nutrient, and hence, growth restriction would lead to increased growth rates during the recovery period. However, the physiological mechanisms of such response are not clear.

Recovery index is one parameter proposed by Wilson and Osbourn (1960) to compare the degree of recovery after a period of restricted growth. It is calculated by following equation:

Equation 2-1: $\text{Recovery index (\%)}: \frac{\text{LW.Dif.1} - \text{LW.Dif.2}}{\text{LW.Dif.1}} \text{ x } 100$

Where LW.Dif.1 is the LW difference between control and restricted group at the end of the restricted period and LW.Dif.2 is the LW difference between the same groups at the end of the compensatory (recovery) period. It is important to note that the recovery index will always be affected by the rate of LWG by controls and the duration of the recovery (compensatory) period, which may limit the use of this index to compare results across different studies.

2.8.3. *Duration of restriction*

Ryan (1990) stated that the severity of restriction is associated with persistence of compensation while increasing the duration of feed restriction (without altering severity) is likely result in higher growth rates during re-alimentation. However, no relationship between the period of nutritional restriction and LWG during compensation was found by Levy et al. (1971) when comparing periods of 30, 75 and 120d; or Hornick et al. (1998) when restricting feed for 115, 239 and 411d. Allden (1968) also investigated the effect of duration of the nutritional restriction in lambs. The experimental design included two periods of restriction (200 and 400 days) and also two control groups (Figure 2-2). The first control group (Group 1) received *ad libitum* feeding from the start of the experiment, whereas the second group (Group 5) were composed of younger lambs and were offered the *ad libitum* diet at the same time and liveweight as the restricted groups. It was observed that during the recovery period, the restricted groups grew at the same rate (in terms of liveweight and body length) and had same dry matter intake (DMI) of the younger unrestricted lambs (Group 5). They also observed that the two control groups (Group 1 and 5) had different performance, demonstrating that comparison between two growth paths in different points in time can be affected by environmental factors and wrongly interpreted. For instance, Peters et al. (1980) have showed an increase in voluntary intake (5.2 vs 4.8 kg DM/head.day) and LWG when increasing artificially the daylight to 16 hours/day compared to heifers under natural daylight of approximately 10 hours/day during the winter in Michigan, USA.

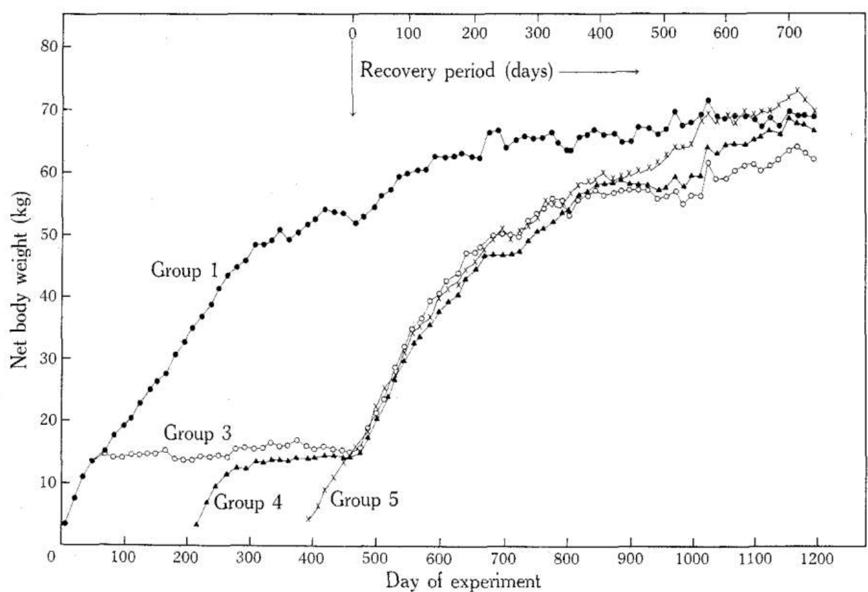


Figure 2-2 Liveweight change of lambs nutritionally restricted[1] for 200 or 400 days (Groups 3 and 4) following a period of *ad libitum* feeding. Groups 1 and 5 received the same *ad libitum* diet throughout the experiment. Reproduced from (Allden, 1968). [1]Restricted lambs received the same diet as control groups (lucerne hay and barley grain 2:3 w/w) but in different quantities in order to promote the differences in growth path.

The results obtained by Allden (1968) which are exhibited in Figure 2-2 do not support the observation made by Ryan (1990) in relation to the effect of the length of restriction. Increasing the length of nutritional restriction without altering the severity does not result in longer periods of compensation. Moreover, it demonstrates the importance of the reference (i.e. controls) when determining compensatory growth.

2.9 Food intake during compensatory growth

Ryan (1990) suggested compensatory growth was only possible if there was more energy available to the animal or if there was a change in the way the energy was partitioned in the animal. Some studies have suggested changes in maintenance energy requirements (Sainz et al., 1995), feed conversion (Drori et al., 1974), tissue deposition pattern (Levy et al., 1971) and feed intake (Hornick et al., 1998).

The most common feature observed in a wide range of species following a period of nutrient restriction is an increase in food intake (Wilson and Osbourn, 1960; Ryan et al., 1993a; Sainz et al., 1995; Skalski et al., 2005) although comparisons of animals at different LW are likely to be biased even when intake is expressed relative to metabolic weight. The relationship between voluntary food

intake and LW from birth to maturity is not common in the literature. Factors such as previous nutritional history, climate fluctuations, digestibility of the diet and physiological state of the animal can potentially affect this relationship. Allden (1968) demonstrated this relationship where intake of weaned lambs increased rapidly from 15 to 25 kg LW and then decreased until maturity (55 kg LW at 1250 days of age) when it stabilized. This change in intake with maturity was consistent across all lambs except those that underwent a more severe restriction (LW loss) which had much higher intake compared to other lambs who maintained or gained LW at a comparable LW (Figure 2-8). Interestingly, these lambs which experienced a change in intake pattern and LW loss also had longer leg length and chest depth than other lambs of a similar LW (Allden 1968). This observation is in agreement with Ashworth (1969) who reported increased LWG and intake of children recovering from nutritional disorders until they achieved the expected weight-for-height.

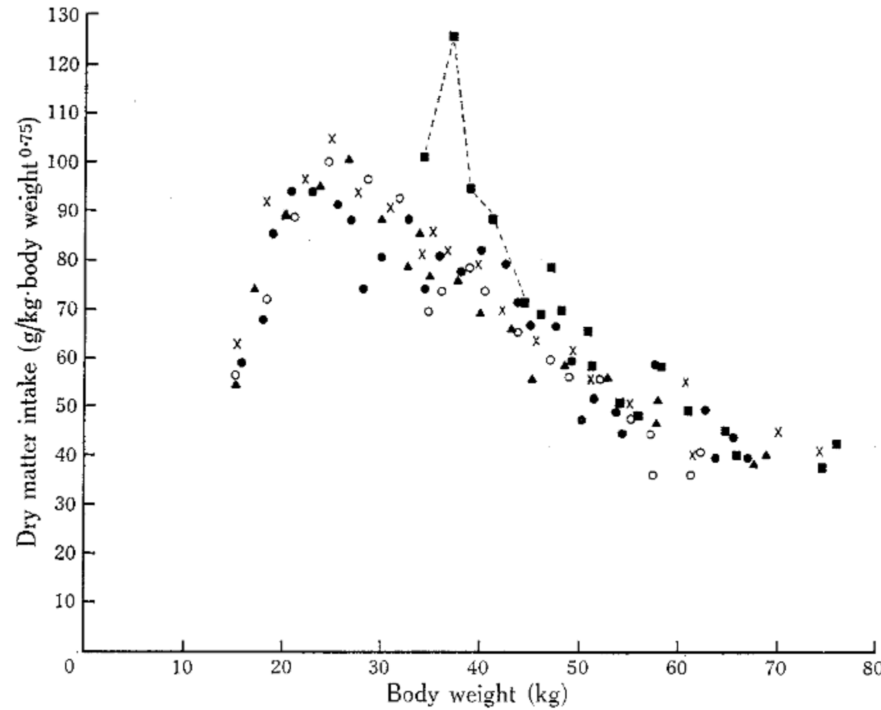


Figure 2-3 Relationship of dry matter intake per unit of metabolic liveweight (liveweight $^{0.75}$) to liveweight of lambs from weaning to maturity. Values represent 56-day means for each treatment group. Reproduced from Allden (1968). Dashed line, solid squares indicate variation of intake of lambs severely restricted during early growth

The mechanism by which food intake responds to differences in the weight-for-height relationship is not defined. However, works on Zucker rats have suggested that appetite control is linked with the impetus for the protein deposition and the rates of retention of lipid and energy appear to be have no effect on voluntary food intake (Radcliffe and Webster, 1976, 1979; Webster, 1993). In addition, research with ruminants have shown that lambs are able to select their diets in order to maximize

growth and meet their crude protein requirements. It also demonstrated that sheep and lamb would adapt the protein intake accordantly to the physiological state such as pregnancy and maturity (Kyriazakis and Oldham, 1993; Cooper et al., 1994). These evidences may suggests that the drive of voluntary intake during compensatory growth is associated with the potential for protein deposition after a period of restricted nutrition.

When dry matter intake expressed per kilo of LW is plotted against time it is often observed that compensating lambs have higher intake when compared to unrestricted animals. Ryan et al. (1993a) showed that previously restricted steers and sheep took 100 days before intake was higher than that of unrestricted counterparts. Steers maintained a higher intake for longer compared to sheep (140 *vs* 35 days; Ryan et al., 1993) and this was attributed to the higher capacity for steers to show compensatory growth for an extended period of time compared to sheep. It is possible that the increased intake following a period of growth restriction may be partially explained by the differences in body size of the groups at the beginning of the compensatory phase since there is clear decrease in voluntary intake as animals approach mature size (Figure 2-3). However, as was demonstrated by Allden (1968), dry matter intake of compensating animals is often higher than controls when compared at the same LW during a transitory period. It's also important to notice that the unit utilized to express intake can lead to divergent interpretations. For instance, Blaxter et al. (1982) showed that continuous growing sheep did not show any change in voluntary intake (i.e. kg of DM/day) over time when comparing the average of 6 months periods over 4 years of experiment. Presumably due to the increase in liveweight over the experiment the authors would have found different result if the intake was expressed as a proportion of liveweight (i.e. g DM/ kg LW) or metabolic weight (i.e. g DM/ kg $LW^{0.75}$). The control of voluntary intake is complex and regulated by physical and metabolic controls and in this case it is difficult to categorically state whether increased growth is a result or cause of increased feed intake in ruminants undergoing compensatory gain (Forbes, 2007).

2.10 Longitudinal bone growth

The process by which a mammal increases in height is through elongation of long bones by endochondral ossification. The control of the whole process is complex and integrates local and systemic mechanisms acting together (Van der Eerden et al., 2003). Growth plate is the cartilage layer between the epiphyseal and metaphyseal distal ends of long bones (Figure 2-4). Endochondral ossification occurs at the growth plate and consists of the differentiation and apoptosis of the chondrocytes and mineralisation. The three stages of differentiation of chondrocytes form the

different zones of the growth plate. The resting zone is composed of stem-like chondrocytes that after differentiation enter into the proliferative zone (Nilsson et al., 2005). Chondrocytes at the proliferative zone undergo rapid cell division and extracellular matrix (ECM) protein production. At this stage ECM proteins are essential for the longitudinal disposition of the cells, which is critical for the formation of bones with elongated shape (Van der Eerden et al., 2003). At any given moment the proliferative chondrocytes differentiate into hypertrophic chondrocytes. At this stage chondrocytes undergo the maximum expansion and the final cell volume drives the rate of longitudinal growth (Breur et al., 1991). Hypertrophic chondrocytes release matrix vesicles which secrete calcium-phosphate, hydroxyapatite and matrix metalloproteinase resulting in the mineralization of the matrix and their surroundings (Abad et al., 2002; Van der Eerden et al., 2003). The extracellular matrix around the hypertrophic chondrocytes is then invaded by blood vessels and osteoblasts and the mineralized chondrocytes undergo apoptosis.

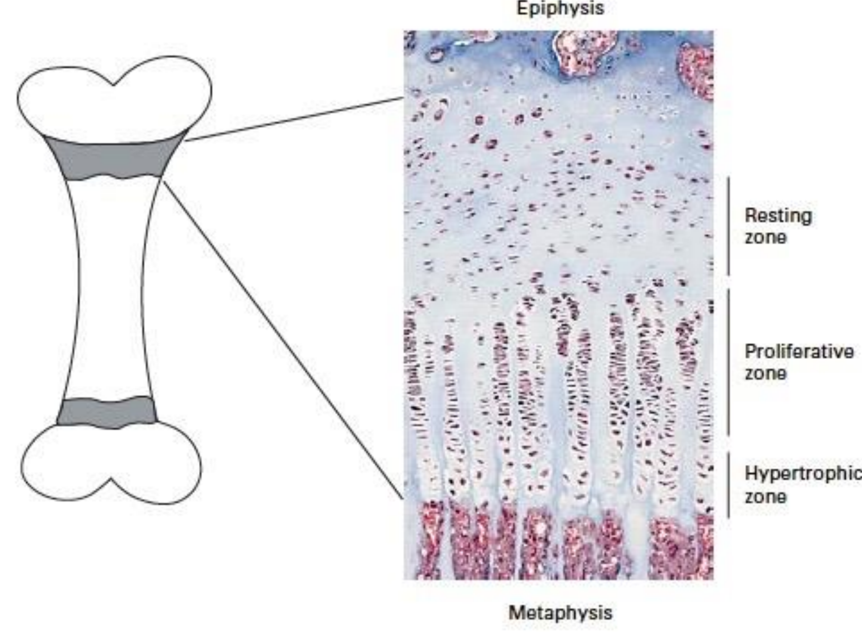


Figure 2-4 Representation of growth plate location and its histology image. Reproduced from: Nilsson et al. (2005)

Nutrition has a marked effect on endochondral ossification and, hence, bone elongation. The nutritional regulation of this process in not completely known but is believed to involve transcription factors, hormones, epigenetic mechanisms and microRNAs (Gat-Yablonski et al., 2013). The most relevant of these controlling factors will be discussed in the following sections of this review.

2.11 Bone turnover

Bone remodelling or turnover is the process whereby the skeleton builds bone structure, resorbs some of it and then rebuilds itself. In ruminants, the effect of nutrition on the remodelling process is mostly associated with mineral deficiency. However, studies with different species have demonstrated that also protein and energy restriction can affect bone turnover and lead to changes in bone structure (Hamrick et al., 2008; Sukumar et al., 2011). Changes in bone turnover can directly affect the mineral requirements of animals, specifically calcium and phosphorus. Therefore, understanding the effect of protein and energy restriction on bone remodelling is an important area to be addressed in animal nutrition.

Bone remodelling is composed of bone resorption by the osteoclast cells followed by bone formation by osteoblast cells. This process is important to maintain the stability and integrity of the skeleton (Proff and Römer, 2009). It occurs in small packs of cells called basic multicellular unit (BMUs) which comprises osteoclasts and osteoblasts (Hill and Orth, 1998). In short, the process of bone turnover involves the following sequential steps: resorption, reversal and formation (Figure 2-5). The control of these steps includes several hormones such as parathyroid hormone (PTH), 1,25-Dihydroxyvitamin D_3, growth hormone (GH), insulin, sex steroids, thyroid hormones and calcitonin as well as cytokines and growth factors (IGF-1 and -2, transforming growth factor beta-1, bone morphogenetic protein, fibroblast growth factor, platelet-derived growth factor, tumour necrosis factor alpha and colony-stimulating factor).

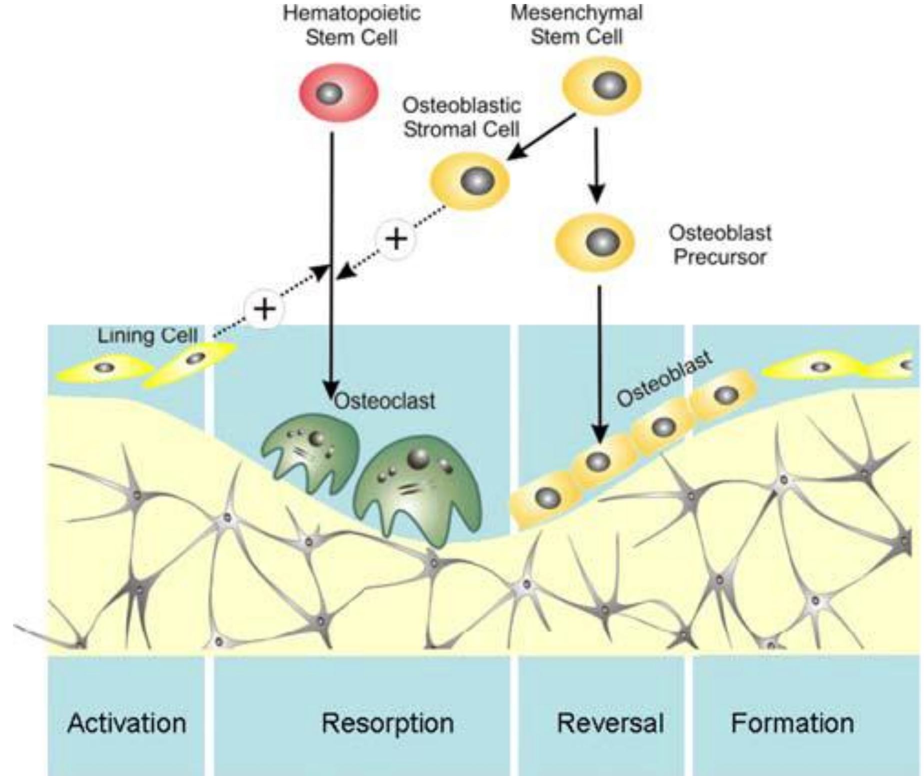


Figure 2-5 Overview of the bone remodelling (turnover) process. Reproduced from Proff and Römer (2009)

Initially osteoclast precursors cells are stimulated by expression of receptor activation of nuclear factor kappa-B ligand (RANKL) and monocyte-macrophage colony-stimulating factors (M-CSF) by bone lining cells, which binds to the RANK that exists as a surface receptor on the membrane of pre-osteoclasts (Boyce and Xing, 2008). The osteoclast progenitors differentiate into osteoclasts and start breaking down bone and liberating bone morphogenetic proteins (BMPs) and insulin-like growth factor II, which will then stimulate osteoblast cells (Proff and Römer, 2009). The transition from the resorption phase to formation is comprised by the reversal phase where there is a discontinuation of osteoclast action by apoptosis followed by the differentiation of osteoblast precursors into active osteoblasts (Hill and Orth, 1998). The active osteoblasts will then lay down new bone (i.e. collagen type I, osteocalcin, osteopontin, osteonectin, bone sialo protein and alkaline phosphatase) until the complete replacement of the bone that was previously degraded by osteoclasts (Proff and Römer, 2009).

The balance between bone formation and reabsorption will ultimately determine bone mineral density (BMD) and bone strength. Nutrition (i.e. protein, energy and minerals), physiological status and aging are known to affect this balance and promote bone loss or accretion (Mazess, 1982; Seibel, 2007). The skeleton is the biggest store of minerals (e.g. Ca and P) in the body and this remodelling process represents a significant way to recycle these minerals.

2.11.1. *Direct assessment of bone turnover*

Bone biopsies enable direct assessment of the balance between bone formation and resorption *in vivo* (Recker, 2013). In cattle, bone biopsies have been mostly used for the diagnosis of mineral deficiency such as Ca, P and Mg (Little, 1972; Read et al., 1986; Malafaia et al., 2017). There are currently two *in vivo* methodologies of bone biopsy being used in cattle namely, rib and coccygeal vertebrae biopsy. The most utilized is the rib biopsy which is performed at the 11th or 12th rib and consists on the removal of the outer cortical rib layer and provides a specimen that can be used for determination of mineral content, specific gravity, thickness and histopathological analysis (Little, 1972). A more recent variation of this methodology collects a larger diameter biopsy (25 *vs* 15 mm) than the original method with the outer, medullary cavity and inner cortical layers collected in-tact (Malafaia et al., 2017). Biopsies collected under the method of Malafaia et al. (2017) can also be utilized for densitometry by X-ray examination. However rib bone contains a much larger proportion of cortical

bone than trabecular bone. Trabecular bone represents only 20% of bone mass but it accounts for 80% of bone surface area. In addition, approximately a quarter of the total trabecular bone that undergoes remodelling every year opposed to only 2 to 3% of cortical bone (Swaminathan, 2001).

Coccygeal vertebrae biopsy is a more invasive method and consists on the surgical removal of the 9th coccygeal vertebrae (Cy9). This methodology has been used for determination of bone density as well as chemical composition (Esser et al., 2009). Efforts have being made to develop non-invasive techniques that correlate with direct measures from Cy9 biopsy. For instance, Keene et al. (2004) used radiographic photometry and dual-energy X-ray absorptiometry to predict mineral composition of samples, however the technique lacked the sensitivity to detect changes in mineral composition. Coates et al. (2016a) recently demonstrated that single photon absorptiometry effectively predicted bone mineral density of Cy9 ($r=0.92$ when compared to laboratory measurements of core ash density). The methodology proposed by Coates appears to provide a good non-invasive method to predict bone density however it does not provide a direct measure of osteoblast and osteoclast activity. In addition, bone density can decrease in the absence of a net mobilization of minerals. This situation is particularly common in growing animals under mineral deficiency where the dimensional size of a bone increases while density decreases (Coates et al., 2016b).

Thus, in order to have a better estimative of the remodelling process in growing cattle it seems necessary to develop a bone biopsy methodology that enables collection of a representative portion of trabecular bone where indicators of bone remodelling can be measured.

2.11.2. *Bone metabolic biomarkers*

An indirect way to assess bone turnover is by measuring components released during bone formation and resorption. These molecules are usually classified as formation or resorption markers. The following section of this thesis is to describe the most relevant biochemical and technical aspects of bone biomarkers which might be measured to assess bone remodelling.

2.11.2.1 Bone-specific alkaline phosphatase

Alkaline phosphatases (AP) are membrane isoenzymes encoded by different genes. Total alkaline phosphatase in the circulation is composed by several isoforms that can be produced in the liver, bone, intestine, spleen, kidney and placenta (Seibel, 2000). Bone-specific alkaline phosphatase (BAP) is a product of osteoblast activity and is released into the circulation (Yang and Grey, 2006). In adult humans, the proportion of AP in the serum is derived approximately 50% from liver and 50% bone.

The proportion of BAP is greater (up to 90%) in children and adolescents probably due to higher rates of bone growth (Van Hoof et al., 1990). Bone-specific AP immunoassays measure the concentration of BAP in the circulation however they typically show ~3-8% cross-reactivity with liver produced AP (Seibel, 2000). Nevertheless, the concentration of BAP in the plasma of humans shows a good correlation (r=0.663, $P<0.0005$) with bone mineral density (Tobiume et al., 1997). In Holstein cows studies have demonstrated BAP decreases with age (Figure 2-3; Sato et al., 2013), parity (Kurosaki et al., 2007) and as cows transitioned from parturition to lactation (Kim et al., 2010) which is similar to changes reported in humans (Van Hoof et al., 1990).

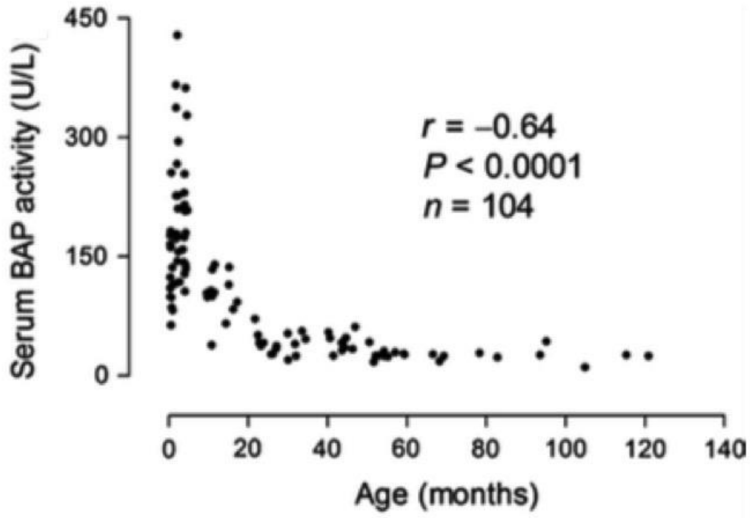


Figure 2-6 Relationship between age and serum bone-specific alkaline phosphatase (BAP) activity in non-peripartum Holstein dairy cows. r = correlation coefficient, n = number of animals. Reproduced from Sato et al. (2013).

The is no available information in the literature about the use of BAP as a bone marker to detect bone loss during nutritional restriction in cattle neither is there a validation of this methodology for comparing the concentration of BAP with structural changes in trabecular or compact bone.

2.11.2.2 Osteocalcin

Osteocalcin (OCN) is a hydroxyapatite-binding protein which represents the largest proportion (~15%) of the non-collagenous matrix proteins in bone (Hannon and Eastell, 2006). It is mostly expressed by osteoblasts but it also can be synthetized by odontoblasts and hypertrophic chondrocytes (Seibel, 2000; Hannon and Eastell, 2006). After being synthetized, most osteocalcin is incorporated into the non-collagenous matrix proteins while the remainder is released into the circulation

(Hauschka and Wians, 1989). Osteocalcin may also be released during bone resorption which lead Hannon and Eastell (2006) to suggest that osteocalcin may be a marker of bone turnover rather bone formation.

Strong relationships between OCN and bone formation rates were reported in patients undergoing haemodialysis (Ureńa et al., 1995) and patients with vertebral osteoporosis (Delmas et al., 1991). In contrast, there was no relationship between OCN concentration and histomorphometric measurements in patients suffering from cholestatic osteopenia (Guichelaar et al., 2002). Importantly, patients in this latter study had low bone volume, reduced bone formation rates and increased eroded surface indicative of bone loss.

In Holstein cows OCN concentration in the plasma decreased during the first 5 weeks (4 ng/mL) of lactation and gradually increased as lactation progressed to peak (16 ng/mL) at 30 weeks in milk (Elizondo Salazar et al., 2013). The decrease in OCN during early lactation is likely to be reflect bone remodelling that is likely to occur in response to the rapid mobilisation of minerals from that skeleton at this time. This result is similar to that reported for humans during the post-partum period (Ritchie et al., 1998) where a high correlation ($r=-0.727$, $P<0.001$) existed between the length of lactation and bone mineral density of the lumbar spine. Further the authors noticed that after weaning there was a spontaneous compensation for the bone loss during pregnancy and lactation.

2.11.2.3 Pyridinium cross-links

Collagen type I consists of two α1 chains and one α2 chain forming a triple-helical strand. The collagen strands are linked by cross-links at the end of a collagen molecule and the helical portion of the adjacent molecule (Yang and Grey, 2006). Pyridinoline (PYD) and deoxypyridinoline (DPD) are the two cross-links found in collagen. During bone formation, osteoblast cells deposit collagen type I into the matrix and cross-links are then formed extracellularly in order to stabilize the collagen structure (Yang and Grey, 2006). Osteoclasts breakdown cross-linked type I collagen during bone resorption and release PYD and DPD into the circulation and before excretion via urine. It has been estimated that 40 to 50% of cross-links are in the free form while 30 to 40% occurs as a very small peptide (<1000 Da) and the remaining as peptide fragments (1-10 kDa) (Swaminathan, 2001). Commercial enzyme immunoassays are available for determination of the free and total form of PYD and DPD in urine and serum and are utilized for medical research and clinical diagnosis (Swaminathan, 2001; Yang and Grey, 2006).

In humans, the concentration of urinary PYD (r=0.35, P<0.05) and DPD (r=0.46, P<0.01) showed a significant correlation with bone resorption measurements as osteoclast surface of patients with vertebral osteoporosis (Delmas et al., 1991). Mark Wilkinson et al. (2003) compared the use of free and total DPD in the urine and concluded that total DPD is a more accurate indicative of bone resorption than free DPD. DeLaurier et al. (2004) reported a significant relationship (r=0.69, P<0.001) between total urinary DPD measured by high performance liquid chromatography (HPLC) and serum total DPD measured by a commercial ELISA kit (MetraTM DPD, Quidel Corporation; CA, USA) in cats. In addition, the authors reported a significant decrease in total DPD excretion in urine with increased age (Figure 2-7) similar to the change in the concentration of BAP with age in dairy cattle. Taken together these results suggest that both bone resorption and formation are accelerated at younger ages during rapid growth and slow down as animals approach their mature skeletal size.

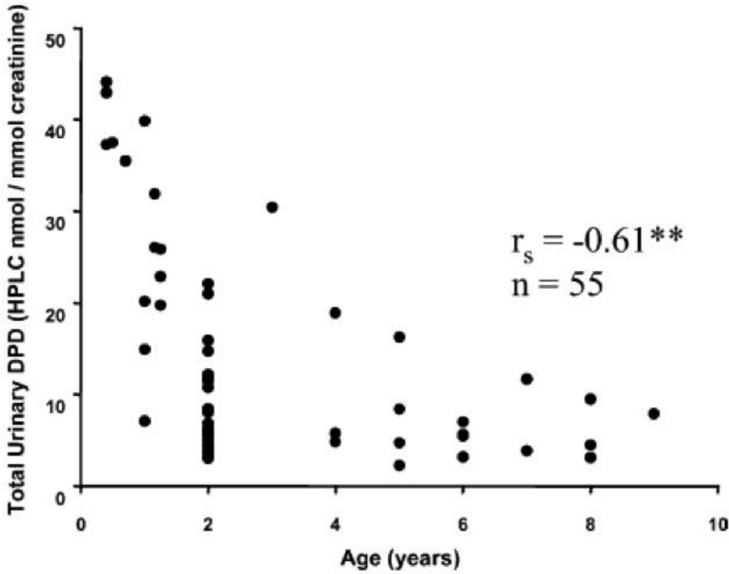


Figure 2-7 Relationship between total deoxypyridinoline (DPD) urinary concentration measured by HPLC and age in cats. r_s= Sperman's correlation coefficient, n = number of samples, ** = P<0.05. Reproduced from DeLaurier et al. (2004).

The concentration of PYD increased and peaked soon after calving in the serum (Elizondo Salazar et al. (2013) and urine (Liesegang et al. (1998) of Holstein cows, decreasing again as lactation progressed. The concentration of PYD was higher in the serum of Holstein cows fed a low Ca diet (4.6 g Ca/kg DM) compared with those fed a high Ca diet (6.4 g Ca/kg DM) during early lactation (Moreira et al., 2009). In contrast, PYD was unresponsive to the P content of the diet fed to Holstein heifers (4.5 to 12 months of age) (Esser et al., 2009). However, bone density was also unresponsive to dietary P content in this study which was attributed to the diets providing adequate P for the growth

of these heifers (2.9 to 3.9 g P/kg DM) (Esser et al., 2009). Whilst the concentration of PYD in serum and urine has been shown to respond with diets and physiological processes that would suggest mineral mobilisation and resorption and bone remodelling no direct links between PYD and bone turnover have been demonstrated in cattle.

2.11.2.4 Cross-linked telopeptide of type I collagen

During the process of collagen breakdown by osteoclast activity approximately 60% of the released cross-links are attached to peptides. C-terminal (CTx) and N-terminal (NTx) are the two cross-link forming sites. At the C-terminal end, two different fragments have been identified as the cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal crosslinked telopeptide of type I collagen (CTX-1). The relative abundance of CTX-1 and ICTP in circulation is dependent on the collagenolytic pathway in action during bone solubilization (Garnero et al., 2003). The release of CTX-1 is associated with Cathepsin K while ICTP is linked with the matrix metalloproteinases (MMPs) MMP-2, -9, -13 or -14 which explains the difference in the abundance of the two markers between different bone pathologies (Garnero et al., 2003). Currently there are commercial immunoassays to measure both molecules in serum and urine and also for ICTP in serum. The urinary marker of CTx and NTx needs to be corrected by urinary creatinine excretion due to the sensitivity to renal clearance rates (Delmas et al., 2000). Nikahval et al. (2016) evaluated the use of a commercial ELISA (Shanghai Crystal Day Biotech Co.; China) kit for determination of CTX-1 in dogs and concluded that it could be use for diagnostic of osteoarthritis. In humans, Garnero et al. (2001) described a significant negative correlation between concentration of CTX-1 in the serum with bone mass at the mid ($r=-0.23$; $P<0.0001$) and distal radius ($r=-0.27$; $P<0.0001$). In previous work, Garnero et al. (1996) reported significant negative correlations between the concentration of NTx and CTx in the urine and bone mineral density of the lumbar spine, total hip and distal radius.

In multiparous Swedish Red and White dairy cows the concentration of CTX-1 is highest at parturition and decreases as lactation progresses (Figure 2-8; (Holtenius and Ekelund, 2005) which is similar to the change in the concentration of PYD in serum (Elizondo Salazar et al., 2013) and DPD in urine (Liesegang et al., 1998) of lactating Holstein cows.

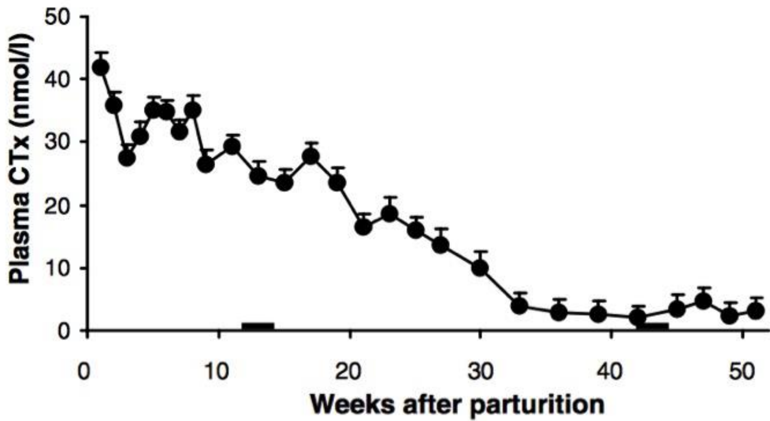


Figure 2-8 Change in plasma concentration of CTx (CTX-1) after parturition. Reproduced from Holtenius and Ekelund (2005)

In a subsequent experiment (Ekelund et al. (2006) with lactating Swedish Red and White dairy cows the concentration of CTX-1 in the plasma declined as in previous work (Holtenius and Ekelund, 2005) but was found to be unresponsive to dietary P content (3.2 vs 4.3 g P/kg DM). The authors concluded that the diet with lower phosphorus concentration was not sufficiently deficient to induce an increase in bone resorption, however no direct measurements on bone remodelling were conducted. Liesegang et al. (1998) found no difference in the concentration of ICTP in the serum of mature dairy cows with and without symptoms of peri-partum milk fever but reported a peak ICTP concentration (~ 40 µg/L) on day 4 of lactation, declining thereafter as lactation progressed which was consistent with the changes in CTX-1 described above.

2.12 Nutritional effects of endocrinal status and its relationship with bone growth and remodelling

This section will review the hormonal effects on bone growth by outlining the effects of a range of hormones affected by nutrition. This will provide the rationale for the selection of a suite of hormones to monitor during the experiments.

2.12.1. *Growth hormone and insulin-like growth factor-1*

The somatotropic axis is composed of growth hormone (GH), insulin-like growth factor-1 (IGF-1) and their carrier proteins and receptors. As its name suggests, GH is considered the main hormone linked with longitudinal growth (Breier, 1999). It is secreted by the anterior pituitary gland by the somatotropic cells. The secretion of this hormone is regulated by the combined action of Growth

hormone-realising hormone (GHRH) and Growth hormone-inhibiting hormone (GHIH, or somatostatin) which are mainly produced in the hypothalamus. The Hepatic IGF-1 production is responsible for 75% of all IGF-1 in the circulation with the remaining 25% is extra-hepatic production derived from virtually every other tissue in the body (Ohlsson et al., 2009). This feature is responsible for the autocrine/paracrine effect of IGF-1 which promotes cell differentiation and proliferation locally, including bone.

The effects of GH on the growth plate are both direct where GH acts locally recruiting chondrocytes in the resting zone to differentiate into proliferative chondrocytes (Isaksson, 1987) and indirect where GH promotes local production of IGF-1 which then stimulates proliferation of proliferative chondrocytes (Isaksson, 1987). Wang et al. (1999) demonstrated that IGF-1 null mice displayed a 35% reduction in skeleton elongation rate compared to wild-type mice, with no difference in hypertrophic chondrocyte number or PZ height. However, HZ thickness was decreased by 35% and terminal hypertrophic chondrocyte height was decreased by 30%. This result is in agreement with previous publications that stated that GH action stimulates formation and proliferation of young chondrocytes whereas IGF-1 actions target chondrocyte hypertrophy (Isaksson, 1987). The importance of local compared with hepatic derived IGF-1 on skeletal growth are demonstrated by a number of transgenic mice lines which target hepatic IGF-1, the acid-labile sub-unit (ALS) and insulin-like growth factor binding protein-3 (IGFBP3) (Sjögren et al., 1999 Ohlsson et al. (2009), Yakar et al., 2009). The concentration of IGF-1 in the circulation of transgenic mice was 2.5 to 25% of that of wild-type mice with only mild reductions in bone length, trabecular bone volume, trabecular thickness and cortical thickness in the transgenic mice.

Models of spatial IGF-1 inactivation were employed to determine the specific role of local IGF-1 on bone growth and remodelling whilst normal concentrations of IGF-1 in the circulation (Govoni et al., 2007a; Govoni et al., 2007b; Sheng et al., 2013). Inactivation of IGF-1 production in chondrocyte collagen type II α-1 cells (i.e. chondrocyte knock-out model) resulted in a mild reduction in body length (7%), cortical bone width (7%) and cortical volumetric bone mineral density (4%) (Govoni et al. (2007a) compared with wild-type counterparts. The small effect on bone in this model compared to the large effect of total IGF-1 inactivation described by Mohan et al. (2003) suggest that circulating IGF-1 or local production of IGF-1 in other bone cells plays a greater role maintaining bone development. Inactivation of IGF-1 in osteoblastic cells (i.e. osteoblast knock-out model) resulted in a reduction in femur length (15%), trabecular bone volume (20%), cortical bone width (20%), cortical volumetric bone mineral density (5%), bone formation rate (BFR, 48%) and mineral apposition rate

(MAR, 40%) compared to wild-type counterparts (Govoni et al., 2007b). In more recent work, Sheng et al. (2013) also observed that disruption of Inactivation of IGF-1 in osteocytes resulted in a reduction in elongation and thickness of the calvarial bone and growth plate with no difference in thickness at the PZ but a thinner HZ compared to wild-type counterparts. It is important to note that none of these chondrocyte, osteoblast or osteocyte IGF-1 inactivated models showed decreases in plasma concentration of IGF-1 and this demonstrates that local production of IGF-1 is essential for bone elongation and maintenance and it cannot be replaced by serum IGF-1.

The somatotrophic axis is sensitive to nutrient supply, with protein and energy intake modulating the concentration and action of GH and IGF-1. Inadequate energy intake reduced linear growth in cattle which was associated with reduced concentration of IGF-1 in plasma (Blum et al., 1985; Yambayamba et al., 1996a). Elsasser et al. (1989) observed a linear increase in plasma concentration of IGF-1 with increasing the CP content of a high energy content diet (12.3 MJ/kg DM). However, steers fed a lower energy content diet (8.2 MJ/kg DM) did not show any increase in IGF-1 concentration when fed an 11 or 14% CP diet but IGF-1 concentration was greater than in steers fed an 8% CP diet. The administration of GH (bST) to well fed cattle is known to increase growth rates, decrease carcass fat and increase IGF-1 production (Moseley et al., 1992). However, during a nutritional restriction, administration of external GH to cattle did not increase the concentration of IGF-1 in the plasma (Elsasser et al., 1989), similarly GH treatment is less effective in malnourished compared to well-fed children. In contrast, the use of external IGF-1 administration during nutritional restriction has been shown to reduce protein loss in sheep (Douglas et al., 1991), cattle (Hill et al., 1999) and mice (O'sullivan et al., 1989). Interestingly, feed restricted mice showed a 40-60% decrease in GH receptors in the growth plate followed by a significant number of GH receptor-positive cells seven days after re-feeding (Gat-Yablonski et al., 2008) and this may explain the difference in action of GH and IGF-1 during nutrient restriction.

2.12.2. *Thyroid hormones*

Thyroid hormones (TH) are involved in basal metabolic processes such as oxygen consumption and lipid, protein and carbohydrate metabolism and also trigger cell differentiation and maturation in several tissues (Squires, 2010). It has also been suggested that thyroid hormones can affect growth by interacting with other growth stimulating hormones. For instance, Matsuo et al., (1990) demonstrated that infusion of Thyroxin (T4) in thyroidectomized rats compensated for the decrease in biding capacity of ^{125}IGF-1, sugesting that TH stimulate IGF-1 receptors in the anterior pituitary gland. Then concentration of thyroid hormones, T4 and 3,5,3'-triiodothyronine (T3) is also affected

by nutrition. The most recognized nutrient to affect the thyroid action is iodine, which represents about 60% of the weight of T4. Adequate secretion of thyroid hormones may still be possible during mild iodine restriction by an increase in thyroid uptake and clearance rate (Delange, 1994). Intakes under the threshold of about 50 µg I/day for humans decrease the absolute uptake of iodine by the thyroid despite the increase of thyroid clearance (Delange, 1994). In cattle, during nutritional restriction there is usually a decrease in concentration in both T3 and T4, which remain lower than normal for few weeks after the start of a recovery period (Blum et al., 1985; Hayden et al., 1993; Yambayamba et al., 1996a; Hornick et al., 2000). Decreased secretion of T4 during fasting is not completely understood but recent studies have indicated that this regulation is at least in part controlled by leptin/neuropeptide-Y pathway mediating suppression of thyrotropin-releasing hormone (THR) and thyroid-stimulating hormone (TSH) secretion (Vella et al., 2011). Changes in concentration are partially mediated by the peripheral conversion of T4 into T3 and reverse T3 (rT3) led by the decrease in hepatic deiodinase type 1 (D1) activity (O'Mara et al., 1993). The deiodination of the outer ring of T4 leads to the activated form T3, whereas the inner ring deiodination of T4 results in the formation of rT3. T3 is the only form of this hormone that can bind to thyroid receptors (TR), thus to become biologically active T4 needs to be converted into T3 (van der Spek et al., 2017).

TH concentration is directly correlated with energy expenditure and caloric loss (Squires, 2010). Yambayamba et al. (1996a) observed that heifers fed a diet that maintained LW had a lower concentration of T4 and T3 and these were also associated with a lower resting metabolic rate (RMR). The lower RMR in previously restricted heifers was still observed on day 10 during a recovery period as well as a significantly lower concentration of TH when compared to control group. A decrease in RMR and energy requirement during feed restriction and also in early stages of re-alimentation has been pointed as one of the physiological changes that could contribute to compensatory growth (Fox et al., 1974). Feed restriction associated with a low-protein / high-carbohydrate diet increased the concentration of T3 in the serum of rats while a high-protein / low-carbohydrate diet decreased the concentration of T3 (Glass et al., 1978). However, it is unknown if thyroid metabolism is responsive to the manipulation of the ratio of ME to CP content of a diet and if this could be involved in LW changes during feed restriction in cattle.

Maglich et al. (2004) studied the effect of the nuclear receptor CAR (NR1I3) on the control of energy homeostasis. They subjected the genetically modified mice without the CAR gene ($Car^{-/-}$) and the wild-type to fasting for 24 h and observed that fasted $Car^{-/-}$ did not show any difference in T3 and T4 concentration as opposed to the wild-type mice. When $Car^{-/-}$ were fed a 40% restricted diet for 12

weeks, these animals lost more than twice as much liveweight when compared to the wild-type. These results demonstrate that CAR expression during feed restriction is necessary for the normal decrease in T3 and T4 levels and these have significant effect on the process of liveweight loss.

Thyroid receptors (TR) were localized to the resting and PZ but not in the HZ of the growth plate of rodents (Robson, 2000), however Abu et al. (2000) reported that mRNA and protein of TRα1, α2, β1 and β2 were widely distributed in all growth plate zones in humans. It has been demonstrated that T3 can induce hypertrophic differentiation by stimulation of ciclin-dependent kinase inhibitors (Quarto et al., 1992). *In vitro* trials have shown that the action of T3 on growth plate chondrocyte proliferation and differentiation has been linked with IGF-1-R activation and Wnt signaling (Wang et al., 2007, 2010). The specific action of T3 in the remodeling process is not well defined. It is unclear if the stimulation of bone formation is by the direct action of T3 on the osteoclasts lineage or the stimulus in osteoclastogenesis and bone resorption are secondary to the action of T3 in osteoblasts, osteocytes, stromal cells or other bone marrow cell lineages (Bassett and Williams, 2016). However, treatment of ovariectomized rats with TSH have shown an increase in bone mass as well as preventing bone loss without increasing levels of T3 and T4 (Sampath et al., 2007).

2.12.3. *Leptin*

Leptin is a protein hormone produced mainly by the white adipose tissue and it is a product of the *obese* (*ob*) gene. It is commonly accepted that circulating levels of leptin in response to nutritional restriction are an important evolutionary adaptation for survival. It can play an important role controlling energy balance, food intake, thyroid activity, protein synthesis, reproduction and skeletal metabolism (Squires, 2010). Leptin levels are positively correlated with body fatness. Increases in the amount of adipose tissue leads to increases in leptin production, which in turn activate the satiety centre in the hypothalamus and decrease food intake (Ahima et al., 1996). The opposite effect is observed when animals are subjected to food restrictions, although the response of plasma leptin level response to food intake varies depending on body fatness. Following a period of fasting, fat animals shows a greater increase in circulating levels of leptin than lean ones during the realimentation phase (Chilliard et al., 2005).

The action of leptin at the anterior pituitary gland is not completely understood but it is known that it can affect the pattern of luteinizing hormone (LH) and GH production, especially under nutritional stress. Underfed rodents treated with leptin do not show an as great decrease in T4, LH and GH when compared to untreated cohorts. It also promoted the maintenance of the oestrous cycle despite nutritional restriction (Ahima et al., 1996). Nagatani et al. (2000) working with gonadectomised male

sheep treated with estrogen, detected that 78 h fasting induced reductions in LH and increases in GH secretion. They also reported that when fasted animals were treated with leptin (50 mg/kg, sc, every 8 h) they overcame the fasting-induced suppression of pulsatile LH. It also leads to further increments in the serum GH concentration. In rats, increases in GHRH and decreases in GHIH with a concomitant rise in GH, have been reported after leptin administration in either well fed and restricted animals (Cocchi et al., 1999; Watanobe and Habu, 2002), but the stimulatory response was more sensitive in fasted animals than in well-fed rats.

A local action of leptin at the bone growth plate has been demonstrated. It seems that the stimulatory growth effect at the growth plate acts synergistically with IGF-1 and TH. Increases in IGF-1 receptor expression at mRNA and protein level due to leptin treatment have been shown in both *in vivo* and *in vitro* studies. The expression of IGF-1 receptor was doubled when T3 was also supplemented, compared with just leptin administration (Maor et al., 2002; Gat-Yablonski, 2004). Interestingly, leptin administration also upregulated expression of thyroid hormone receptor (TRα) in growth plate chondrocytes. Maor et al. (2002) working with *in vitro* culture of mandibular condyles of mice found that leptin stimulate in a dose-dependent manner the width of proliferative zone and condyle size. They also reported that these effects were completely blunted when anti-IGF-1 antibodies were applied to the culture. This response may indicate that leptin plays a role mediating the stimulatory action of IGF-1 and has a synergic effect with T3. This dose-dependent characteristic of leptin action in bone metabolism was also demonstrated in a *in vivo* study by Martin et al. (2007). The result shows that administration of low-dose of leptin (50 μg/kg.day) to tail suspended rats inhibited BMD loss in trabecular and cortical bone. It also supressed the negative effects on bone growth increasing the femoral length and cortical area, although, rats that received the high-dose of leptin (500 μg/kg.day) showed lower BMD, trabecular connectivity and femoral growth independent of suspension condition. Serum IGF-1 concentration in low-dose treatments remained unchanged which could suggest an independent action of this hormone at the bone level. However, the findings of Maor et al. (2002) and Gat-Yablonski (2004) suggest that the link between these hormones could be through the stimulation of expression of IGF-1 and T3 receptors.

2.12.4. *Insulin*

The main function of insulin is to regulate blood glucose concentration by controlling the uptake by tissues and storage as glycogen or lipid. It is produced in the pancreas by the β cells in the islets of Langerhans (Squires, 2010). The most important mechanism by which insulin is secreted is mediated by glucose-stimulated K_{ATP} channel dependent pathway, which is a response to the concentration of

glucose and its precursors such as propionic acid in the bloodstream (Miki et al., 1998). Insulin's response to nutritional changes tends to be similar to that of IGF-1; decreased during periods of restriction and increased after a shift to adequate or excess nutrition. Hornick et al. (2000) reviewing several works showed that insulin is one of the first hormones to return to the same concentration as control groups in animals undergoing compensatory growth after a period of nutrient restriction. The counter-regulatory hormone to insulin is glucagon, which is produced by the pancreas and produces opposite effects of insulin.

The action of insulin and glucagon during negative energy balance plays important role in the physiological adaptation of the body. During negative energy balance, the concentration of insulin and glucose in the circulation are reduced. Lower glucose concentration leads to decreased amounts of glycerol, which is precursor for lipogenesis. Low availability of glycerol and insulin increases triglycerides breakdown (lypolysis) over synthesis or resynthesis in the adipocytes, and this process increases the concentration of NEFAs (non-esterified fatty acids) in the bloodstream (Herdt, 2000). In the liver, increased glucagon stimulates NEFA movement into the mitochondria through *carnitine palmityl transferase I* (CPT I) action. The decreased amount of glucose flowing into the Krebs cycle stimulates β-oxidation of NEFA and the production of both glucose and ketone bodies (e.g. acetone, acetoacetate and beta-hydroxybutyrate) (Chow and Jesse, 1992). Ketone bodies enter the circulation and are delivered to tissues, especially muscle, where they are reconverted to acetyl-Coa which will feed the Krebs cycle for energy production (Herdt, 2000; Adewuyi et al., 2005).

Apart from the well-known importance of the insulin axis to the regulation of homeostasis and adaptation for periods of nutritional stress discussed previously, recent work has demonstrated the direct effect of this hormone during bone remodelling and also a link between skeletal homeostasis and energy regulation (Rosen and Motyl, 2010). Avnet et al. (2012) demonstrated that insulin receptor isoforms IR-A and IR-B are expressed by human osteoblasts. The type A receptors were found in pre-osteoblasts and type B receptors in mature osteoblasts which may suggest that insulin plays a role stimulating differentiation from marrow stromal cells (Klein, 2014). The deletion of insulin receptors in osteoblasts of mice demonstrated that insulin promotes bone formation through inhibition of Twist2 and also stimulates OCN production (Ferron et al., 2010). The uncarboxylated active OCN generated by insulin signalling in the osteoblasts promotes insulin sensitivity in peripheral organs and enhances insulin production by β cells in the pancreas in a feed-forward loop fashion (Clemens and Karsenty, 2011).

2.13 Conclusions

The above review described factors which influence compensatory and catch-up growth responses in a range of animal models and the cellular and endocrine changes which may be involved in these responses. Specific attention was paid to the role and response of bone in animals undergoing compensatory and catch-up growth. Reasons for the variability in which ruminants respond during recovery periods after nutrient restriction were explored throughout.

A distinction was made between growth of soft tissue during and after nutritional restriction (called compensatory growth after nutritional restriction was alleviated) and growth of bone during and after nutritional restriction (called catch-up growth after nutritional restriction was alleviated). This was to avoid the confusion and interplay of terms used in the literature. Much more work has been done on the compensatory growth aspect in livestock than on the catch-up phenomenon and conclusions had to be drawn from the vast amount of work with humans and rodent models. The mechanism by which bone elongation responds to nutrition and its subsequent catch-up response after nutritional restriction has not been documented in livestock species but is of huge importance in understanding how the common phenomenon of compensatory growth occurs in cattle within tropical grazing systems. The lack of information about skeletal development and its relationship with nutrition isimportant for developing new strategies in agricultural production and may also contribute to scientific knowledge in basic bone biology and human health.

The current rib bone biopsy technique does not provide enough trabecular bone tissue to allow histomorphometric analysis of bone formation and resorption. On the other hand, coccygeal vertebrae biopsy could potentially allow histomorphometric assessments however this methodology is more invasive raising animal welfare concerns. Moreover, coccygeal vertebrae biopsy does not allow repetitive sampling on the same animal. In order to assess endochondral ossification in live cattle a methodology to obtain samples of the growth plate is needed. Based on this review of literature such a technique does not exist. Bone metabolic biomarkers are the main tools currently used to assess bone turnover in cattle, however the relationship between the concentration of such markers and changes in bone formation and resorption were assumed by experiments conducted with other species with little direct validation in cattle.

The concept put forward in this thesis is that muscle growth responds to skeletal elongation (the stretch mechanism) and this implies that there is an allometric relationship between skeletal dimensions and LW of the animal since muscle mass is the major driver of LW. It follows that

changes in rate of bone elongation (which can be simply measured as changes in hip height in the field) may be affected by level of nutrition and hence exert an effect on change in LW. The cellular mechanism by which changes in the chondrocytes of the long bones bring this about have been described as chondrocyte proliferation and chondrocyte hypertrophy but the quantitative changes and quantitative responses have not been described, especially those changes in response to seasonal changes in - diet quality and quantity. These cellular changes are presumably influenced by various hormones which respond to nutrients and this mechanism is not well understood. This thesis will investigate the skeletal and LW response of cattle to nutritional restriction and re-alimentation and the cellular and hormonal changes that occur in response to these treatments in order to understand the mechanism of the changes in skeletal elongation rate in cattle. The practical significance of the work was to identify if compensatory growth could be enhanced by increasing skeletal elongation under nutritional restriction through dietary (protein and energy) manipulation and if the skeleton also exhibits catch-up growth in cattle.

Chapter 3. General Materials and Methods

3.1 Introduction

Experimental and analytical procedures used more than once are described in this chapter. Procedures that were conducted in only one experiment or where variation in the procedure exists between experiments are described in each experimental chapter.

3.2 Experimental procedures

3.2.1. *Blood sample collection*

Blood samples were collected from the jugular vein into lithium heparin coated vacutainers (Becton Dickinson; Franklin Lakes, NJ, USA). Immediately after collection the vacutainers were slowly inverted 6 to 8 times and then placed on ice for approximately 30 min prior to centrifugation at 1700 *g* for 10 min at 4°C, plasma was collected and stored at -20°C until analysis.

3.2.2. *Bone sample collection*

The biopsy site was clipped and scrubbed with chlorhexidine surgical scrub (Perrigo; Balcatta, WA, Australia) and wiped clean with Chlor-hex C (Jurox; Rutherford, NSW, Australia) in methylated spirits (Recochem; Lytton, QLD, Australia). The skin and deeper tissue over the tuber coxae were infiltrated with 35 to 40 mL of lignocaine hydrochloride (20 mg lignocaine hydrochloride/mL; Troy laboratories; Glendenning, NSW, Australia) and left for 5 min for effect. An incision approximately 80 mm in length was made and skin and any overlying muscle were retracted. A single biopsy 10-20 mm deep was obtained from the most central part of the tuber coxae of the ilium. A 10-20 mm bone hand-trephine (Sontec Instruments Inc.; Centennial, CO, USA) was used to start the biopsy and then a 16 mm metal hole-saw was used to obtain a deeper sample. An elevator was used to separate the sample from the parent bone. Overlying muscle was sutured with absorbable sutures (2/0 PDS) and the external incision closed with skin staples, the surgical site was then cleaned and sprayed with Chloromide antiseptic spray (Troy laboratories). The bone cores were divided lengthwise using a scalpel blade and sub-samples were fixed with either 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) and placed on ice prior to transfer to the laboratory. The samples remained

in fixative for 24 h at 4ºC and were then transferred to 70% ethanol and stored at 4°C for approximately 4 months until they were sectioned.

3.3 Analytical procedures

3.3.1. *DM and ash*

Sub-samples of feed offered, feed residues and faeces were dried in duplicate to a constant weight at 65ºC for DM determination, usually for 48 hours. Samples were then ground through a 1 mm screen (Retsch ZM 200; Haan, Germany), dried for 24 h at 105°C to determine residual DM content and then combusted in an electric muffle furnace (Modutemp Pty. Ltd.; Perth, WA, Australia) for 8 h at 550°C to determine ash and organic matter (OM) content.

3.3.2. *Total N and crude protein*

The N content of feeds offered was measured by the Kjeldahl method using an auto-digestor (Tecator 2520, FOSS; Hillerød, Denmark) and a N analyser (Kjeltec 8400, FOSS; Hillerød, Denmark) following the manufacturers guidelines (Foss, 2003). Crude protein content was calculated using the conversion factor 6.25 x N.

3.3.3. *Ash-free NDF*

The content of ash-free NDF in feeds offered were measured following the procedure described by Van Soest et al. (1991) using a fibre analyzer (A200, Ankom; Macedon, NY, USA). The digestion solution was made using anhydrous sodium sulphite (0.5 g/50 mL of neutral detergent solution), 100 mL/bag of neutral detergent solution and 4 mL of alpha-amylase. The extraction solution was then drained and the residue was rinsed 3 times with hot water 70-90°C (4 mL alpha-amylase added to the first 2 washes) followed twice by acetone. The filter bag with residues was air-dried before oven drying at 60°C for 24 h. The filter bag was weighed and ashed in a furnace at 500°C for 4.5 h. The ash content was determined, and ash-free NDF content was calculated.

3.3.4. *Mineral content*

The mineral content of feeds offered was determined on an ICP-OES spectrometer (Optima 7300 DV, PerkinElmer; Waltham, MA, USA) after a nitric-perchloric acid digestion. The nitric-perchloric

solution was prepared using 6 mL of nitric acid, 2 mL of perchloric acid and 12 mL of reversed osmosis water.

3.4 Hormone analyses

All hormone assays were performed using either commercial radioimmunoassays (RIA) or immunoradiometric assay (IRMA) kits and a brief description of each procedure is provided in the following sections. An automatic gamma counter (2470 Wizard 2, Perkin Elmer; Waltham, MA, USA) was used to measure gamma radiation from assay tubes. Radioactivity (counts per minute, cpm) was then processed using the software AssayZap (Biosoft; Cambridge, UK) in order to generate a curve using known concentration of standards, and to calculate the concentration of unknown samples.

3.4.1. *Insulin*

The concentration of insulin in plasma was determined using a human IRMA assay kit (DIAsource INS-IRMA Kit, DIAsource; Louvain-la-Neuve, Belgium). Briefly, 50 µL of sample (unknown), control and standard and 50 µL of tracer (^{125}Iodine labeled anti-insulin antibody) was added to tubes coated with monoclonal anti-insulin antibodies and incubated for 2 h at room temperature. Fifty. After incubation, the content of all tubes (except total counts) were decanted and washed twice using the wash solution. The inter- and intra-assay coefficients of variation were 9.7% and 3.3 % for a quality control of 16.2 µIU/mL.

3.4.2. *Total insulin-like growth factor-1 (IGF-1)*

The concentration of total insulin-like growth factor-1 (IGF-1) in plasma was determined using an IRMA assay (A15729, Immunotech, Beckman Coulter; Prague, Czech Republic). All samples and quality controls were first dissociated in order to release IGF-1 from binding proteins to permit the measure of total IGF-1 in the sample. Briefly, 50 µL of dissociated sample (unknown) or quality control and standard (dissociation not required) and 300 µL of tracer (^{125}Iodine labeled anti-IGF-1 antibody)were added to anti-IGF-1 antibody-coated tubes. Tubes were then vortexed and incubated for 1 h at room temperature on an orbital shaking platform (Edwards Instrument Company; Narellan, NSW, Australia) at 180 rpm. After incubation, the tubes were decanted before being washed twice with 2 mL of wash solution. The inter- and intra-assay coefficients of variation were 8.4% and 5.2% for a quality control of 359 ng/mL.

3.4.3. *Total triiodothyronine (T3)*

The concentration of total triiodothyronine (T3) in plasma was measured using a RIA kit (IM1699, Immunotech, Beckman Coulter; Prague, Czech Republic). Briefly, 25 µL of sample (unknown), quality control and standard were added to anti-T3 monoclonal antibody coated tubes, followed by 200 µL of ^{125}I labeled T3, the tubes were vortexed and incubated for 1 h at room temp on an orbital shaking platform (180 rpm). Following incubation all tubes (except total counts) were decanted and the remaining radioactivity in each tube counted. The inter- and intra-assay coefficients of variation were 5.3% and 6.4% for a quality control of 2.03 nmol/L.

3.4.4. *Total thyroxine*

The concentration of total thyroxine (T4) in plasma was measured using a RIA kit (IM1447, Immunotech Beckman Coulter; Prague, Czech Republic). Briefly, 20 µL of sample (unknown), quality control and standard were added to anti-T4 monoclonal antibody coated tubes, followed by 500 µL of ^{125}I-labeled T4 tracer. Tubes were vortexed and incubated for 1 h at room temperature on an orbital shaking platform (180 rpm). After incubation all tubes (except total counts) were decanted and the remaining radioactivity in each tube counted. The inter- and intra-assay coefficients of variation were 3.7% and 7.2% for quality controls of 5.0 and 42.6 nmol/L.

3.4.5. *Leptin*

The concentration of leptin in the plasma was determined using a multi-species RIA kit (XL-85K, Millipore Corporation; St Charles, MO, USA). Briefly, 100 µL of sample (unknown), quality control and standard were added to tubes (Labserv, ThermoFisher Scientific; Scooresby, VIC, Australia) with 100 µL of antibody (ginea-pig anti-leptin antibody) and 100 µL of assay buffer (0.05M PBS, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, 0.05% Triton X-100, and 1% bovine serum albumin). Tubes were vortexed and incubated for 24 h at 4°C. On the following day 100 µL of ^{125}Iodine-Human Leptin was added and tubes were again vortexed and incubated for 24 h at 4°C. After incubation, 1 mL of cold precipitating reagent (goat-anti guinea pig IgG serum, with 3% polyethylene glycol, 0.05% Triton X-100 in 0.05M PBS with 0.025M EDTA) was added to all tubes, except total count tubes. The tubes were then centrifuged at 4°C for 40 min at 3,000 *g* (Allegra X-12R centrifuge, Beckman Coulter, Inc.; Fullerton, CA, USA). Immediately after centrifugation the supernatant from all tubes (except total count) was decanted and the radioactivity in the remaining

pellet counted. The inter- and intra-assay coefficients of variation were 6.0% and 4.2% for quality controls of 5.4 and 20.4 ng/mL.

3.4.6. *Adiponectin*

The concentration of adiponectin in plasma was determined usinga RIA kit (HADP-61HK, Millipore Inc.; Billerica, MA, USA). Briefly, 100 µL of sample (unknown), quality control and standard were added to tubes with 100 µL of assay buffer (100 mM phosphate buffer, pH 7.5, with 0.08% sodium azide and 1% BSA), 100 µL of adiponectin antibody (rabbit anti-adiponectin) and 100 µL of tracer (^{125}I-labeled adiponectin). Tubes were vortexed and incubated overnight (20 h) at room temperature. The following day 10 µL of rabbit carrier (30% normal rabbit serum) and 1 mL of precipitating reagent (goat-anti rabbit IgG serum in 0.05M PBS and 0.025M EDTA with 3% PEG, 0.05% Triton X-100 and 0.08% sodium azide) were added. All tubes were then vortexed, incubated for 20 min at 4°C and (with the exception of total count tubes) were centrifuged at 3000 *g* for 40 min at 4°C. After centrifugation, tubes were decanted and radioactivity in the remaining pellet counted. The inter- and intra-assay coefficients of variation were 6.8% and 3.2% for quality controls of 2.3 and 16.7 ng/mL.

3.5 Bone marker analyses

Bone metabolism biomarker assays were performed using commercial enzyme immunoassays (EIA) and a brief description of each procedure is provided in the following sections. For all assays, optical density of assay wells was determined using a plate reader spectrometer (Sunrise Absorbance Microplate Reader, Tecan; Pheonix, CA, USA) with XFluor software (Tecan). The software AssayZap (Biosoft; Cambridge, UK) was used to generate a standard curve and to calculate the concentration of unknown samples.

3.5.1. *Bone-specific alkaline phosphatase (BAP)*

The concentration of bone-specific alkaline phosphatase (BAP)in plasma was determined using an EIA kit (MicroVue BAP 8012, Quidel; San Diego, CA, USA). Briefly, 20 uL of sample (unknown), quality control and standard and 125 µL of assay buffer were added to assay wells coated with murine monoclonal anti-BAP IgG antibody. The plates were incubated for 3 h at room temperature with gentle agitation (Titramax 100, Heidolph Instruments; Schwabach, Germany). Wells were washed four times with buffer (a buffer solution containing non-ionic detergent and 0.05% sodium azide) before 150 µL of substrate solution (3.5% 2-amino-2-methyl-1-propanol) was added and incubated

for 30 min at room temperature. The reaction was stopped with 100 µL of 0.5N NaOH and optical density was measured at 405 nm. The inter- and intra-assay coefficients of variation were 3.6% and 4.2% for aquality control of 53.9 U/L.

3.5.2. *Osteocalcin (OCN)*

The concentration of osteocalcin (OCN) in tplasma was determined using an EIA kit (MicroVue 8002 Osteocalcin, Quidel). Twenty-five µL of sample (unknown), quality control and standardand 125 µL of murine monoclonal anti-OCN antibody were added into assay wells and incubated for 2 h at room temperature. The plate was then washed 3 times using assay buffer, incubated with 150 µL of enzyme conjugate solution (goat anti-mouse IgG conjugated to alkaline phosphatase) for 1 h at room temperature followed by150 µL of substrate solution (p-nitrophenyl phosphate in diethanolamine) for 40 min at room temperature. The reaction was stopped with 50 µL of 0.5N NaOH and optical density was measured at 405 nm. The inter- and intra-assay coefficients of variation were 6.6% and 2.7% for a quality control of 126.7 ng/mL.

3.5.3. *Pyridinoline Crosslinks (Pyd)*

The concentration of plasma pyridinoline crosslinks (PYD) in plasma was determined using an EIA kit (MicroVue 8019 Serum PYD, Quidel). This EIA kit has been validated by Quidel for the determination of PYD concentration in plasma despite the name of the kit. Prior to the commencement of the assay plasma samples were filtered (30k MWCO Spinfilter) with centrifugation at 3,000 *g* for 30 min to remove large molecular weight (MW) proteins, such as albumin and haemoglobin that are known to interfere in the assay. Twenty-five µL of filtered sample (unknown), quality control and standard, 50 µl of reagent solution (glycine solution with indicator dye, and 0.05% Proclin preservative) and 75 µL of pyridinoline antibody solution (rabbit polyclonal anti-Pyd) were added to assay wells. . The plate was incubated overnight (20 h) at 4°C. After incubation the plates were washed3 times using wash buffer solution, 150 µL of substrate solution (p-nitrophenyl phosphate in diethanolamine) was added with a further incubation for 40 min at room temperature, with the reaction stopped with 50 µL of 0.5N NaOH and optical density was measured at 405 nm. The inter- and intra-assay coefficients of variation were 7.8% and 4.6% for a quality control of 5.5.nmol/L

3.5.4. *Total Deoxypyridinoline Crosslinks (tDPD)*

The concentration oftotal deoxypyridinoline crosslinks (tDPD) in plasma was determined using an EIA kit (MicroVue 8032 Total DPD, Quidel). Plasma samples were hydrolysed by combining 100 µL of sample with 100 µL of total DPD acid (6N HCl plus solubilizing agent) and centrifuging at 10,000 *g* for 5 min. The resultant supernatant (100 uL) was then placed in a hydrolysis plate, sealed and incubated at 99°C for 20 h. Hydrolyzed samples were cooled at 4°C before the addition of 25 µL of assay buffer (PBS pH 7.2)and 25 µL of assay base (10N NaOH) to neutralize the solution. For the assay, 50 µL of hydrolysed and neutralized sample (unknown), quality control and standard and 50 µL of assay buffer were added to assay wells coated with murine monoclonal anti-DPD antibody and incubated for 30 min at 4°C in the dark. Following incubation 50 µL of enzyme conjugate solution (DPD conjugated to alkaline phosphatase) was added and incubated for a further 2 h at 4°C in the dark. Plates were then washed three times using wash buffer solution before 150 µL of substrate solution (p-nitrophenyl phosphate in diethanolamine) was added and incubated for 2 h at room temperature. The reaction was then stopped with 0.5 NaOH and optical density was measured at 405 nm. The inter- and intra-assay coefficients of variation were 8.6% and 4.6% for a quality control of 16.4 nmol/L.

3.5.5. *C-terminal Telopeptides of Type I Collagen (CTX-1)*

The concentration of C-terminal telopeptides of type I collagen (CTX-1) in plasma was determined using an EIA kit (Immunodiagnostic Systems Ltd; Boldon, UK). Briefly, 50 µL of sample (unknown), quality control or standard and 150 µL of antibody solution (biotinylated monoclonal murine antibody) were added to the assay plate and incubated for 2 h at room temperature on a shaking platform (Hedolph Titramax 100) at 250 rpm. After incubation, plates were washed five times with 300 µL of wash buffer solution per wash, 100 µL of substrate solution (tetramethylbenzidine substrate in acidic buffer) was added and plates was incubated for a further 15 min at room temperature. The reaction was stopped with 100 µL of 0.18M H_2SO_4 was added and and absorbance was measured at 405 nm with reference at 650 nm. The inter- and intra-assay coefficients of variation were 2.9% and 5.8% for a quality control of 2.4 ng/mL.

3.6 Blood metabolites

The concentration of plasma glucose, calcium, inorganic phosphorus, urea and total protein were measured using an Olympus AU400 auto-analyser (Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). A brief description of each method is provided in the following sections.

3.6.1. *Glucose*

The concentration of glucose in plasma was determined by an enzymatic UV method (hexokinase method) (OSR6121; Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). The method consists of the glucose phosphorylation in the presence of hexokinase, adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). G-6-P reacts with NAD^+ to form NADH and glucose-6-phosphate dehydrogenase. Concentration of glucose is proportional to the NADH increase in absorbance at 340 nm.

3.6.2. *Calcium*

The concentration of total calcium in plasma was determined by a photometric colour method (OSR6121; Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). In this method, calcium ions (Ca^{2+}) react with Asenazo III (2,2'-[1,8-Dihydroxy-3,6-disulphonaphthylene-2,7-bisazo]- bisbenzenear-sonic acid) to form a purple colour complex in which the absorbance is directly proportional to the calcium concentration of the sample.

3.6.3. *Inorganic phosphorus*

The concentration of inorganic phosphorus in plasma was determined by a photometric UV method (OSR6122; Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). The methodology of this assay is a modification of that developed by Daly and Ertingshausen (1972). The inorganic phosphate reacts with molybdate to form a heteropolyacid complex and its absorbance is directly related to the concentration of inorganic phosphate in the sample.

3.6.4. *Urea*

The concentration of urea in plasma (PUN) was determined by a kinetic UV test (OSR6134; Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). In this method, the urea in the sample is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The

ammonia combines with 2-oxoglutarate and NADH in the presence of glutamate-dehydrogenase to produce glutamate and NAD^+. The concentration of urea is proportional to the decrease in NADH absorbance per unit of time.

3.6.5. *Total protein*

The concentration of total protein in plasma was determined by a photometric colour method (OSR6132; Beckman Coulter Diagnostic Systems Division, Melville, NY, USA). This assay is based on a modification of the methodology described by Weichselbaum (1946). In this assay, proteins and polypeptides containing at least two peptide bonds react with cupric ions (Cu^{2+}) to form a violet colour complex. The concentration of protein in the sample is directly proportional to the absorbance of the final complex.

3.7 Histomorphometry

Full details of the bone biopsy procedure and processing are provided in the following experimental chapters. This section only describes the decalcification, sectioning, staining and measurement procedures undertaken which were common across all experiments.

Bone specimens were decalcified to remove the inorganic components from the hydroxyapatite complex. All specimens were transferred to an individual biopsy cassette and submerged in a bucket with 10% EDTA (pH 7.0) solution. The solution was replaced every two weeks until decalcification of the samples was complete. The determination of the end point of the process was done manually accessing the pressure necessary to slice a reference bone specimen using a scalpel. Once the decalcification was complete, samples were embedded in paraffin and the blocks were sectioned transversely in order to obtain multiple 5 µm thick transverse sections.

The sections were stained with toluidine blue and masson trichrome and photographed twice with a 4X objective using a Olympus BX41 microscope (Olympus America Inc.; Melville, NY, USA) equipped with a digital camera Q-Imaging camera (Qimaging Corporation; Surrey, BC, Canada). Five measurements were taken per picture for determination of proliferative (PZ) and hypertrophic (HZ) zone heights, number of hypertrophic chondrocytes (HC) per column and diameter of terminal hypertrophic chondrocytes. The measurements sites were evenly distributed across each zone and were taken by a single observer blinded to treatment group. Height and diameters measurements were taken parallel to the long axis of the bone and a mean of ten measurements per animal was utilized

for analysis. One representative section for each animal was selected for measures of trabecular bone parameters. The pictures were taken at a standardised distance from the growth plate (300 µm). The images were then analysed using the software ImageJ (Schneider et al., 2012) and the plugin BoneJ (Doube et al., 2010) to obtain values for bone volume (BV/TV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th) and bone surface (BS). These parameters are extensively used in bone research and have been previously defined by Dempster et al. (2013). Bone volume is expressed as BV/TV and represents the proportion of total volume (TV) occupied by bone tissue (BV). Trabecular thickness represents the mean thickness of trabecula's of a given sample and trabecular separation represents the mean distance between trabecula. Bone surface represents a length measure of bone tissue in a given sample.

Chapter 4. The effect of a high crude protein diet during energy restriction and re-alimentation in cattle on skeletal elongation rate

4.1 Introduction

The effect of nutritional restriction and subsequent replenishment of restricted nutrients leads to a phenomenon commonly known in cattle as compensatory growth. Nutritional restriction also impairs skeletal development and when the limiting factors are corrected animals commonly demonstrate a faster skeletal growth when compared to unrestricted counterparts of the same age which may be different when comparing animals at the same height; this is commonly referred as catch-up growth. In tropical regions compensatory growth in cattle is most commonly observed as increased LWG during the wet season after a period of nutritional restriction during the previous dry season (Kidd and McLennan, 1998). The practical importance of this is that animals may be supplemented during the dry season to maintain a high LWG, but the compensatory growth of unsupplemented cattle in the subsequent wet season may negate any benefits of supplementation and pastoralists may have essentially wasted their money on expensive supplements for cattle to attain a similar LW at the end of the wet season. Whilst this issue has been investigated previously, few studies have examined the effect of restriction of specific nutrients on subsequent compensatory growth or the effect on skeletal growth and its interaction with LW.

The somatotropic axis (GH together with IGF-1) is the major endocrine regulator of endochondral ossification and bone turnover (Nilsson et al., 2005; Mohan and Kesavan, 2012). Increased level of CP intake stimulates IGF-1 in cattle but the IGF-1 response to CP intake is decreased when ME intake is decreased (Elsasser et al., 1989). However it is unknown if higher CP intake will stimulate IGF-1 production during a severe ME restriction in cattle. In an early study with rodents, Frandsen et al. (1954) showed that higher protein intake during severe caloric restriction (i.e. less than 50% of control intake) increased the number and size of chondrocytes at the epiphyseal growth plate and this was associated with increased length of the trunk and tail when compared to pair fed caloric restricted cohorts.

In humans, caloric restriction during anorexia nervosa leads to osteopenia and increased risk of fractures (Munoz and Argente, 2002). In addition, experiments with rodents have shown that pair-fed rats consuming low protein diets have lower bone mineral density, cancellous bone mass and

trabecular thickness (Bourrin et al., 2000b). These results suggests that both, protein and energy dietary restriction, may impair bone turnover leading bone loss. Studies utilizing bone markers as parameters of bone formation and resorption in humans have shown that treating osteopenic patients with rhIGF-1 decreased the concentration of bone resorption markers (Grinspoon et al., 1996; Misra et al., 2009). Sukumar et al. (2011) also demonstrated that higher protein intake during caloric restriction in humans attenuated the process of bone loss and this was linked with increased concentration of IGF-1 and lower levels of the bone resorption marker deoxypyridinoline. It is unknown if a higher CP diet could decrease bone resorption during caloric restriction in cattle. In addition, despite bone metabolism biomarkers having been used in research with bovines, very few studies compared these measures with direct measures on bone (Allen, 2003). Moreover, there is no available information about the use of bone metabolism biomarkers in *Bos indicus* cattle. In sheep, changes in bone turnover can partially supply mineral requirements (e.g. Ca and P) during high physiological demand and replenish the storage once the demand is lower than dietary intake (Braithwaite et al., 1969, 1970). Therefore, understanding the effect of protein and energy restriction on bone turnover in cattle may have direct consequences on mineral mobilization and thus practical applications to mineral nutrition.

The present study aims to explore the effect of a high or low CP diet during ME restriction and also its effect during re-alimentation with a high CP and high ME diet in two distinct cattle genotypes on skeletal elongation rate, as measured by hip height. Furthermore by examining hormonal and cellular changes it aims to determine the impact of these nutritional treatments on the growth plate and trabecular bone histomorphometry of the tuber coxae bone during and after nutrient restriction. In addition, various hormones as well as bone metabolism biomarkers are evaluated in order to provide an assessment of the physiological changes that accompany these nutritional challenges and also to evaluate the use of bone metabolism biomarkers as tools to indicate bone metabolism in cattle.

4.2 Hypotheses

This experiment tested several hypotheses:

1 - Higher CP intake will stimulate endochondral ossification during energy restriction by increasing the concentration of circulating IGF-1 resulting in an increase in skeletal elongation rate as measured by hip height. It is also expected that the increased IGF-1 will decrease bone resorption at the trabecular bone without increasing bone formation. The impact of nutrition on the bone remodelling

process is expected to be associated with changes in the concentration of bone metabolism biomarkers.

2 - Re-alimentation, with restricted animals having access to a high CP and ME diet, will result in catch-up growth in length of bones as measured by hip height.

3 - Greater skeletal size associated with lower LW will result in greater compensatory growth of LW during re-alimentation.

4 – HF steers are expected to have greater rates of skeletal growth and liveweight gain than BX steers when CP and ME are not limiting. Plasma hormone concentration may differ between the genotypes, although is expected a similar pattern of change in response to nutritional restriction.

4.3 Materials and Methods

This experiment was conducted at the Queensland Animal Science Precinct at The University of Queensland (Gatton, QLD, Australia). The experiment was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by The University of Queensland Animal Ethics Committee.

4.3.1. *Experimental design, animals, diets and feeding*

Fifteen *Bos indicus* (Brahman crossbred, BX) steers and fifteen *Bos taurus* (Holstein-Friesian, HF) steers were fed a Rhodes (*Chloris gayana*) grass and Dolichos lablab (*Lablab purpureus*) mixed hay [905 g organic matter (OM), 88 g CP, 644 g ash-free neutral detergent fibre (NDF), 3.9 g P and 7.9 g Ca/kg dry matter (DM)] *ad libitum* in group pens for 23 days and in individual pens for 7 days prior to the commencement of the experiment. All steers were treated for internal and external parasites [Cydectin (5 g Moxidectin/L), Fort Dodge; Baulkham Hills, NSW, Australia) and vaccinated for bovine ephemeral fever (Webster BEF, Zoetis; West Ryde, NSW, Australia) and clostridial diseases (Ultravac 5-in-1, Zoetis; West Ryde, NSW, Australia) prior to commencement of the experiment. The Brahman crossbred steers were sourced from the Queensland Department of Agriculture and Fisheries (QDAF) Spyglass research station, Charters Towers and are a stabilised cross of approximately 5/8 *Bos indicus* and 3/8 *Bos taurus* genotype.

At the commencement of the experiment (day 1) the HF steers [230 ± 34 kg liveweight (LW); mean ± standard deviation (SD)] and BX steers (178 ± 6 kg) were ranked and blocked on LW from heaviest to lightest within each genotype and allocated to one of five blocks of adjacent individual pens (n=6 pens/block). Three steers from the same LW ranking block within each genotype were randomly allocated to individual pens within each block of pens. Within each block of pens the steers from each genotype were then randomly allocated one of three nutritional treatments, high CP and high ME intake (HCP-HME), high CP and low ME intake (HCP-LME) and low CP and low ME intake (LCP-LME). The individual steer was considered the experimental unit.

The experiment consisted of two phases. During Phase 1 (93 days duration) the steers were offered one of the three diets: HCP-HME, HCP-LME and LCP-LME. The nutritional composition of each diet is presented in Table 4-1. Diets HCP-HME and HCP-LME consisted of lucerne (*Medicago sativa*) chaffed hay (chop length approximately 5-10 mm) and LCP-LME of Mitchell grass (*Astrebla* spp.) hay [chop length approximately 50 mm (Jaylor 4350 Feed Grinder, McIntosh and Son; Dalby, QLD, Australia)]. Diets HCP-HME and LCP-LME were offered *ad libitum* and the daily quantity offered was calculated based on the previous day's intake plus 10% and 20% respectively on an as fed basis. Steers fed HCP-LME had limited access to the diet. The feed allowance for steers offered diet HCP-LME was calculated from the mean ME intake of all steers within the corresponding genotype fed LCP-LME during the previous week (MJ ME/kg LW.day) in a such manner that steers fed HCP-LME and LCP-LME had the same ME intake but distinct CP intake. Metabolizable energy in each nutritional treatment was estimated by the equation $M/D = 0.172DMD - 1.707$ described in Freer et al. (2007). Steers allocated to the LCP-LME treatment were offered 50 g cottonseed meal (CSM; 924 g OM, 42.8 g CP, 237 g NDF, 14.6 g P and 2.5 g Ca/kg DM)/kg Mitchell grass hay as fed from day 42 until the end of Phase 1. The addition of CSM was adopted in order to stabilize the LW loss trend in this treatment (-0.21 and -0.17 kg/day for BX and HF respectively). Steers allocated to the HCP-LME treatment were offered 84 mg mono-sodium phosphate (MSP; 240 g P/kg DM)/kg LW.day such that steers were provided with adequate dietary P required to achieve an equivalent LWG to that achieved by the HCP-HME steers. During Phase 2 (103 days duration) all steers were offered lucerne chaff *ad libitum*. The steers remained in the same pens throughout the experiment and had access to drinking water at all times.

Table 4-1 Composition of diet treatments offered during Phase 1.

Nutrient profile	**HCP-HME**	**HCP-LME**[1]	**LCP-LME**[2]
MJ /kg DM			
ME	9.7	9.7	5.1
g/kg DM			
OM	896	896	901
CP	199	199	38
NDF	356	356	631
ADF	271	271	248
P	3.2	3.2	1.8
Ca	12.2	12.2	4.7

[1] Steers were offered CSM at 50 g/ kg of hay as fed from day 42 until end of Phase 1.
[2] Steers were offered MSP at the rate of 84g/ kg LW.day

Feed residues were collected and weighed at 0730 h each day and steers were offered feed at approximately 0800 h each day. Sub-samples of feed offered were collected at feeding each day and bulked over seven consecutive days. Feed residues were weighed daily and bulked over seven consecutive days for each steer. Duplicate sub-samples of feed offered and feed residues for each steer were collected at the end of each seven day period.

4.3.2. *Faecal, urine and rumen fluid collection*

Two cohorts of steers (n=15/cohort) were moved into individual metabolism crates on days 19 and 33 of Phase 1 of the experiment in the same sequence as their individual pens. Each cohort remained in the metabolism crates for 9 consecutive days, which included a 2 day adaptation period and a 7 day collection period. This is a standard adaptation period in this laboratory and animals spend time in metabolism crates in the training period. Total faecal and urine output and feed residues were collected and weighed daily. Daily urine output of each steer was acidified to pH 3 with 5% sulphuric acid and a 5% sub-sample was collected each day, placed at 4ºC and bulked over the collection period. At the end of the collection period the bulked urine was mixed well and aliquots were collected into 5 mL tubes and stored at -20ºC for subsequent analysis. A 10% sub-sample of faecal output was

collected each day, placed at 4ºC and bulked over the collection period. At the end of the collection period the bulked faeces was mixed well and triplicate sub-samples were collected for subsequent analysis. Triplicate sub-samples of feed offered and residues were also collected for subsequent analysis.

Upon the completion of each faecal and urine collection period the steers were returned to their individual pens and rumen fluid was collected prior to feeding per *os* using a stomach tube attached to a hand pump. Rumen fluid sub-samples were acidified with 20% sulphuric acid or 20% metaphosphoric acid with internal standard (i.e. 4 methyl n- valeric acid) and stored at -20°C for subsequent analysis of NH_3N and volatile fatty acids (VFA) respectively.

4.3.3. *Liveweight and body dimension measurements*

Liveweight was measured prior to feeding every seven days throughout the experiment. HH was measured using a measuring stick on days -10, 7, 35, 57, 70, 85, 103, 112, 125, 140, 154, 168, 182, 196 and 203 of the experiment. Point of shoulder to point of olecranon (SLD-OLC), point of olecranon to accessory carpal bone (OLC-ACB), tuber coxae to tuber isheii (TC-TI), point of shoulder to tuber coxae (SLD-TC) and tuber coxae to tuber coxae (TC-TC) measurements were taken using a measuring tape on the same days as HH measurements except for day 112 when no measurements were taken (Figure 4-1). Liveweight gain and body measurement growth rates were calculated by regressing each measurement over time within each phase of the experiment.

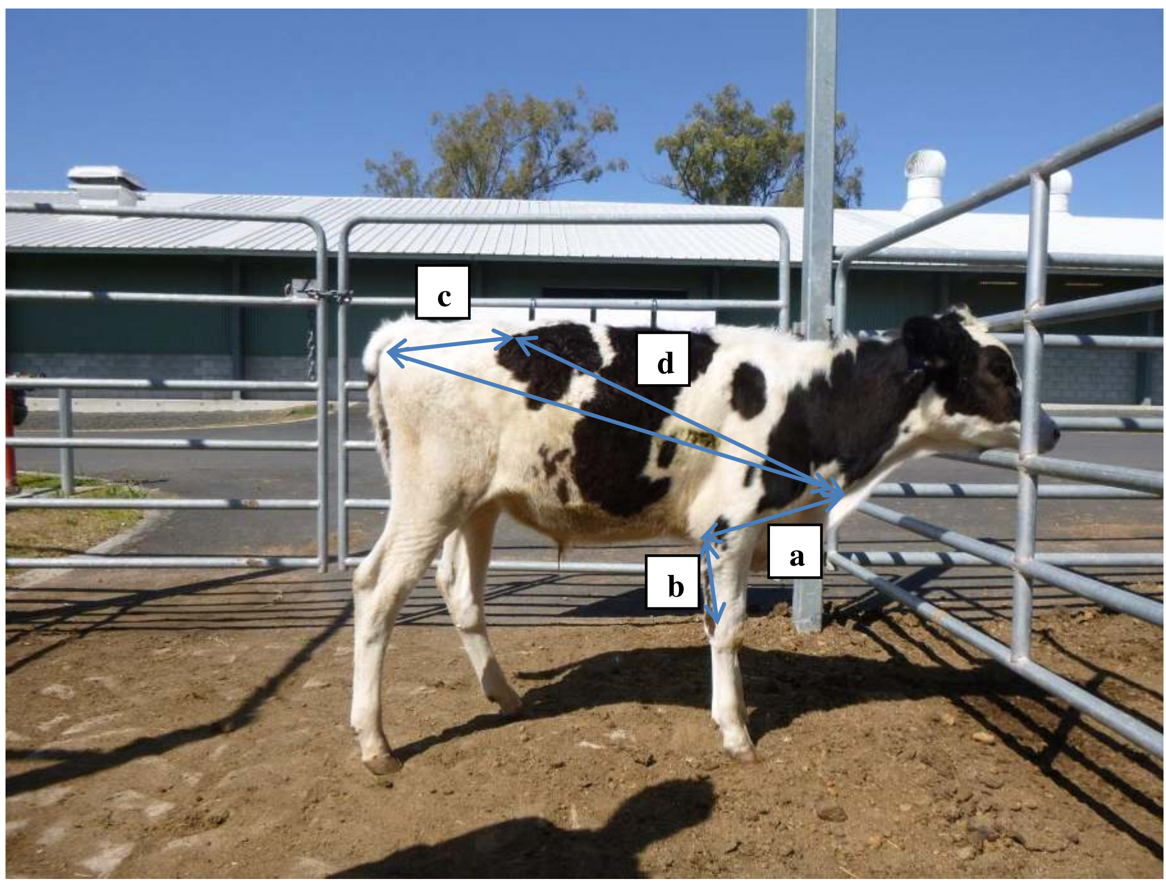


Figure 4-1 Measurement sites of body dimensions: (a) Point of shoulder to point of olecranon (SLD-OLC), (b) point of olecranon to accessory carpal bone (OLC-ACB), (c) tuber coxae to tuber isheii (TC-TI) and (d) point of shoulder to tuber coxae (SLD-TC).

4.3.4. *Blood samples*

Blood plasma samples were collected on days -10, 78, 103, 175 and 203. The methods utilized for collection, storage and analysis of these samples are described in Chapter 3.

The ratio between triiodothyronine (T3) and thyroxine (T4) was calculated as previously described by Wilkin and Isles (1984) using Equation 4-1.

Equation 4-1: $T3{:}\,T4 = \frac{\text{T3 (nmol/L)}}{\text{T4 (nmol/L)}} \text{x}1000$

4.3.5. *Bone samples*

Bone biopsies were collected from the tuber coxae on the left, right and left side of each steer on days -9, 104 and 207 of the experiment respectively. On day 104 the biopsies were collected before the change in diet from Phase 1 to 2. Data collection (i.e. DMI, body measurements and blood samples)

was finished on day 203, however steers were kept being fed the same Phase 2 *ad libitum* diet until day 207. The biopsy collected on day 207 was conducted on the same side as the first biopsy. The analysis of the biopsies collected on day 207 showed that growth plate structure and morphology was completely recovered after the first biopsy taken from the same side of the animal. In addition, all comparisons between growths plates were made within collection date, which means that all treatments would have been equally affected by any carry-over effects of the first biopsy, if they existed.

The procedures of sample collection as well as the methodology used for processing and analysing the samples are described in Chapter 3.

4.3.6. *Laboratory analysis*

The methodology utilised for chemical analysis of the feed offered and residues as well as blood metabolites, hormone analysis and bone metabolism markers are described in Chapter 3.

Thawed urine sub-samples were filtered through a 0.2 μm syringe filter (Phenex-RC, Phenomenex, Torrance, CA, USA) and the concentration of purine derivatives was determined using a high-performance liquid chromatograph method described by Balcells et al. (1992). Microbial crude protein production was then estimated using the method of Chen and Gomes (1995) using the endogenous purine derivative (PDE) values for *Bos indicus* and *Bos taurus* cattle described by Bowen et al. (2006). The equations utilized in for this calculation are summarised below:

Equation 4-2: $PDE = Allantoin\ (mmol/d) + Uric\ Acid\ (mmol/d) + Xanthine\ (mmol/d)$

Equation 4-3: $Bos\ taurus\ Y = \frac{PDE - 0.414LW^{0.75}}{0.85}$

Equation 4-4: $Bos\ indicus\ Y = \frac{PDE - 0.190LW^{0.75}}{0.85}$

Equation 4-5: $Microbial\ N\ (Mic.N;\ g\ N/d)\ \frac{Y\ (mmol/d) \times 70}{0.116\ x 0.83 \times 1000} = 0.727Y$

Equation 4-6: $Microbial\ crude\ protein\ (MCP;\ g/d) = Mic.N \times 6.25$

The concentration of NH_3N in the rumen fluid was measured by titration with 0.01 *M* HCl using a TIM 840 Titration workstation manager (Radiometer Analytical SAS; Villeubanne Cedex, France) after distillation using a semi-automatic distillation unit (UDK 139, Velp Scientifica; Usmate, MB, Italy). The concentration of individual VFA's in the rumen fluid were analysed by gas liquid chromatography (GC-2010, Shimadzu; Kyoto, Honshu, Japan) fitted with a polar capillary column (ZB-FFAP, Phenomenex; Lane Cove, NSW, Australia).

4.4 Statistical analysis

All statistical analyses were conducted using the open-source software R version (R Core Team, 2013) and the linear mixed models procedure of the package "nlme" (Pinheiro et al., 2013). Prior to analysis all data was checked for normality and homoscedasticity and, if necessary, data was transformed according to the box-cox procedure (Osborne, 2010). Liveweight gain and rate of change of body measurements were calculated by regressing each measurement over time for each phase separately. For these parameters the initial LW and HH of each phase were used as covariates. All the other parameters were compared within the time-point at which they were collected. Nutrition and genotype treatments and their interaction were included in the model as fixed effects and steers within block were included as random factors. When analysis of variance showed a significant effect of nutrition treatment or the interaction between nutrition treatment and genotype, a tukey test *post hoc* was performed to explore the differences between groups. If the interaction was not significant ($P>0.05$) it was removed from the model and data was reanalysed including only main factors. Liveweight gain and hip height gain (HHG) were also compared across phases. These models included genotype, nutrition and phase in order to compare LWG and HHG of restricted treatments during Phase 2 with control groups (HCP-HME) during Phase 1. Stepwise linear regression analysis was conducted to assess the effect of ME intake and CP intake on LWG and HHG. The initial models included ME intake, CP intake and genotype as explanatory variables and they were removed when not significant ($P>0.05$). Metabolisable energy intake and CP intake were highly correlated ($r=0.95$) which could violate the collinearity assumption if both variables were included in the final model. However, in none of the models were both variables (i.e. ME and CP intake) shown to be significant simultaneously.

4.5 Results

4.5.1. *Dry matter, metabolizable energy and crude protein intake, liveweight gain, hip height gain, feed conversion ratio, liveweight:hip height and rate of change in body measurements*

The appearance of experimental steers at the end of Phase 1 is shown in Figure 4-2. During Phase 1, steers offered the HCP-HME treatment had higher DM, ME and CP intake than steers offered the HCP-LME and LCP-LME treatments (Table 4-2) while steers offered the LCP-LME treatment had higher DM intake but comparable ME intake and lower CP intake to steers offered the HCP-LME treatment, as planned. During Phase 2, steers that had a low ME intake during Phase 1 (HCP-LME and LCP-LME) had higher DM intake than steers fed HCP-HME during Phase 1 (Table 4-3). When offered *ad libitum* HCP-HME diet, the previously ME restricted steers showed a transitory greater ME intake when compared to control groups at the same LW and genotype (Figure 4-3). The pattern of ME intake over the experimental period showed a decrease as steers increased LW in both genotypes, but HF had a markedly higher ME intake than BX during the whole period (Figure 4-3).

Table 4-2. Dry matter (DM) intake (DMI; g/kg LW.day), metabolizable energy (ME) intake (MEI; MJ/kg LW.day), crude protein (CP) intake (CPI; g CP/kg LW.day), feed conversion ratio (kg DMI/kg LWG), LWG (kg/day), hip height gain (HHG; mm/100 day), liveweight:hip height (LW:HH; kg/cm) and rate of change in distance between other points on the body (TC, OLC, ACB, SLD and TI; mm/100 day) of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments[1] (Phase 1) [2,3]

	Nutrition (N)					**Genotype (G)**				**N x G**	
	HCP-HME	**HCP-LME**	**LCP-LME**	SEM	*P*	**HF**	**BX**	sem	*P*	SEM	*P*
				Phase 1							
DMI	31.5 ^{c}	12.1 ^{a}	20.3 ^{b}	0.60	<0.001	23.6	18.9	0.6	<0.001	0.90	0.06
MEI	0.30 ^{b}	0.12 ^{a}	0.11 ^{a}	0.05	<0.001	0.19	0.16	0.01	<0.05	0.01	0.46
CPI	6.13 ^{c}	2.00 ^{b}	0.84 ^{a}	0.27	<0.001	3.15	2.82	0.22	0.15	0.22	0.06
FCR	6.3 ^{a}	30.2 ^{b}	47.2 ^{b}	10.1	<0.001	29.3	27.0	9.0	0.32	11.3	0.10
LWG	1.27 ^{b}	0.16 ^{a}	0.14 ^{a}	0.03	<0.001	0.58	0.47	0.04	<0.05	0.06	0.02
HHG	104.7 ^{c}	41.8 ^{b}	30.3 ^{a}	3.10	<0.001	63.0	54.4	2.53	<0.05	6.20	0.01
LW:HH[4]	2.1 ^{c}	1.5 ^{a}	1.7 ^{b}	0.04	<0.001	1.96	1.65	0.08	<0.001	0.15	0.41
TC-TC[5]	8.4 ^{b}	1.7 ^{a}	1.6 ^{a}	0.3	<0.001	4.5	3.3	0.3	0.58	0.4	0.29
OLC-ACB	1.82 ^{b}	0.48 ^{a}	0.04 ^{a}	0.3	<0.001	0.8	0.8	0.2	0.97	0.4	0.67
SLD-TC	18.6 ^{b}	4.5 ^{a}	3.5 ^{a}	2.4	<0.001	10.1	7.6	1.9	0.37	3.4	0.41
TC-TI	5.0 ^{b}	0.9 ^{a}	0.6 ^{a}	0.2	<0.001	3.0	1.4	0.2	<0.001	0.4	0.44
SLD-OLC	3.6 ^{b}	1.5 ^{a}	2.1 ^{ab}	0.4	<0.05	2.6	2.2	0.4	0.45	0.7	0.42

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)]

[2]Data are least squares means with standard error of the mean (SEM)

[3]Means within a row with different superscripts differ ($P<0.05$)

[4]LW:HH at end of Phase 1

[5]Tuber coxae (TC), Tuber isheii (TI), Olecranon (OLC), Accessory carpal bone (ACB) and point of shoulder (SLD).

Table 4-3. Dry matter (DM) intake (DMI; g/kg LW.day), metabolizable energy (ME) intake (MEI; MJ/kg LW.day), crude protein (CP) intake (CPI; g CP/kg LW.day), feed conversion ratio (kg DMI/kg LWG), LWG (kg/d), hip height gain (HHG; mm/100 day), liveweight:hip height (LW:HH; kg/cm) and rate of change in distance between other points on the body (TC, OLC, ACB, SLD and TI; mm/100 day) of *Bos taurus* (HF) and *Bos indicus* (BX) steers undergoing re-alimentation (Phase 2)[2,3]

	Nutrition (N)[4]					**Genotype (G)**				**N x G**	
	HCP-HME	**HCP-LME**	**LCP-LME**	SEM	*P*	**HF**	**BX**	SEM	*P*	SEM	*P*
				Phase 2							
DMI	26.6 [a]	32.6 [b]	32.2 [b]	0.5	<0.001	32.1	28.8	0.4	<0.001	0.6	0.27
MEI	0.25 [a]	0.31 [b]	0.30 [b]	0.03	<0.001	0.30	0.27	0.01	<0.001	0.02	0.20
CPI	4.41	5.39	5.34	0.12	<0.001	5.33	4.77	0.10	<0.001	0.17	0.24
FCR	11.2 [a]	6.8 [b]	6.1 [b]	0.56	<0.001	9.2	6.8	0.36	<0.001	0.49	0.74
LWG	0.93 [a]	1.34 [b]	1.45 [b]	0.04	<0.001	1.3	1.2	0.04	0.08	0.07	0.04
HHG	75 [a]	89 [b]	89 [b]	5.76	<0.05	92.0	77.1	7.06	<0.05	7.0	0.38
LW:HH[5]	2.9 [b]	2.1 [a]	2.2 [a]	0.07	<0.001	2.6	2.2	0.06	<0.001	0.07	0.32
TC-TC[6]	7.7 [a]	9.1 [ab]	9.8 [b]	0.5	<0.05	9.3	8.5	0.4	0.24	0.7	0.63
OLC-ACB	2.2	2.9	2.6	0.4	0.42	2.6	2.6	0.3	0.94	0.5	0.80
SLD-TC	4.0 [a]	10.8 [b]	13.4 [b]	1.5	<0.001	8.0	10.8	1.3	0.16	3.3	0.89
TC-TI	4.2 [a]	5.8 [b]	6.4 [b]	0.5	<0.001	5.6	5.3	0.3	0.44	0.5	0.05
SLD-OLC	3.0	3.4	3.3	0.6	0.93	2.82	3.62	0.5	0.31	0.9	0.22

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)]

[2]Data are least squares means with standard error of the mean (SEM)

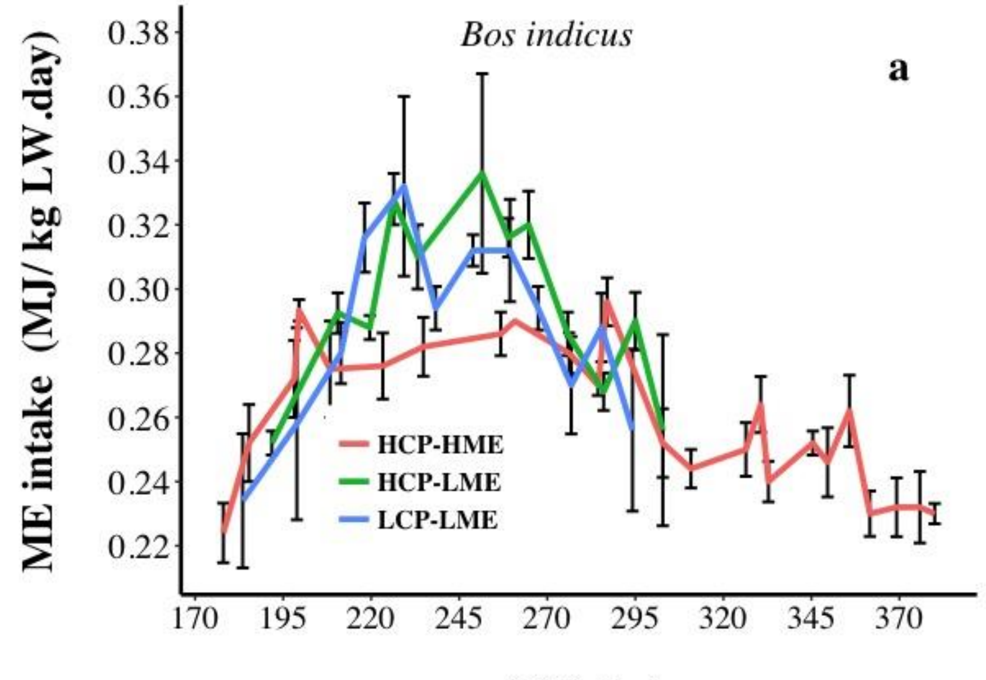
[3]Means within a row with different superscripts differ ($P<0.05$)

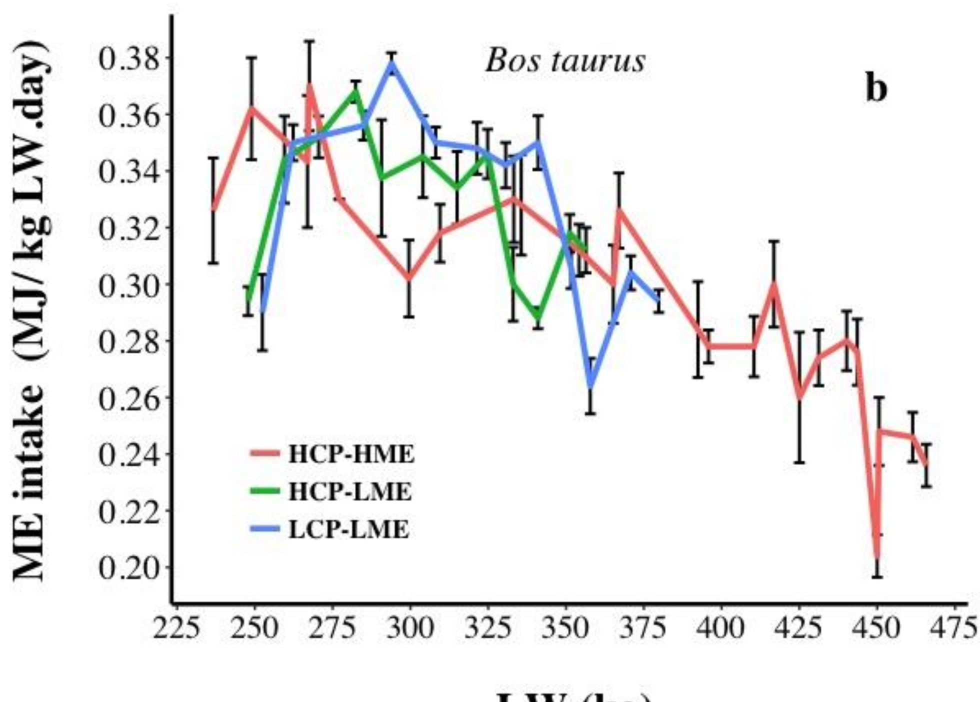
[4]Nutritional treatment provided during Phase 1 of the experiment

[5]LW:HH at end of Phase 2

[6]Tuber coxae (TC), Tuber isheii (TI), Olecranon (OLC), Accessory carpal bone (ACB) and point of shoulder (SLD)

Figure 4-2. Appearance of *Bos indicus* (a, b and c) and *Bos taurus* (d, e and f) steers at the start of Phase 2, after approximately 100 days being fed HCP-HME (a. and d.), HCP-LME (b. and e.) and LCP-LME (c. and f.) respectively.


Bos indicus
a
ME intake (MJ/ kg LW.day)
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
HCP-HME
HCP-LME
LCP-LME
170 195 220 245 270 295 320 345 370
LW (kg)


Bos taurus
b
ME intake (MJ/ kg LW.day)
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
HCP-HME
HCP-LME
LCP-LME
225 250 275 300 325 350 375 400 425 450 475
LW (kg)

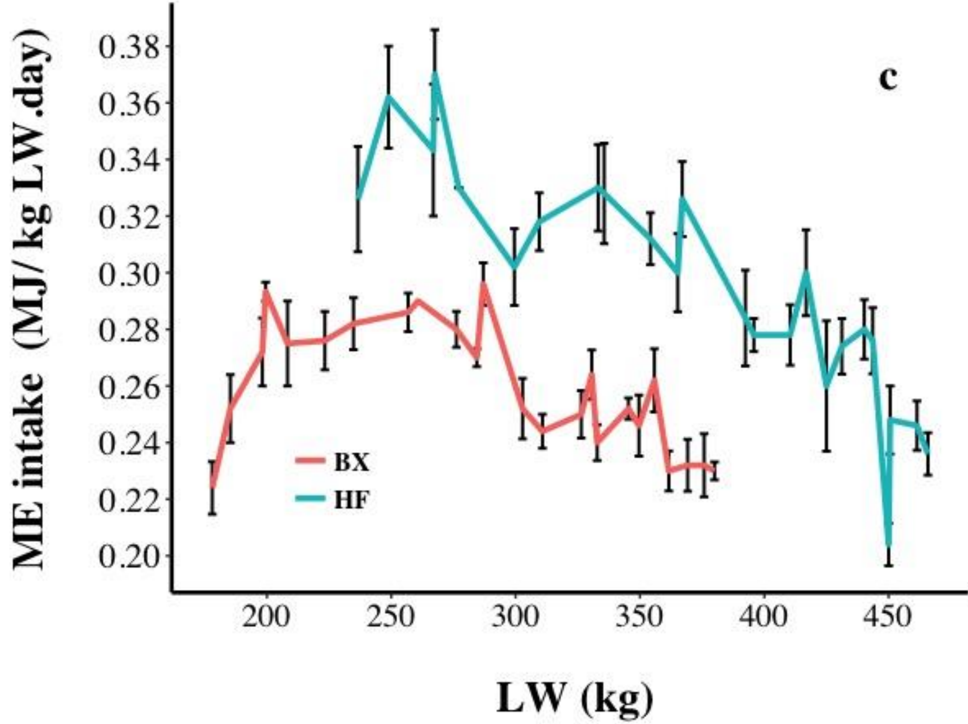


Figure 4-3. Relationship between metabolizable energy (ME) intake and liveweight (LW) of *Bos indicus* (a) and *Bos taurus* (b) steers fed different nutritional treatments in Phase 1[1] and HCP-HME in Phase 2, and the relationship between ME intake of *Bos indicus* (BX) and *Bos taurus* (HF) fed HCP-HME throughout the whole experimental period (c).

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)]. Each point is the arithmetic mean of 5 steers with standard error of the mean.

Liveweight gain of HCP-HME steers was higher in Phase 1 and lower in Phase 2 compared with HCP-LME and LCP-LME steers, with no difference in LWG between HCP-LME and LCP-LME steers in either phase (Table 4-2, Table 4-3, Figure 4-4). Holstein-Friesian steers had higher LWG than BX steers in Phase 1 but not Phase 2 of the experiment (Table 4-2 and Table 4-3). There were significant interactions between nutritional and treatment genotype in both phases of the experiment (Figure 4-5 and Figure 4-6). HF steers consuming lucerne chaff *ad libitum* grew faster than BX on the same nutritional treatment. HF steers fed LCP-LME during Phase 1 showed a tendency (P=0.07) for greater LWG than HCP-LME during Phase 2. This was the only group that showed greater (1.3 *vs* 1.5; P<0.05) LWG during Phase 2 when compared to HCP-HME steers during Phase 1. Steers fed ME restricted diets (HCP-LME and LCP-LME) had a much less efficient feed conversion compared to HCP-HME during Phase 1. During Phase 2, the previously restricted steers converted DM into LW more efficiently than steers fed HCP-HME throughout the experiment.

The rate of skeletal growth for all dimensions measured was higher in HCP-HME steers than HCP-LME and LCP-LME steers during Phase 1 (Table 4-2 and Figure 4-5). Hip height gain was higher in HCP-HME but also higher in HCP-LME steers compared with LCP-LME steers but no other differences in rate of change of body dimensions were observed in response to the additional CP intake by these steers during Phase 1. A significant interaction between nutritional treatment and genotype was found in Phase 1, the differences followed the same pattern of LWG (Figure 4-5). During Phase 2 the rate of HH gain was greater in HCP-LME and LCP-LME steers compared to HCP-HME steers, with changes in TC-TC, TC-TI and TC-SLD reflecting changes in HH (Table 4-3). The HH gain of restricted steers (i.e. HCP-LME and LCP-LME) during Phase 2 was not significantly different from HCP-HME steers during Phase 1. Hip height gain was higher in HF steers compared with BX steers in both phases of the experiment but differences in rate of change of other body dimensions was generally similar between genotypes during both phases.

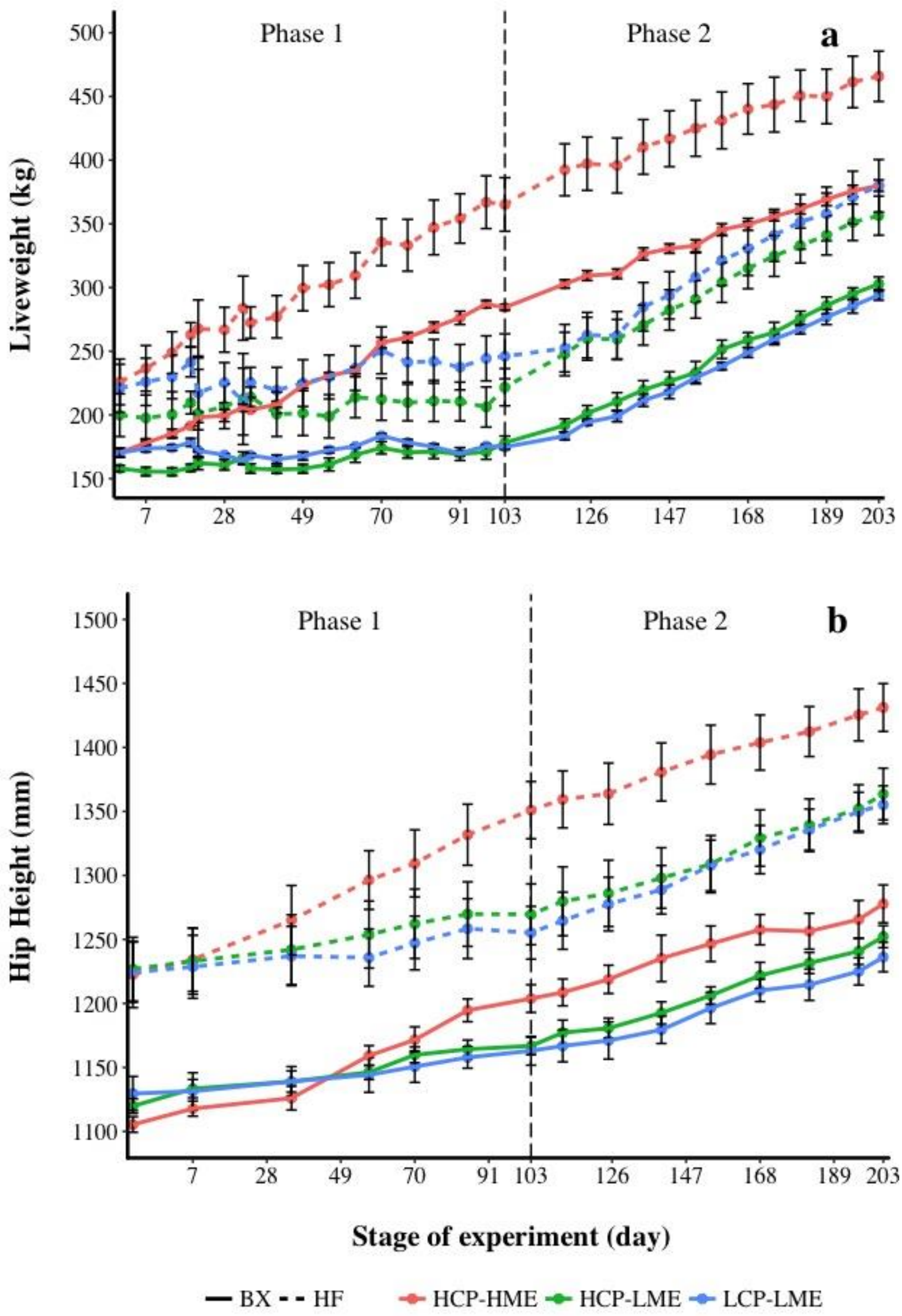


Figure 4-4. Change in liveweight (a) and hip height (b) of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1) and undergoing re-alimentation (Phase 2)[1].

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)].

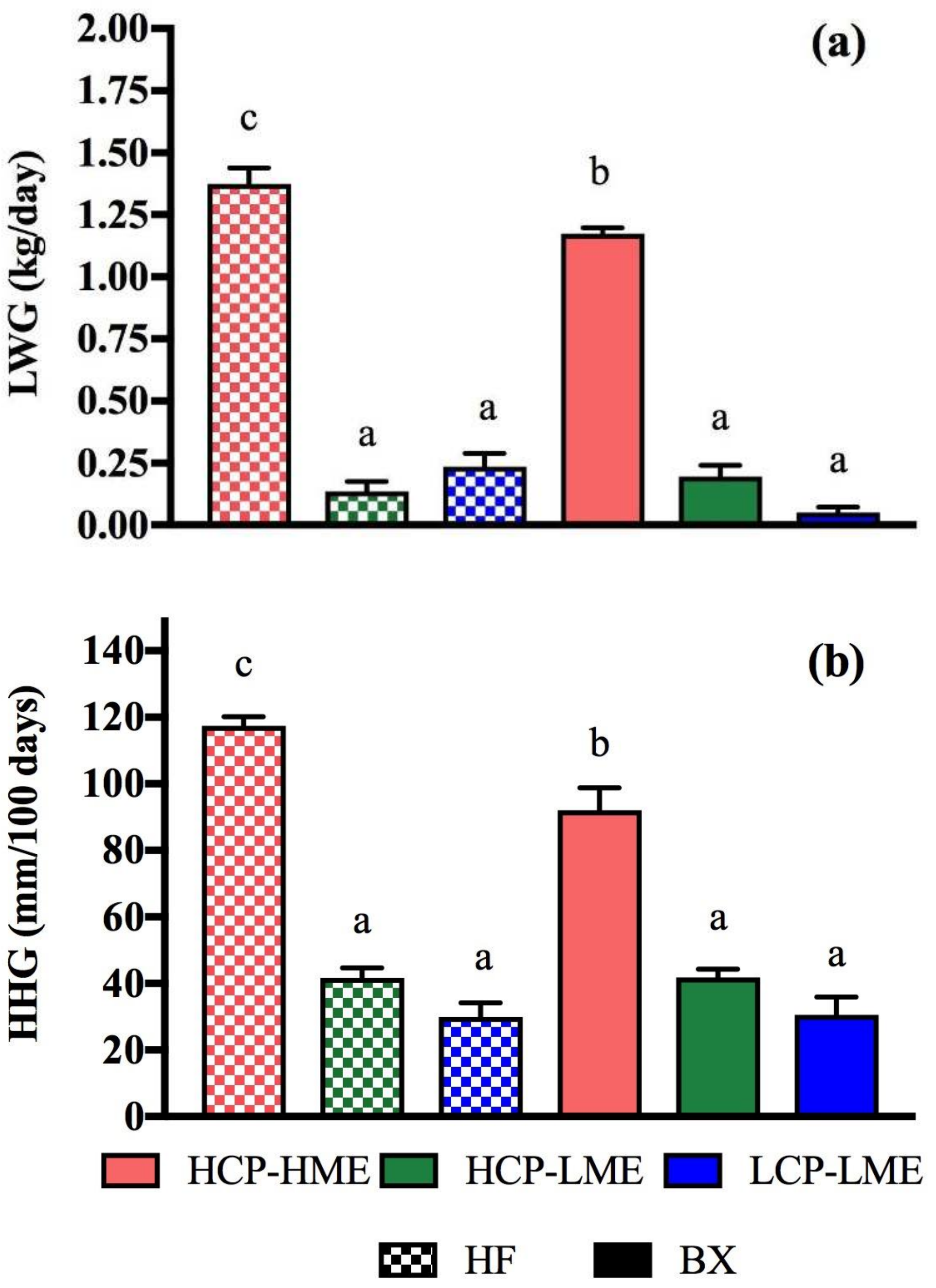


Figure 4-5. Liveweight gain (LWG; a) and hip height gain (HHG; b) of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)].

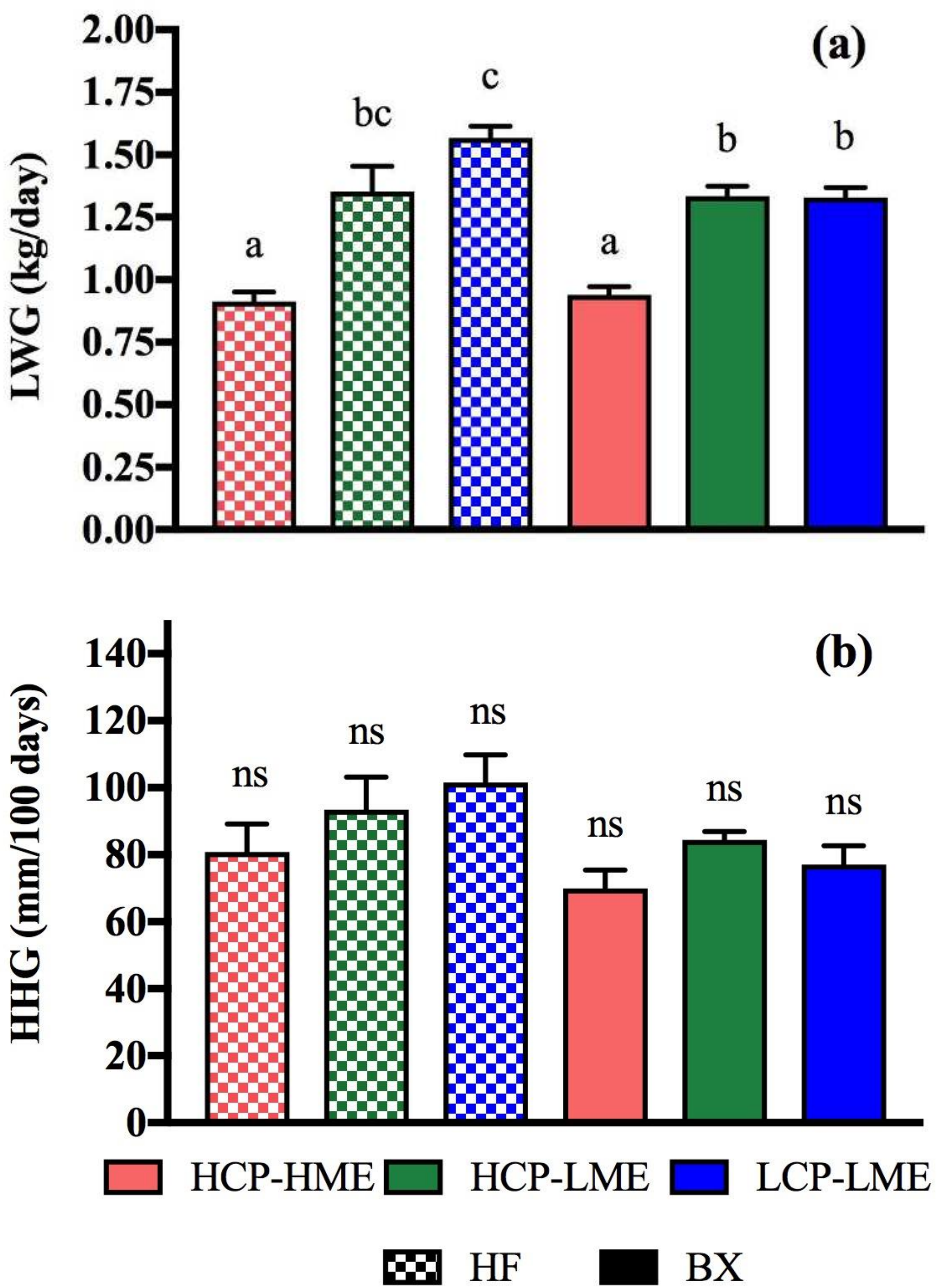


Figure 4-6. Liveweight gain (LWG; a) and hip height gain (HHG; b) of *Bos taurus* (HF) and *Bos indicus* (BX) steers undergoing re-alimentation (Phase 2). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, P<0.05)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

4.5.2. *Rumen parameters and microbial protein production*

The digestibility of the lucerne chaff did not differ with intake in the current experiment [65.6% and 67.2% at *ad libitum* (HCP-HME) and restricted intakes (HCP-LME)] but was significantly higher ($P<0.01$) than the digestibility of the Mitchell grass hay (40.1%) consumed by steers offered the LCP-LME treatment; digestibility was unaffected by genotype. Steers fed the Mitchell grass hay based diet (LCP-LME) had a lower concentration of NH_3N in the rumen and produced less MCP and had a lower EMCP than steers fed the lucerne chaff based diet (HCP-HME and HCP-LME) regardless of DM and ME intake (Table 4-4). HF steers fed the LME-LCP treatment diet had a lower concentration of NH_3N in the rumen than BX steers fed the same nutritional treatment (Figure 4-7). Microbial protein production but not the EMCP was greater in steers fed lucerne chaff *ad libitum* (HCP-HME) compared with steers fed a restricted amount of lucerne chaff (HCP-LME). Steers with restricted DM intake (HCP-LME) had a lower concentration of VFA in the rumen than steers fed *ad libitum* (HCP-HME and LCP-LME) regardless of the CP content of the diet. The interaction between genotype and nutritional treatments showed that this effect was only observed in HF steers (Figure 4-7).

Table 4-4. The dry matter digestibility (DMD; %), pH, concentration of ammonia (NH_3N; mg/L) and volatile fatty acids (VFA, mmol/L) and the molar percentage of acetic, butyric and propionic acids in the rumen fluid and the microbial protein (MCP) production (g/kg LW.day) and the efficiency of MCP production (EMCP; g MCP/kg DOMI) of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments[1] during Phase 1 of the experiment [2,3]

	Nutrition (N)					Genotype (G)				N x G	
	HCP-HME	**HCP-LME**	**LCP-LME**	SEM	*P*	**HF**	**BX**	SEM	*P*	SEM	*P*
DMD	65.6 ^{b}	67.2 ^{b}	40.1 ^{a}	1.3	<0.001	56.7	58.6	1.6	0.11	2.0	0.59
pH	7.20 ^{b}	7.27 ^{b}	6.90 ^{a}	0.1	<0.001	7.14	7.11	0.1	0.65	0.1	0.16
NH_3N	131.4 ^{b}	130.9 ^{b}	49.7 ^{a}	11.0	<0.001	87	121	6.4	<0.005	11.0	0.04
MCP prod.	2.17 ^{c}	0.62 ^{b}	0.32 ^{a}	0.1	<0.001	1.15	0.92	0.1	0.77	0.1	0.45
EMCP	91.0 ^{b}	72.6 ^{b}	32.2 ^{a}	9.7	<0.001	65.8	64.7	5.4	0.88	8.5	0.38
Total VFA	47.8 ^{b}	36.5 ^{a}	51.7 ^{b}	2.5	<0.001	45.2	45.4	2.0	0.96	3.5	0.04
Acetic	74.1 ^{a}	74.2 ^{a}	78.1 ^{b}	0.5	<0.001	76.7	74.2	0.5	<0.001	0.7	0.21
Propionic	13.0 ^{a}	12.3 ^{a}	14.4 ^{b}	0.3	<0.001	13.2	13.3	0.2	0.76	0.4	0.40
Butyric	8.4 ^{c}	4.15 ^{b}	2.5 ^{a}	0.6	<0.001	6.5	3.4	0.5	<0.001	0.8	0.45

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

[2]Data are least squares means, with standard error of the mean (SEM)

[3]Means within a row with different superscripts differ (P<0.05)

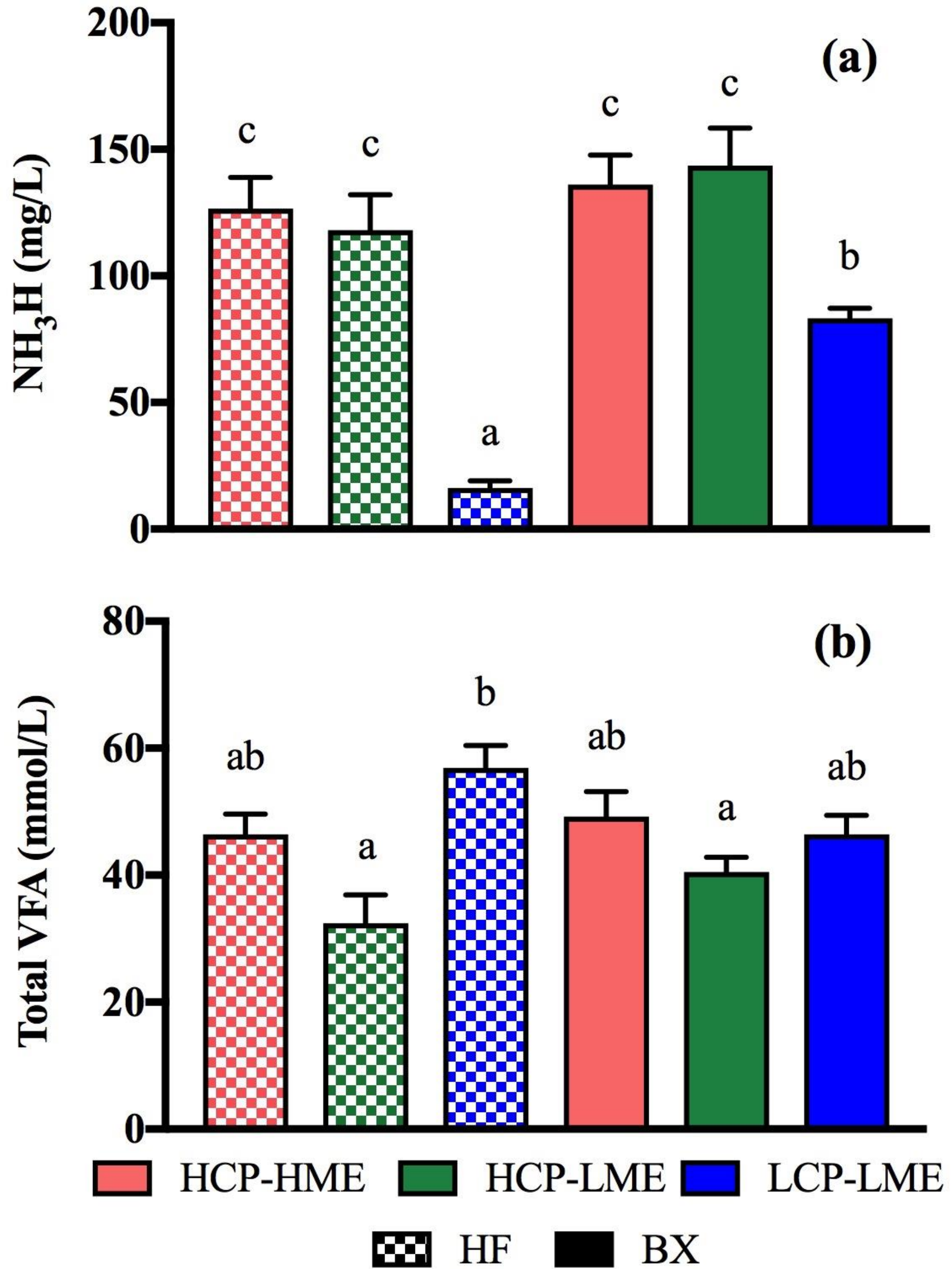


Figure 4-7 Concentration of rumen ammonia (NH_3H; a) and total volatile fatty acids (VFA; b) in *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

4.5.3. *Plasma metabolites, hormones and bone metabolism markers*

The concentration of blood metabolites, hormones and bone metabolism markers were statistically analysed within collection date for the effect of nutritional treatments, genotype as well as the

interaction between both factors. All main factors are shown in separate Figures as well as the significant interactions.

The plasma concentration of glucose and Ca were both affected by nutrition during Phase 1 of the experiment (Table 4-5), which were higher in steers consuming HCP-HME diet. Urea-N concentration was higher in the plasma of steers fed high CP treatment regardless of ME intake. Nutritional treatment had no effect on the concentration of total protein, NEFA or inorganic P in the plasma during Phase 1 of the experiment. There were no significant differences in any of the metabolites analysed during Phase 2 of the experiment when steers were consuming the same diet. The concentration of NEFA and PUN was higher in the plasma of BX steers compared with HF steers at the end of both phases of the experiment.

In Phase 1 the concentration of insulin and IGF-1 were higher (P<0.0001) in the plasma of steers fed the HCP-HME diet compared with steers fed the LME diets (

Figure 4-8), with no difference in concentration in steers offered the HCP-LME and LCP-LME diets. Leptin concentration was not affected by the nutritional treatments. BX had initially higher concentration of leptin than HF but no differences were observed between two genotypes after the imposition of the nutritional treatments. During Phase 1, the plasma T3 hormone showed an additive response to dietary CP and ME intake (Figure 4-9). It was highest in steers fed HCP-HME followed by HCP-LME and lowest in LCP-LME group. No significant differences were found when comparing the concentration of T3 between the genotypes (2.3 vs 2.0 nmol/L for HF and BX respectively; P=0.82; results not shown). The adiponectin concentration was not different between genotypes throughout the experiment. Interestingly, the nutritional restriction treatments had no effect on the concentration of adiponectin by the end of the Phase 1, but there was a significant higher (P=0.02) adiponectin concentration in steers that consumed HCP-HME diet compared to HCP-LME by the end of Phase 2. Adponectin was the only hormone that showed significant different response to the nutritional treatments imposed during Phase 1. There was also no genotype effect on any hormone at the end of either phases of the experiment (Figure 4-10). However, a significant interaction between genotype and nutritional treatments at the end of Phase 1 was found for T4, with HF steers having significantly (P=0.004) lower concentrations of T4 when submitted to reduced intake of CP and ME while BX steers were not affected (Figure 4-11). The decrease in T4 in HF fed low CP and ME diets led to a rise in T3:T4 ratio which was significantly higher than BX and HF steers fed high protein low ME and also BX steers fed low CP and low ME diet (Figure 4-12).

Table 4-5 The concentration of glucose (mmol/L), total protein (g/L), non-esterified fatty acids (NEFA; mEq/L), plasma urea nitrogen (mmol/L), inorganic P (mmol/L) and total Ca (mmol/L) in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments[1] (Phase 1) and undergoing re-alimentation (Phase 2)[2,3]

	Nutrition (N)					**Genotype (G)**				**N x G**	
	HCP-HME	**HCP-LME**	**LCP-LME**	SEM	*P*	**HF**	**BX**	SEM	*P*	SEM	*P*
					Phase 1						
Glucose	5.2 [b]	3.9 [a]	3.7 [a]	0.09	<0.001	4.2	4.3	0.07	0.82	0.14	0.36
Total Protein	65.7	66.1	64.7	1.4	0.62	67	64	1.2	0.05	1.8	0.46
NEFA	0.20	0.16	0.11	0.03	0.24	0.10	0.22	0.02	0.001	0.04	0.45
PUN	7.1 [b]	7.5 [b]	1.2 [a]	0.2	<0.001	4.8	5.8	0.2	0.007	0.4	0.92
P	2.3	2.1	2.3	0.09	0.22	2.2	2.3	0.08	0.62	0.14	0.85
Ca	2.4 [b]	2.1 [a]	2.1 [a]	0.03	<0.001	2.2	2.2	0.02	0.90	0.04	0.28
					Phase 2						
Glucose	4.8	4.9	5.0	0.1	0.19	4.9	4.8	0.08	0.28	0.14	0.63
Total Protein	67.2	65.0	65.9	1.2	0.52	67.4	64.6	1.0	0.06	1.8	0.35
NEFA	0.14	0.16	0.13	0.03	0.74	0.09	0.19	0.02	0.007	0.04	0.35
PUN	7.7	7.8	7.4	0.2	0.64	7.0	8.3	0.2	<0.001	0.3	0.04
P	2.2	2.3	2.3	0.08	0.70	2.3	2.3	0.07	0.81	0.12	0.84
Ca	2.0	2.1	2.2	0.05	0.23	2.1	2.0	0.04	0.17	0.07	0.61

[1]See materials and methods for description of nutritional treatments [high CP intake and high ME intake (HCP-HME), high CP intake and low ME intake (HCP-LME) and low CP intake and low ME intake (LCP-LME)]

[2]Data are least squares means with standard error of the mean (SEM)

[3]Means within a row with different superscripts differ ($P<0.05$)

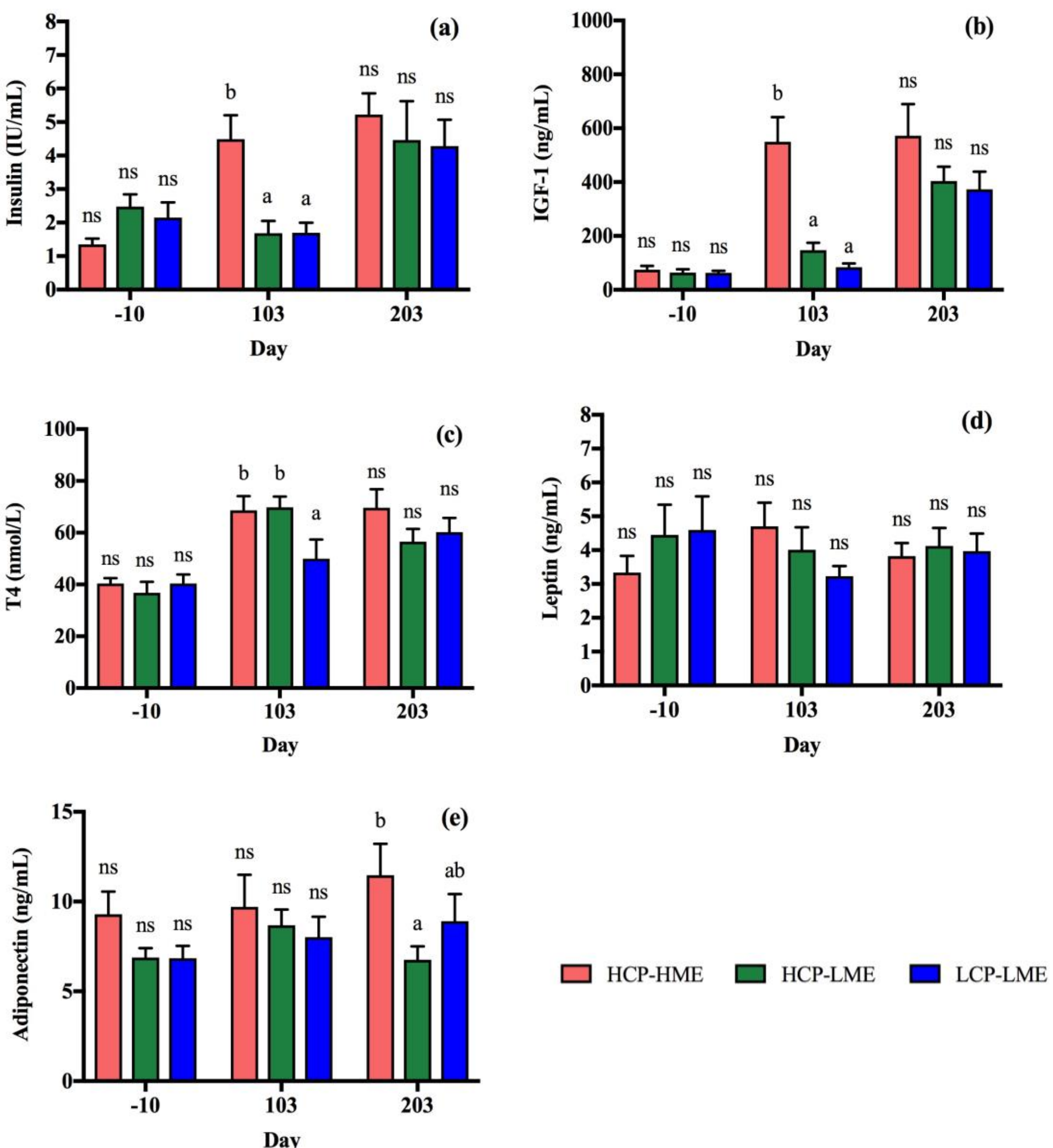


Figure 4-8 The plasma concentration of insulin (IU/mL; a), insulin-like growth factor-1 (IGF-1; ng/mL; b), thyroxine (T4; nmol/L; c), leptin (ng/mL; d) and of adiponectin (ng/mL; e) of steers fed different nutritional treatments prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, P<0.05) and ns is non-significant[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

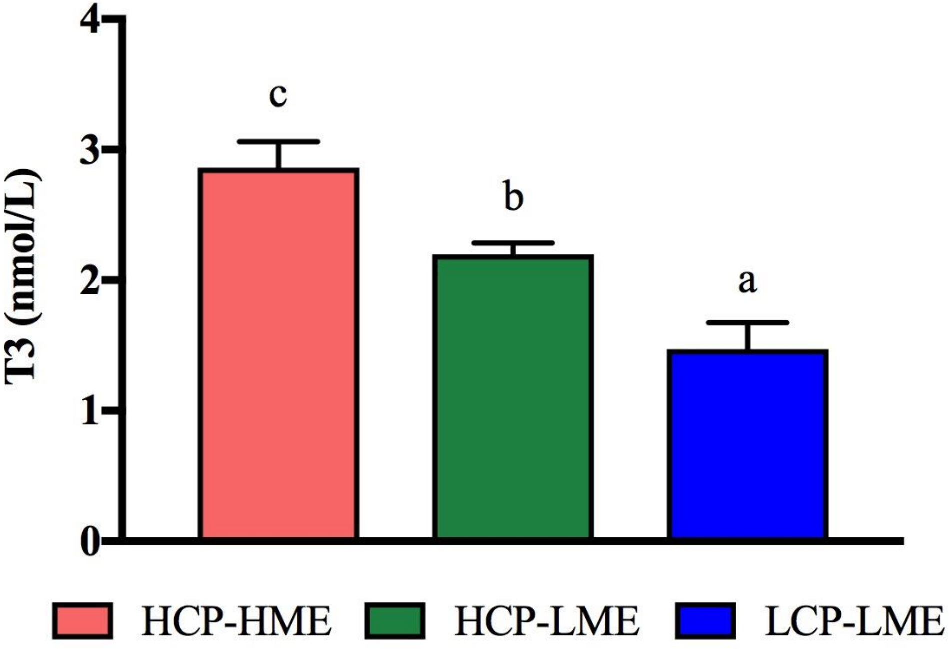


Figure 4-9 The plasma concentration of triiodothyronine (T3; nmol/L) of steers fed different nutritional treatments at the end of Phase 1 (day 103). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

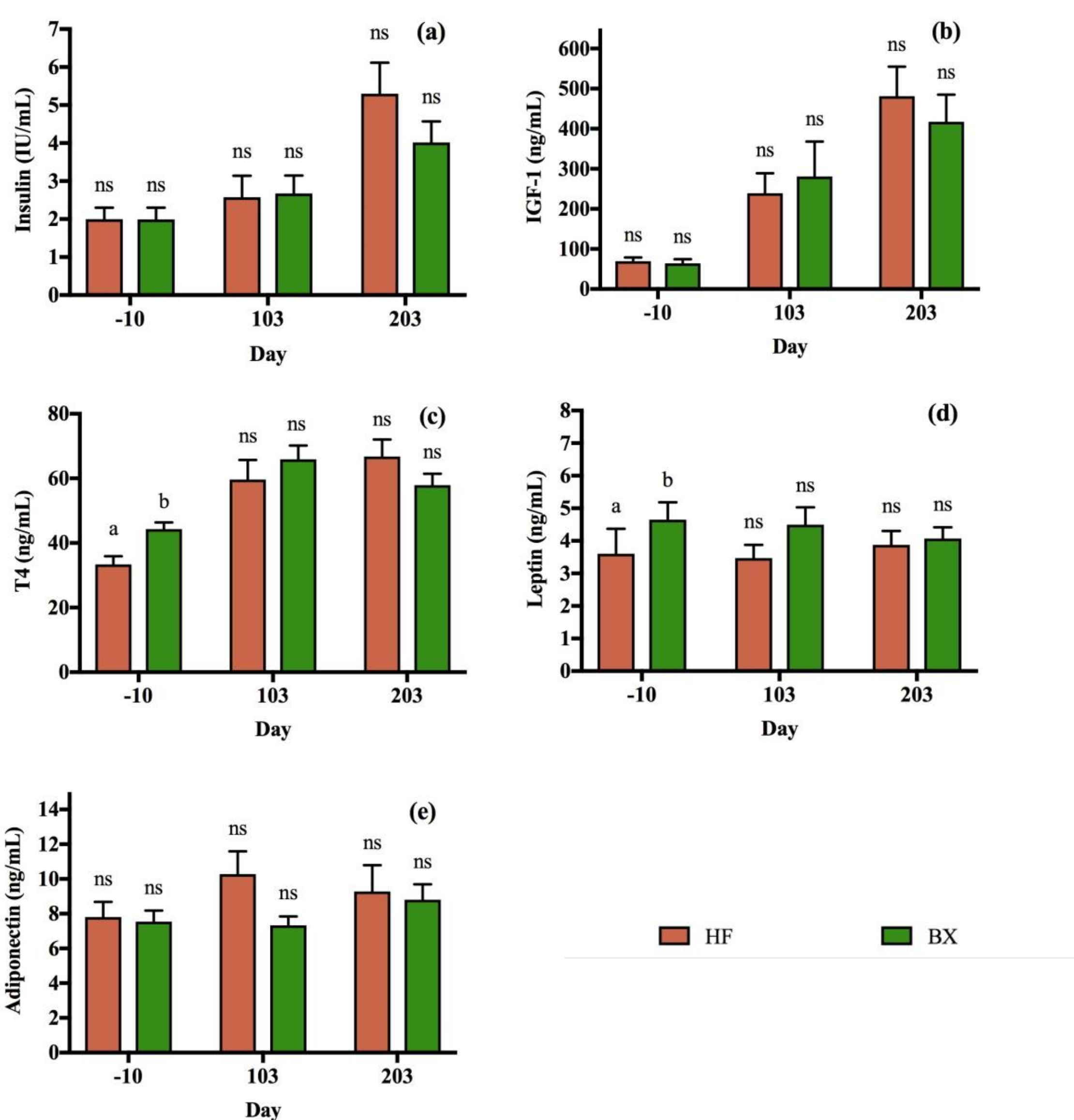


Figure 4-10 The plasma concentration of insulin (IU/mL; a), insulin-like growth factor-1 (IGF-1; ng/mL; b) thyroxine (T4; nmol/L; c), leptin (ng/mL; d) and adiponectin (ng/mL; e) of *Bos taurus* (HF) and *Bos indicus* (BX) steers prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant

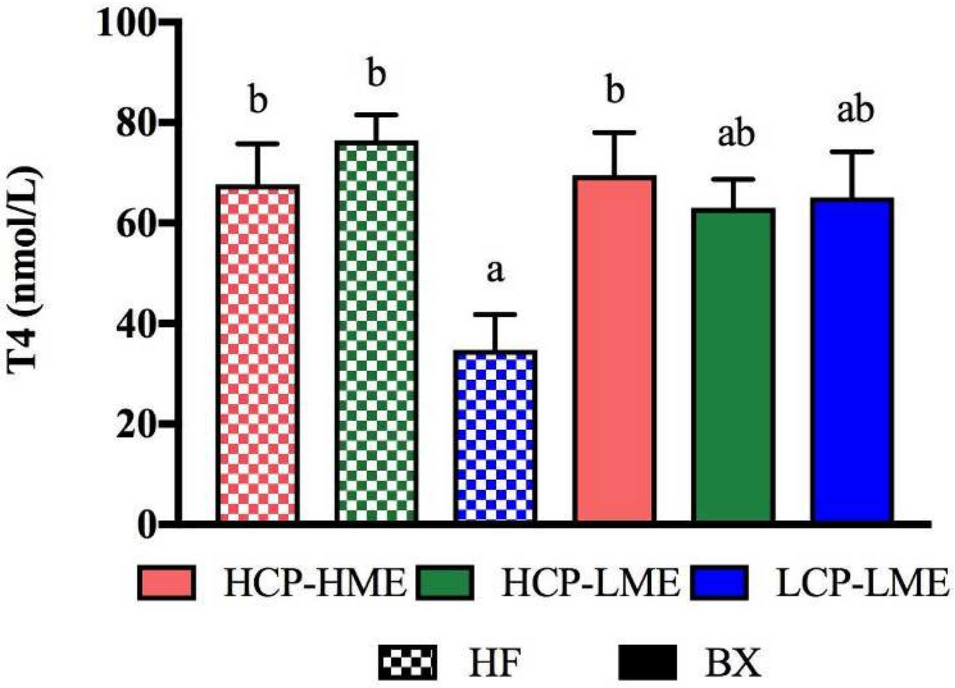


Figure 4-11 Concentration of thyroxine (T4) in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

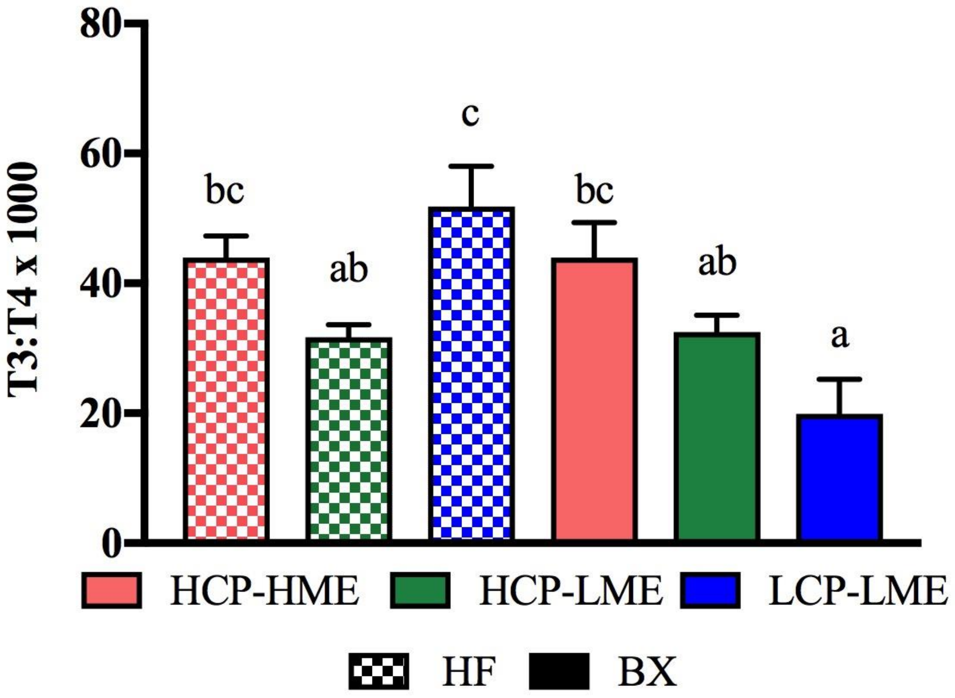


Figure 4-12 Triiodothyronine (T3) and thyroxine (T4) ratio in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

During Phase 1, BAP concentrations increased markedly in HCP-HME steers compared to steers consuming low energy diets (HCP-LME and LCP-LME) (Figure 4-13). In contrast, so significant nutritional effects on plasma OCN concentrations were found. For HCP-HME steers, tDPD was increased at the end of Phase 1, being significantly ($P<0.05$) different from HCP-LME steers, but not LCP-LME steers. Further, a significant interaction between nutritional and genotype effects was found for tDPD concentration. There was lower plasma tDPD concentrations in HCP-LME compared to LCP-LME, at the end of Phase 1, and this effect was observed in HF but not BX steers (Figure 4-15). For PYD, LCP-LME steers had significantly ($P<0.05$) higher PYD than HCP-LME steers, but HCP-HME steers were not different from either other group. Overall there was no significant effect of genotype on bone biomarker concentrations in Phase 1, but at the end of Phase 2 (day 203) HF had higher plasma PYD and OCN but lower concentration of tDPD concentrations than BX steers (Figure 4-14). In general nutritional treatments applied in Phase 1 had no effect on the concentration of most of the bone metabolism markers by the end of the re-alimentation phase (Phase 2 except *Bos taurus* steers fed LCP-LME during Phase 1 showed a higher concentration of BAP on Phase 2 than steers of the same genotype offered HCP-LME (Figure 4-16).

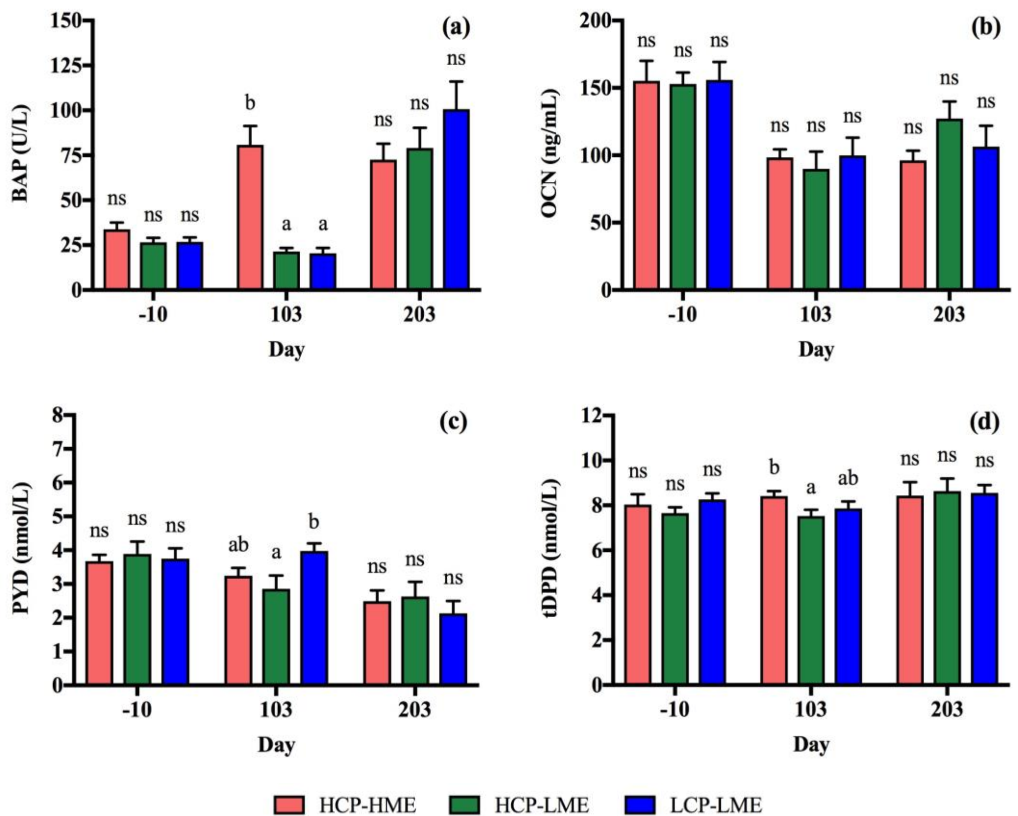


Figure 4-13 The plasma concentration of bone alkaline phosphatase (BAP; U/L; a), osteocalcin (OCN; ng/mL; b), pyridinoline (PYD; nmol/L; c) and total deoxypyridinoline (tDPD; nmol/L; d) of steers prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

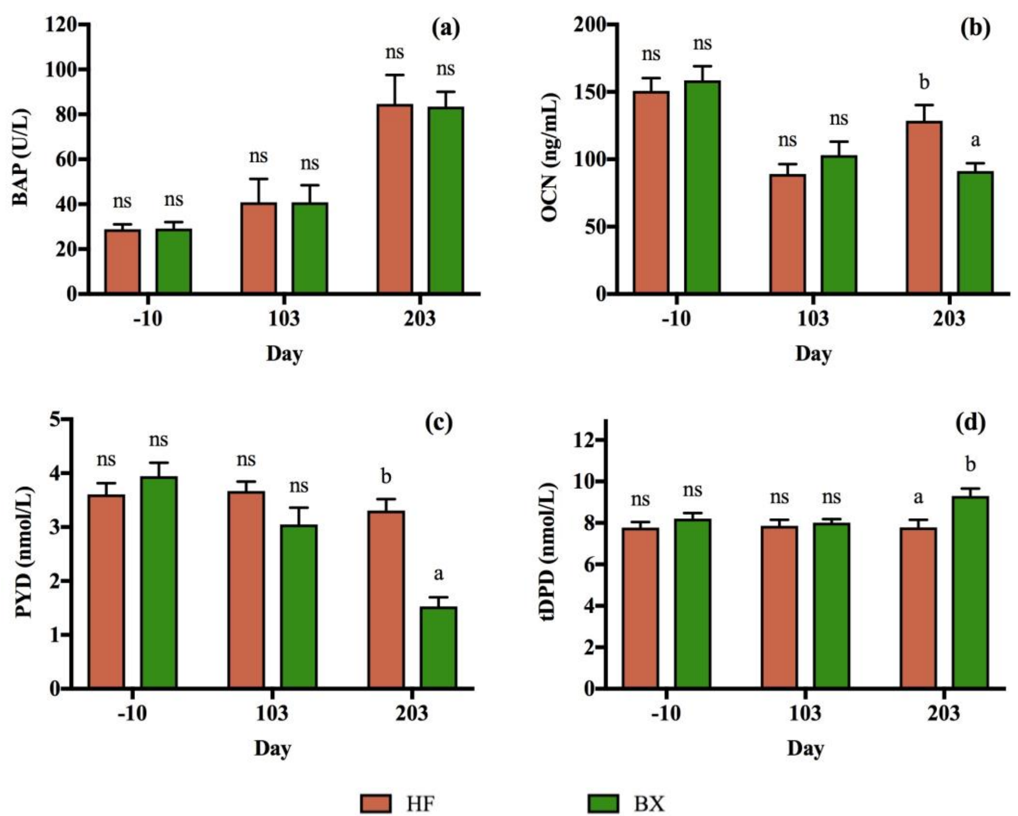


Figure 4-14 The concentration of bone alkaline phosphatase (BAP; U/L; a), osteocalcin (OCN; ng/mL; b), pyridinoline (PYD; nmol/L; c) and total deoxypyridinoline (tDPD; nmol/L; d) in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant.

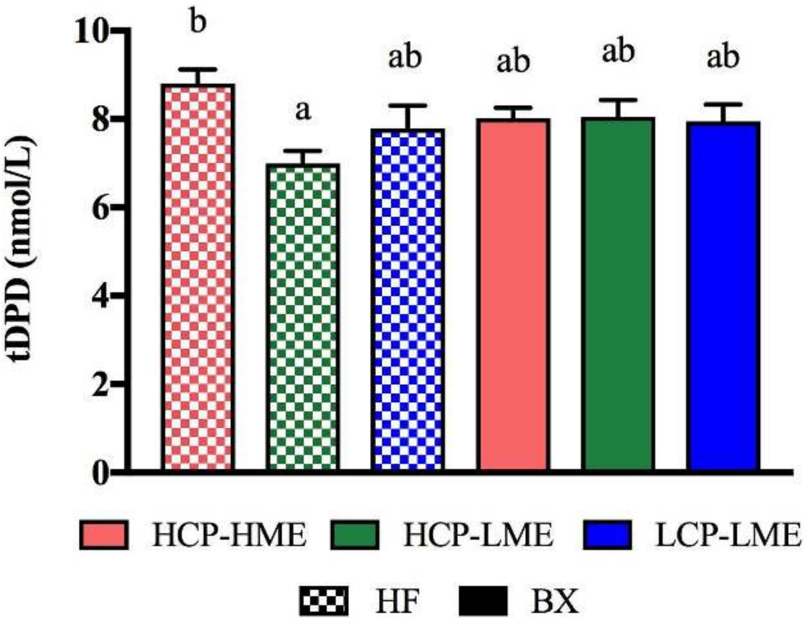


Figure 4-15 Concentration of total deoxypyridinoline (tDPD) in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, P<0.05)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

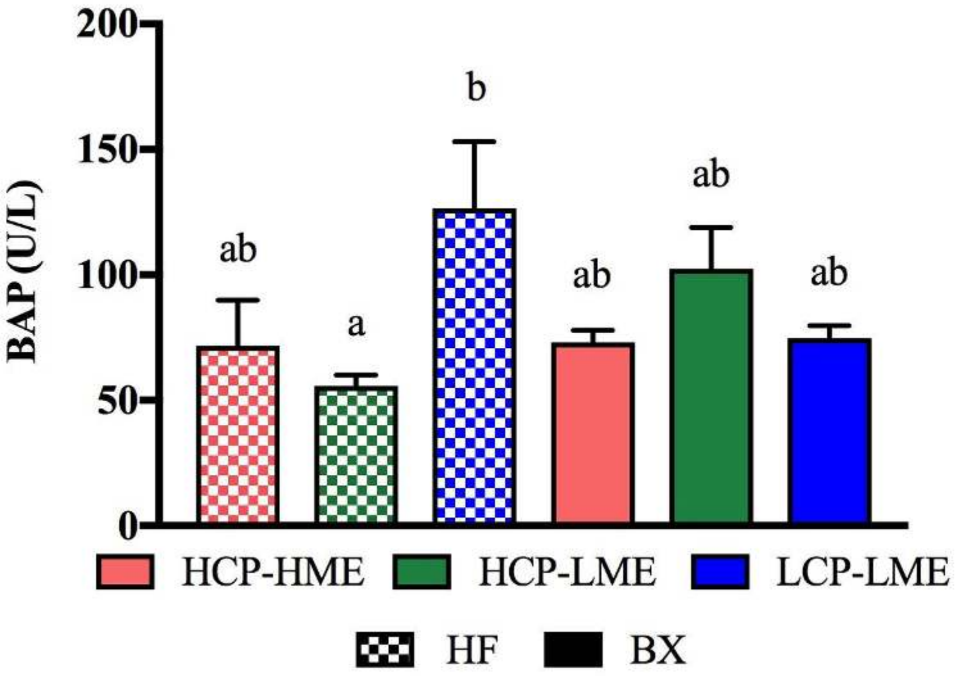


Figure 4-16 Concentration of bone alkaline phosphatase (BAP) in the plasma of *Bos taurus* (HF) and *Bos indicus* (BX) steers undergoing re-alimentation (Phase 2). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$)[1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

4.5.4. *Growth plate and trabecular bone histology*

Bone histological changes in trabecular bone were monitored in biopsies taken from the tuber coxae bone and bone slices examined histologically to determine proliferative and hypertrophic zones and their characteristics. Reduced ME intake decreased the height of the hypertrophic zone of the growth plate independent of the level of CP intake (Figure 4-17 and Figure 4-18). Conversely, height of the proliferative phase was only affected in *Bos taurus* but not in *Bos indicus* steers (Figure 4-20). The diameter of terminal hypertrophic chondrocytes showed an additive effect for CP and ME intake. There were more chondrocytes per column at the hypertrophic zone of HCP-HME than HCP-LME but no differences were found between LME treatments. At the end of Phase 2, the proliferative zone of steers fed LME in Phase 1 was higher than HCP-HME. In addition, there was a tendency ($P=0.07$) for the hypertrophic zone to be significant thicker in these treatments. In all collection dates, proliferative and hypertrophic zones were thicker in HX steers (Figure 4-20). The number of hypertrophic chondrocytes per column and diameter of terminal hypertrophic chondrocyte were also greater in *Bos taurus* steers but only on the first collection date.

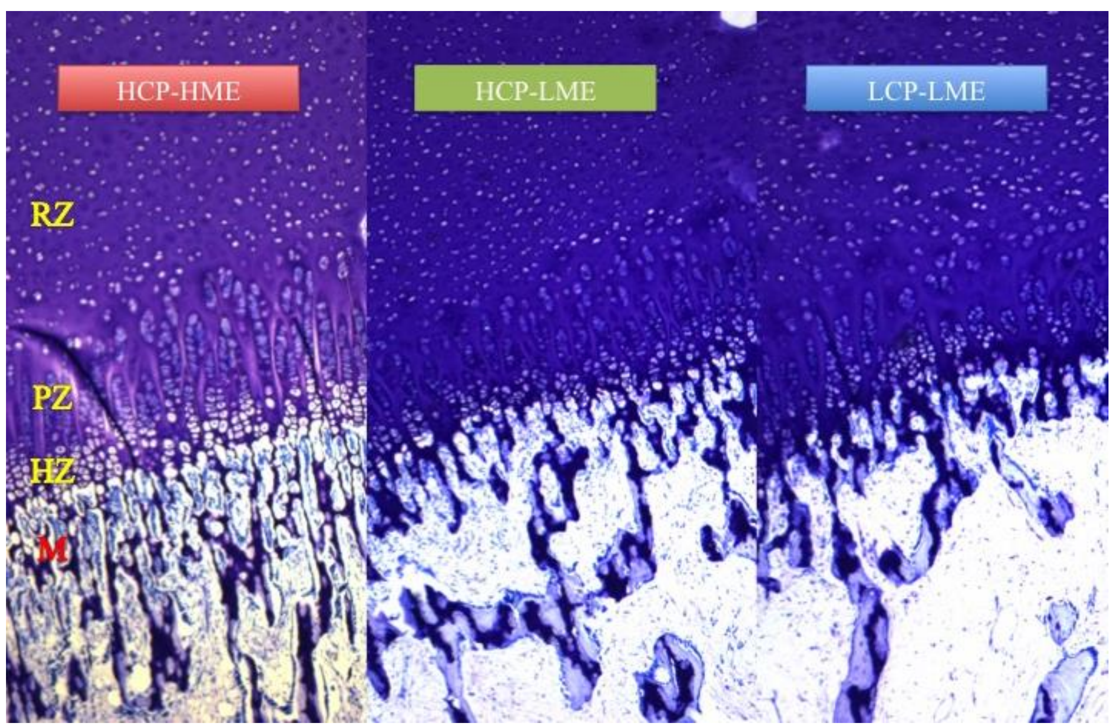


Figure 4-17 Tuber coxae growth plate structure of steers fed HCP-HME, HCP-LME and LCP-LME respectively at the end of Phase 1. Sections were stained with toluidine blue and photographed at 4X using an Olympus BX41 microscope (Olympus America Inc.; Melville, NY, USA). Resting, proliferative, and hypertrophic zones and metaphysis are assigned as: RZ, PZ, HZ and M respectively.

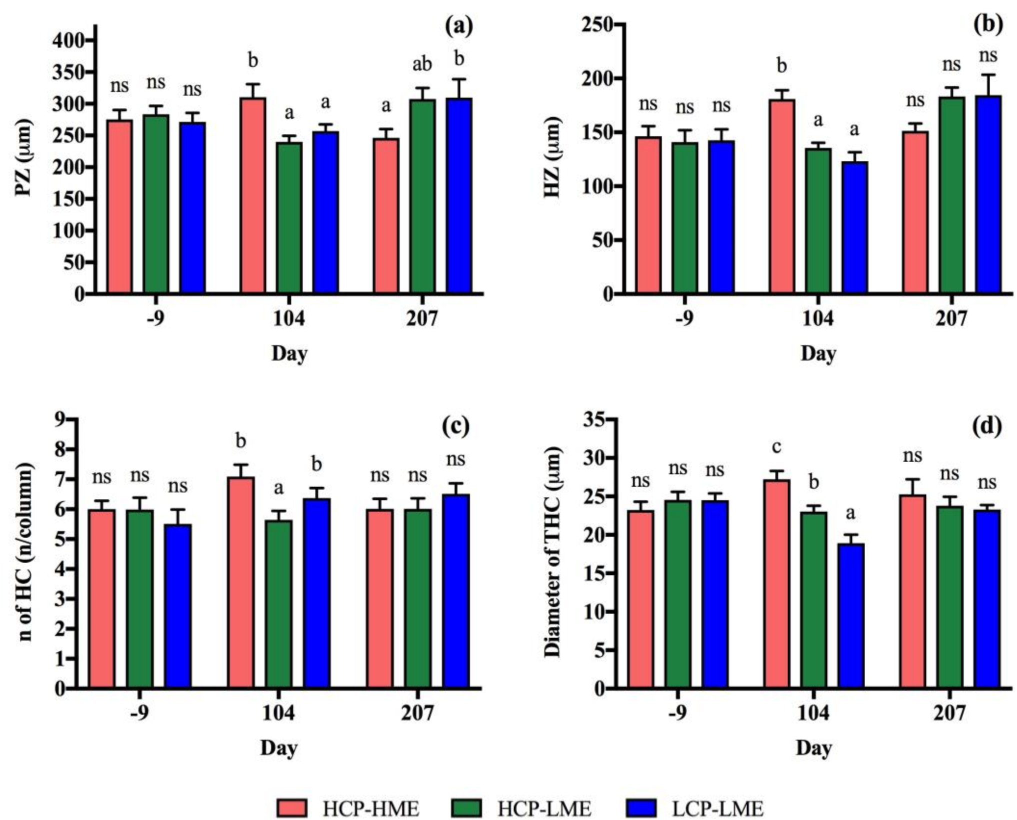


Figure 4-18 Height of proliferative (PZ; a) and hypertrophic zone (HZ; b), number of hypertrophic chondrocytes (HC) per column (n of HC; c), diameter of terminal HC (THC; d) of steers prior the start of the experiment (day -9), at the end of Phase 1 (day 104) and at the end of Phase 2 (day 207).

Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant [1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

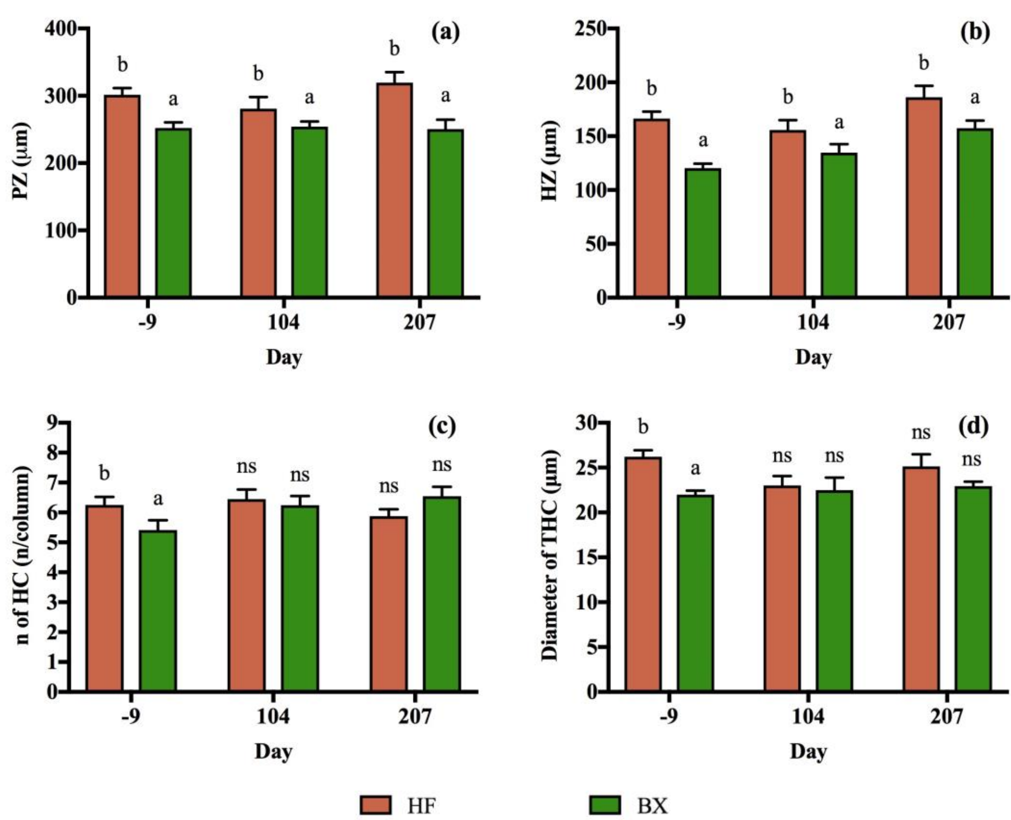


Figure 4-19 Height of proliferative (PZ; a) and hypertrophic zone (HZ; b), number of hypertrophic chondrocytes (HC) per column (n of HC; c), diameter of terminal HC (THC; d) of *Bos taurus* (HF) and *Bos indicus* (BX) steers prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant.

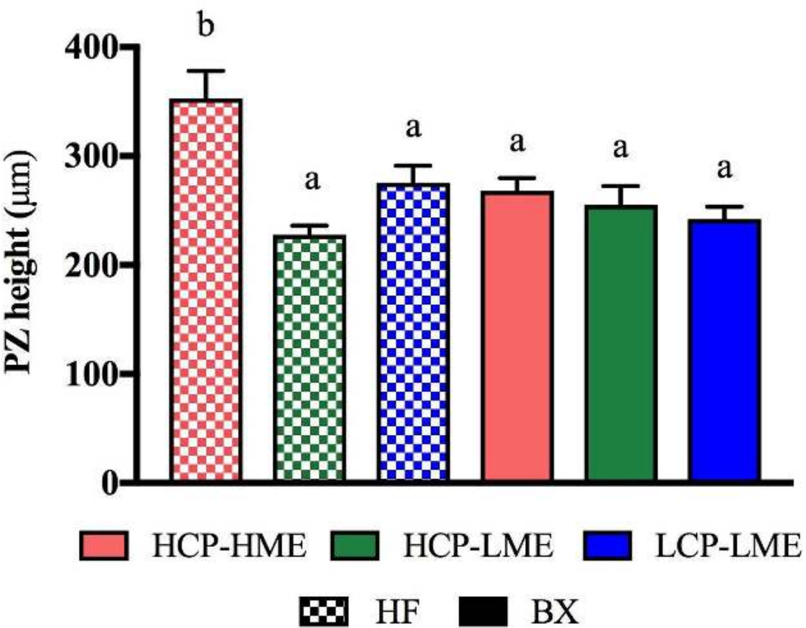


Figure 4-20 Height of the proliferative zone (PZ) of tuber coxae samples collected from *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$) [1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)].

A significant difference between nutritional groups was found in bone volume prior the imposition of the diets on day -9 (Figure 4-22). This variable was then analysed using the initial measurement as a covariate of the percentage of change during each phase (Figure 4-24). Prior to the start of nutritional treatments, HF steers had significantly higher bone volume and trabecular thickness than BX (Figure 4-23). In addition at the end of Phase 1, *Bos indicus* steers showed a bigger trabecular separation and smaller bone surface than *Bos taurus* steers. Trabecular bone of steers fed HCP-HME had higher volume, surface and also smaller separation at the end of Phase 1 than steers consuming low ME (Figure 4-21). Only BX steers showed reductions in trabecular thickness due to reduced ME intake but there was no effect of CP intake during ME restriction (Figure 4-24). Steers fed LCP-LME had significant loss of trabecular bone during Phase 1 when compared to HCP-HME (Figure 4-25). The differences in trabecular parameters found in Phase 1 were no longer evident at the end of Phase 2. There was a significant interaction of genotype and nutritional effects for percentage of change in trabecular bone volume during Phase 2 (Figure 4-25). Brahman-cross steers fed LME diets during Phase 1 had a significant increase in bone volume when fed HCP-HME *ad libitum.* Trabecular

separation was bigger and bone surface smaller in BX than in HF at the end of Phase 1 but trabecular thickness was higher in BX at the end of the following phase.

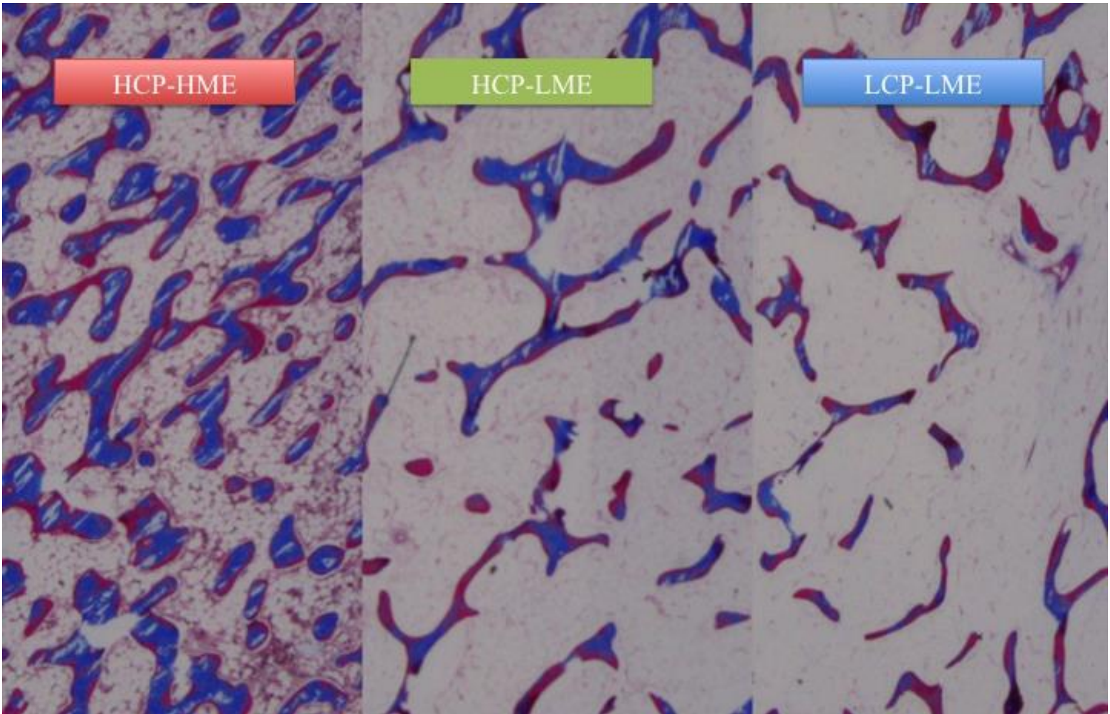


Figure 4-21 Trabecular bone structure of steers fed HCP-HME, HCP-LME and LCP-LME respectively at the end of Phase 1. Sections were stained with masson trichrome and photographed at 4X using an Olympus BX41 microscope (Olympus America Inc.; Melville, NY, USA).

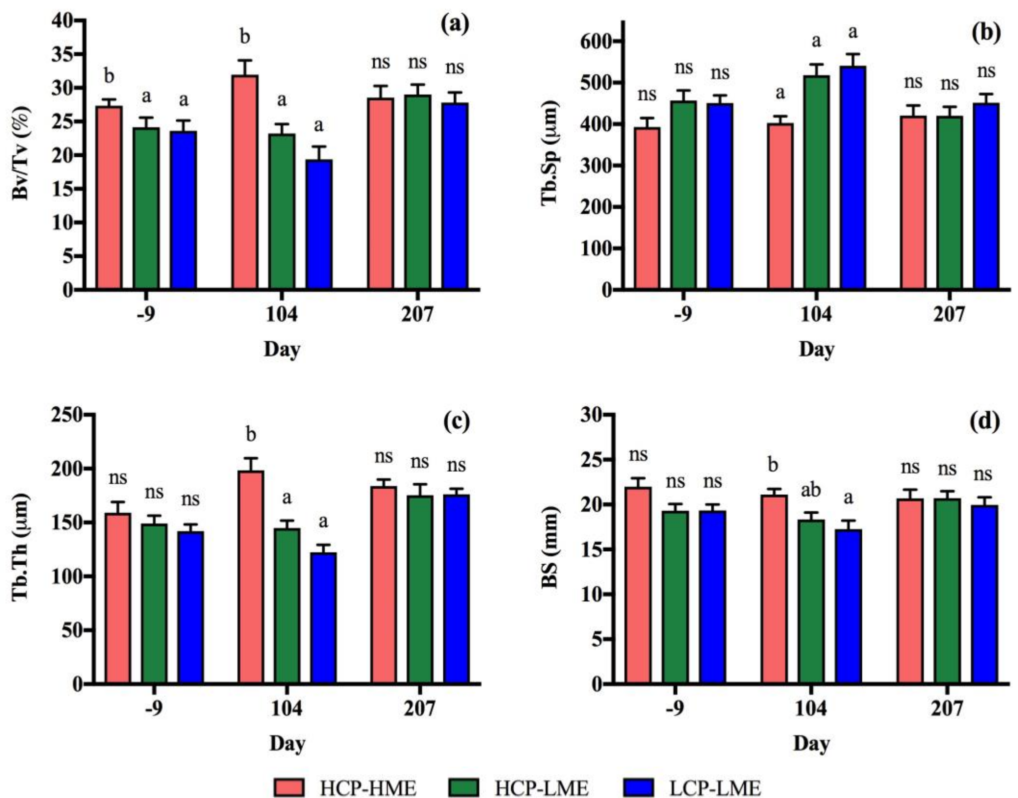


Figure 4-22 Bone volume (Bv/Tv; %; a), trabecular separation (Tb.Th; µm; b), trabecular thickness (Th.Sp; µm; c) and bone surface (BS; mm; d) of steers prior the start of the experiment (day -9), at the end of Phase 1 (day 104) and at the end of Phase 2 (day 207). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant [1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

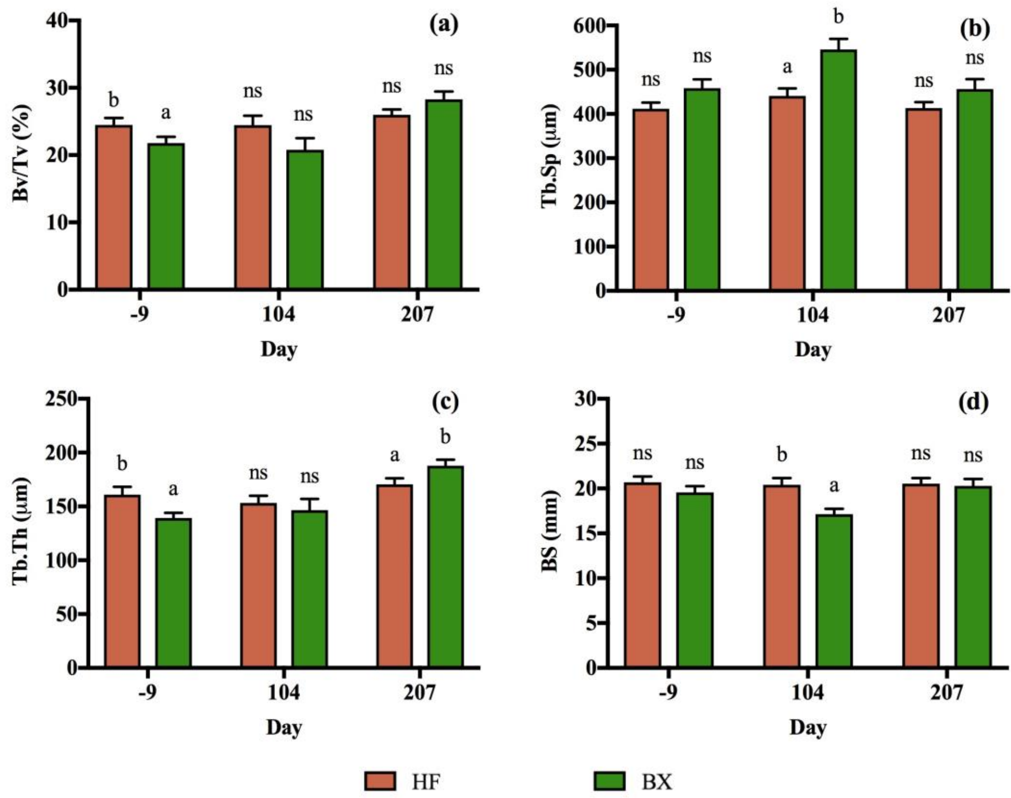


Figure 4-23 Bone volume (Bs/Ts; %; a), trabecular separation (Tb.Th; µm; b), trabecular thickness (Th.Sp; µm; c) and bone surface (BS; mm; d) of *Bos taurus* (HF) and *Bos indicus* (BX) steers prior the start of the experiment (day -10), at the end of Phase 1 (day 103) and at the end of Phase 2 (day 203). Data are expressed as means ± SEM. Means with different superscripts on given days are significantly different (Tukey, $P<0.05$) and ns is non-significant.

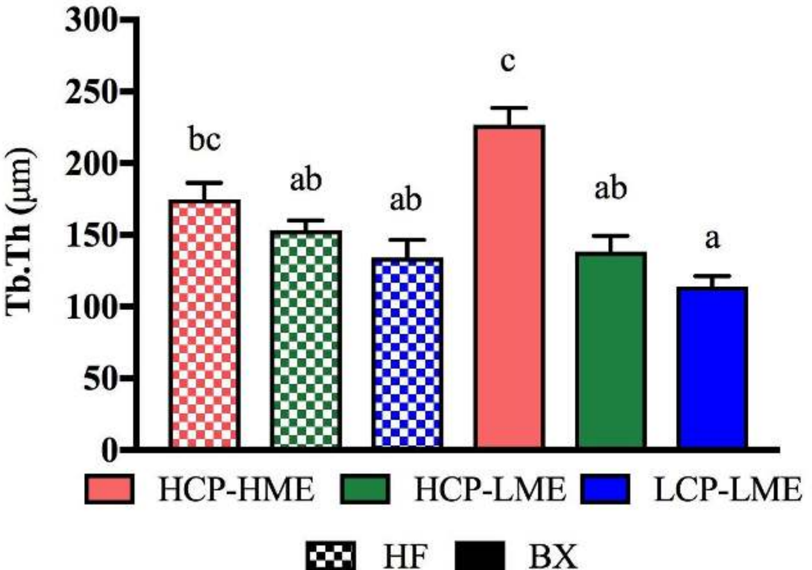


Figure 4-24 Trabecular thickness (Tb.Th) of tuber coxae samples collected from *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1). Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$) [1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

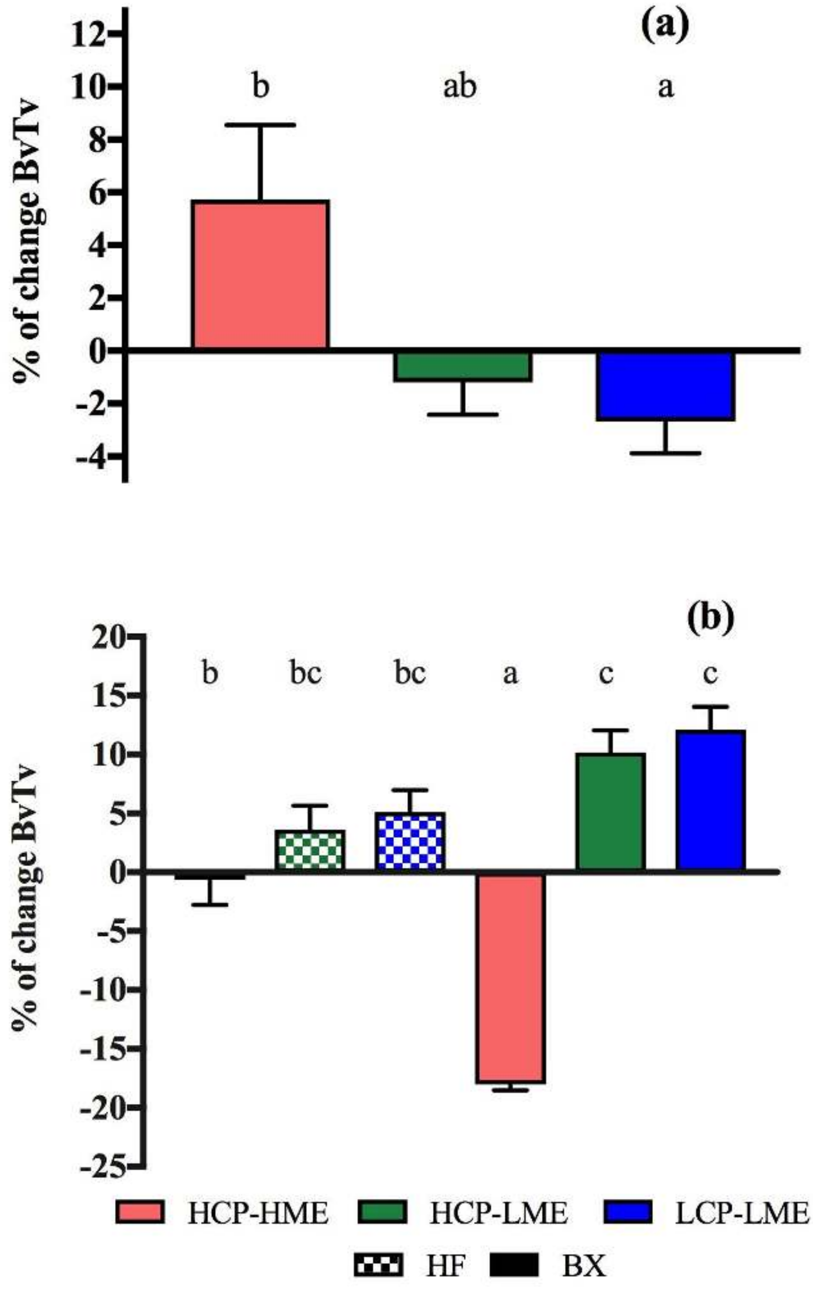


Figure 4-25 Percentage of change in trabecular bone volume of tuber coxae samples collected from *Bos taurus* (HF) and *Bos indicus* (BX) steers fed different nutritional treatments (Phase 1; a) and during re-alimentation (Phase 2; b). During Phase 1, the interaction between genotype and nutritional

factors was not significant so the means of both genotypes were pulled by nutritional treatment. Data are expressed as means ± SEM. Means with different letters are significantly different (Tukey, $P<0.05$) [1].

[1]See materials and methods for description of nutritional treatments [high crude protein intake and high MEI (HCP-HME), high crude protein intake and low MEI (HCP-LME) and low crude protein intake and low MEI (LCP-LME)]

4.6 Discussion

The current experiment investigated the effect of protein/energy ratio in the diet on LWG and skeletal elongation in cattle undergoing periods of nutritional restriction and re-alimentation. The experiment found that LWG in cattle responded primarily to ME intake while skeletal elongation was also stimulated by CP intake, as indicated by change in hip height and histomorphometric assessments of the growth plate. Moreover, plasma T3 concentrations were increased by CP intake during energy restriction with no differences between genotypes while T4 concentrations were reduced in *Bos taurus* steers consuming the low CP and ME diet. Bone resorption at the trabecular bone was slightly reduced in cattle consuming a higher CP diet during energy restriction, however energy restriction severely reduced bone formation independent of the CP content of the diet. The experiment also demonstrated that the skeleton shows catch-up growth with an increasing growth rate in body measurements when compared to unrestricted cohorts at same age but growth rates were not different of control cattle at the same HH. Moreover, catch-up growth of previously restricted cattle was associated with morphological changes at the epiphyseal growth plate as well as normalization of hormone concentrations that were comparable to unrestricted cattle at similar growth rates. The morphological and physiological changes and implications of these findings are investigated and discussed.

4.6.1. *Nutritional parameters*

The type of nutrient restriction (i.e. protein, energy or minerals) has been assumed to be one of the factors affecting compensatory growth (Wilson and Osbourn, 1960). In ruminants, the ingested protein is partially degraded in the rumen yielding ammonia, amino acids and peptides. The concentration of ammonia in the rumen directly affects MCP production and consequently the rate of digestion of the substrate (Satter and Slyter, 1974). A decrease in digestibility leads to reductions in rate of passage and consequently DM intake. Thus in ruminant nutrition a CP deficiency in the diet is usually accompanied by reduced energy intake from which it is difficult to separate specific protein and energy effects. In this study, the design of the nutritional treatments allows the differentiation

between the effects of a high and low CP content diet during energy restriction. This assumption can be verified by comparing ME and CP intake between treatments. The CP intake was different across all three nutritional treatments while the ME intake was the same between the two restricted groups (i.e. HCP-LME and LCP-LME).

The ME content of a given feedstuff is directly related to the DMD (Freer et al., 2007). Increases in digestibility of DM, ADF and starch have been reported during restricted feeding in high concentrate diets (i.e. >90% of concentrate) (Murphy et al., 1994). In this study the DMD of lucerne chaff was not affected by intake nor by genotype allowing the use of the same value of ME content for restricted (HCP-LME) and *ad libitum* fed cattle (HCP-HME). Similarly, Pearson et al. (2006) found no difference in DMD when feeding lucerne hay *ad libitum* or restricted at 0.75 of *ad libitum* intake.

Microbial crude protein production provides most of the metabolizable protein intake and its measurement provides a better description of the metabolizable protein/ME intake by steers fed the various diets than simply CP content of the diet. Microbial protein is most often limited by the supply of rumen degradable protein (RDP) in tropical pastures. The RDP supply is a function of the CP intake and the ruminal degradability of the protein (Freer et al., 2007). In the present study, steers fed HCP-LME showed higher MCP production when compared to steers offered the LCP-LME diet when both groups did not differ in ME intake. The higher MCP production in HCP-LME is most likely due to an increase of RDP supply caused by the higher CP content of lucerne as well as the greater degradability of protein of this legume when compared to Mitchell grass hay. Moreover, the efficiency of MCP production expressed as EMCP (g MCP/kg DOMI) was independent of the difference in intake between HCP-HME and HCP-LME. These results are in agreement with Panjaitan et al. (2010) who concluded that EMCP was not associated with differences in DM intake and MCP production was directly related to RDP supply when RDP was limiting.

Steers consuming high CP (i.e. HCP-HME and HCP-LME) diets had higher concentrations of rumen NH_3H than cattle consuming the low CP content diet regardless of genotype. However, *Bos indicus* cattle had a substantially greater concentration (83.3 *vs* 16.2 mg/L) of NH_3N than *Bos taurus* when both genotypes were consuming Mitchell grass hay (i.e. LCP-LME). Similarly, Hunter and Siebert (1985) observed that Brahman steers fed spear grass (38 g CP/kg DM) had a higher concentration of NH_3N in the rumen (40 *vs* 16 mg/L) than Hereford steers but this genotype difference disappeared when a urea and sulphur supplement (30 g N/kg DOM) was offered. Moreover, the authors showed

that higher concentration of NH_3N was related to a slower digestion rate in non-supplemented *Bos taurus* cattle.

Plasma urea nitrogen concentration did not reflect the concentration of NH_3H in the rumen, instead it was more related to the relationship between CP to ME content in the diet. In fact Hammond (1997) suggested that PUN could be used as guide to monitor the DOM to CP ratio (DOM:CP) and thus a tool to make adjustments in feeding or grazing management. The DOM:CP of steers fed HCP-HME and HCP-LME in the present experiment was 3.3 and 3.5 respectively, while the PUN concentration was 7.1 and 7.5 mmol/L. Cattle fed LCP-LME had a much greater DOM:CP (12.1) and lower PUN concentration 1.2 mmol/L. In addition, it has been suggested that a DOM:CP of approximately 7:1 and higher corresponds to a limitation of RDP and hence a response to N supplementation is expected (Moore et al., 1995; Moore et al., 1999). Alternatively, the energy and protein relationship in the diet can be expressed as g CP/kg DOM, the inverse of the DOM:CP used by Moore et al 1995. Using this later parameter, the nutritional treatments of this experiment had 297, 284 and 82 g CP/kg DOM for HCP-HME, HCP-LME and LCP-LME respectively. Regardless of how this is expressed, the HCP-HME and HCP-LME diets provided a much higher metabolizable protein:ME and a much higher CP intake than the low LCP-LME diet. This approach allows ME and CP effects to be separated for their influence on LWG, skeletal growth, bone histological parameters and the concentration of various hormones.

Nonesterified fatty acids (NEFA) is one indicator of negative energy balance in cattle (Adewuyi et al., 2005). Increased mobilization of NEFA from the adipose tissue has been reported in cattle during feed restriction (Hayden et al., 1993; Yambayamba et al., 1996a). Interestingly, the restricted cattle (i.e. LCP-LME and HCP-LME) in this experiment did not show different concentration of NEFA when compared to unrestricted cohorts (i.e. HCP-HME). However, BX cattle showed higher concentration of NEFA than HF in Phase 1 and 2. This difference between breeds may be related to the greater proportion of carcass fat in BX than HF cattle (Fox and Black, 1984).

Overall the nutritional treatment design of this experiment was successful in imposing a clear separation between high and low CP diets during energy restriction. This distinction was confirmed not only by the differences in ME and CP intake and the DOM:CP but also by the glucose and PUN concentration in the plasma. Moreover, the similarity in the concentration of Ca and P in the plasma (Table 4-5) of steers fed the ME restricted treatments (i.e. HCP-LME and LCP-LME) indicates that

the nutrition effects discussed below on performance and bone metabolism were independent of any mineral limitation.

4.6.2. *Endochondral ossification during nutritional restriction and re-alimentation*

Liveweight gain was, in general, more responsive to the different planes of nutrition and re-alimentation than body measurements especially height (Figure 4-4). This is in agreement with previous studies, which examined the effect of feed restriction and re-alimentation on body development (Pálsson and Vergés, 1952; Lawrence and Pearce, 1964a; Young, 1988; Keogh et al., 2015). Although the results of the current experiment showed that there was an overall increase in skeleton elongation rate during the re-alimentation phase the magnitude of compensation varied for each body dimension. An increase in bone elongation after a period of growth restriction when compared to unrestricted cohorts have been demonstrated in rabbits (Baron et al., 1994; Gafni et al., 2001) and rats (Marino et al., 2008) but this is the first time it has been shown in cattle linked with histomorphometry analysis of the growth plate. Nevertheless, it has been previously described in the literature that restricted cattle have the capacity to increase growth of body dimensions over unrestricted cattle and achieve complete recovery in terms of skeleton size (Lawrence and Pearce, 1964a, b).

The morphological changes observed in this experiment at the growth plate level are in agreement with the *growth plate delayed senescence* hypothesis (Lui et al., 2011) supported by other authors (Baron et al., 1994; Gafni et al., 2001; Nilsson and Baron, 2004; Gat-Yablonski et al., 2008; Marino et al., 2008; Jobling, 2010). In summary, the growth rate of long bones is rapid in prenatal and early postnatal life and decreases naturally as animals grow older. The reason for the decrease in growth rate in rats is due to a reduction in chondrocyte proliferation and size of terminal hypertrophic chondrocytes which provide a dimensional explanation for changes in length although, the exact mechanisms that lead to these changes are not well understood. During a period of growth inhibiting conditions, such as nutritional restriction, the growth rate of bones are reduced as well as the pace of the growth plate senescence. Once the growth limiting conditions are resolved the growth plate of restricted animals are more “physiologically immature” than unrestricted counterparts of the same age. This effect in turn leads to an increased rate of bone elongation in these previously restricted animals suggesting that the pace of senescence development is not driven by time but at least partially by growth itself. In addition, it has also been demonstrated that the mechanism that controls these changes are intrinsic to the growth plates. Growth plates transplanted between rabbits in different ages exhibit growth rates in accordance to the donors rather than the recipient (Stevens et al., 1999).

In the current experiment, the limited ME intake during Phase 1 is assumed to have reduced the pace of growth plate senescence as well as reducing the height of the PZ and HZ and diameter of the terminal hypertrophic cells. During the re-alimentation phase the restricted animals would have a more immature growth plate so they were able to show greater bone elongation rate when compared to unrestricted counterparts of the same age. However HHG of previously restricted steers was not different to the HHG of the HCP-HME steers in Phase 1 suggesting that there is a maximum upper limit to the rate of skeletal elongation measured as HHG. It is also interesting to note that differences at growth plate morphology at the end of the re-alimentation were not related with any endocrine and metabolite differences in the plasma demonstrating that apart from the well-known endocrine factors that regulate linear growth there are other local mechanisms acting and these are intrinsic to the growth plate.

The magnitude of reduction in diameter of terminal hypertrophic chondrocytes during nutritional restriction is not able to explain the decrease in skeletal growth rate, so proliferation rate of proliferative chondrocytes is assumed to also be reduced to contribute to the reduction in skeletal growth that was evident. For instance, BX steers showed reductions of 12 and 35% in diameter of terminal hypertrophic chondrocytes in HCP-LME and LCP-LME respectively while HHG of cattle in these treatment groups was reduced by 54 and 67% respectively. This suggests that at least part of the reduction in bone elongation during the nutritional restriction was due to a decrease in chondrocyte proliferation. Conversely, Breur et al. (1991) reported high correlations between the volume of terminal hypertrophic chondrocytes and bone elongation rates in rats (r=0.98) and pigs (r=0.83). However, in the latter experiment both pigs and rats were not subjected to any type of nutritional restriction. Alternatively, Wilsman et al. (1996) suggested that the relative contribution of proliferation, matrix synthesis and hypertrophy to growth rates is different between growth plates of bones growing at different rates. The description made by Wilsman and colleagues is in agreement with the results of the present experiment. Moreover, it is in accordance with the delayed senescence hypothesis since a reduction in proliferation rate would result in decreased rate of senescence.

Restricted ME intake reduced the height of HZ in both genotypes but only *Bos indicus* steers showed reductions in the PZ due to nutritional treatment. Despite that, body measurements of growth rates of both genotypes under nutritional restriction were very similar. In the current study there was a significant increase in HHG in steers consuming diets with a higher content of CP during ME restriction. This effect was accompanied by a significant increase in the diameter of hypertrophic cells and also plasma T3 concentration. Thyroid hormones are directly related to body growth and

energy metabolism. Thyroxin is the most abundant in the bloodstream but T3 is the most active form of thyroid hormones.

It is not known if higher plasma T3 concentrations in steers consuming high CP diets could exert a direct stimulatory effect on chondrocyte hypertrophy and consequently growth rates. Hypothyroidism in humans is known to affect growth rates and skeletal maturation (Shao et al., 2007). The induction of hypothyroidism in rats by administration of propylthioracil leads to a decrease in height of the PZ and HZ, terminal hypertrophic cells and growth rates (Marino et al., 2008). Interestingly, limited ME intake did not affect the concentration of T4 in *Bos indicus* steers and it only decreased the concentration in *Bos taurus* steers when it was coupled with limited CP content in the diet. This may indicate that energy and protein balance of the diet affects the deiodination process. In addition, it also indicates a different physiological response between genotypes. Interestingly, investigations in caloric restricted rats fed low protein diets show an increase in plasma T3 hormone concentration above controls (Glass et al., 1978; Sawaya and Lunn, 1985; Ramos et al., 2000; Passos et al., 2001) which is not explained by enhanced conversion of T4 to T3 nor by the maximum binding capacity of T3 in the liver or binding affinity with receptors (Smallridge et al., 1982). In cattle, there is no information about the effect of a low or high protein diet during energy restriction on the concentration of thyroid hormones. In *Bos indicus* cattle T3:T4 decreased during energy restriction and was further reduced in steers consuming a low CP diet. Conversely, T3:T4 was higher (51.8 *vs* 19.8; $P<0.001$) in *Bos taurus* steers fed LCP-LME diets compared to *Bos indicus* steers consuming the same treatment diet. In humans, T3:T4 is lower in patients suffering from protein-calorie malnutrition and it is increased once given access to a high caloric diet (Chopra and Smith, 1975).

In relation to the effect of protein intake on the endochondral ossification process, the results observed in the present investigation are in agreement with the work reported by Frandsen et al. (1954). Diets with decreasing protein content (6, 3 and 0% of casein) as well as pair-fed (24% of casein) limited to the same food intake of littermates on low protein diets were fed to rodents (Frandsen et al., 1954). The authors reported a more severe retardation in terms of skeletal growth, as measured by trunk and tail length, tibial length, tibial width through the proximal epiphysis and the middle portion of the diaphysis, and width of the tibial proximal epiphyseal cartilage plate in rodents fed the low protein diets than pair-fed groups. In addition, it was also observed that the number of chondrocytes per row was very similar between treatments, but the size of the cells in protein restricted animals was greatly reduced. No information was provided in regards to the hormone concentrations of rodents in this experiment. However taken into consideration the previous studies discussed above (Glass et al.,

1978; Sawaya and Lunn, 1985; Passos et al., 2001) which found increased concentration of T3 in rodents fed low protein diets during energy restriction one could argue that the results observed by Frandsen et al. (1954) couldn't be justified by increases in T3 concentration. Alternately, the stimulatory effect of a higher CP diet on endochondral ossification could be mediated by autocrine-paracrine action of local IGF-1 production. This explanation is based on the observation that dietary protein intake stimulates non-hepatic IGF-1 production (Naranjo et al., 2002). Moreover, in mice with a loss of IGF-1 production in chondrocytes and osteoblasts changes in endochondral ossification occur without changes in plasma IGF-1 concentration (Govoni et al., 2007a; Sheng et al., 2013). It is interesting to note that both chondrocyte and osteoblast IGF-1 inactive models resulted in rodents presenting with lower rates of bone elongation than control groups, which was associated with decreases in HZ thickness similar to the results observed in this work.

4.6.3. *Trabecular bone structure*

Experiments with rodents have shown that both energy and protein restriction affect bone structure leading to an osteoporotic state (Bourrin et al., 2000a; Hamrick et al., 2008). In elderly women the occurrence of osteoporosis is increased by insufficient protein intake (Bonjour et al., 1997). In a study with postmenopausal women Sukumar et al. (2011) have shown that a calorie restricted diet with high protein content reduces bone loss. During this experiment, the subjects lost 6.6 and 7.4% of their initial body weight in the high and low protein content diets (24% vs. 18% of total calories of the diet as protein). The authors also found significantly higher concentration of IGF-1 and IGFBP-3 and lower concentrations of DPD in subjects offered the high protein diet. In the current experiment there was no difference in IGF-1 concentration due to higher CP intake when ME intake was restricted. However, a reduction in total DPD concentration was also observed in steers fed the HCP-LME diet but this was not different from the other ME intake restricted group. Conversely, PYD concentration was significantly lower in HCP-LME than LCP-LME. In addition, the percentage change in bone volume (BV/TV; Figure 4-25) during Phase 1 also indicated that the loss of trabecular bone volume was slightly reduced in steers fed high CP during ME restriction.

Pando et al. (2014) have demonstrated that after only one day of re-alimentation there was a significant increase in collagen fibre deposition in the trabecular bone of rats, and this was accompanied by a rise in BAP and IGF-1 to the same concentration as rats in the control group. This shows that bone formation is a very dynamic process and highly responsive to nutrition and a change in nutrition. In the current experiment, there was also no difference in BAP and IGF-1 concentration when comparing between the energy restricted treatments at the end of the re-alimentation period

(100 days). Also all the differences in trabecular parameters which resulted from the previous different types of nutrient restriction were corrected by increased bone formation over the 100 day re-alimentation period. The percentage change in BV/TV during Phase 2 (Figure 4-25) also provides evidence of this. Steers fed restricted ME treatments had significantly ($P<0.05$) and tendency towards significantly ($P=0.07$) thicker PZ and HZ than HCP-HME at the end of Phase 2. These morphological differences are in agreement with the increments in body dimension growth rates during Phase 2. Moreover, the lack of difference in terms of diameter of the terminal hypertrophic chondrocyte at the end of Phase 2 between previously restricted steers (i.e. HCP-LME and LCP-LME) and non-restricted cattle (i.e. HCP-HME) suggests that catch-up growth during Phase 2 appeared to be due to increased proliferation rate but this parameter was not specifically measured in the current experiment.

4.6.4. *Liveweight gain during re-alimentation*

Energy-restricted steers had higher DM intake and LWG during re-alimentation when compared to steers that had *ad libitum* access to lucerne chaff during the entire experimental period. The pattern of DM intake during re-alimentation was strikingly similar between the previously restricted groups of steers (Figure 4-26). Steers fed restricted diets during Phase 1 had increased DM intake and LWG over Phase 2 reaching a peak approximately 50 days after the start of the re-alimentation period. Interestingly, HF steers fed LCP-LME in Phase 1 showed a transitory higher LWG than HF steers fed HCP-LME during re-alimentation, without any increment in DM intake. In addition, this group of steers (i.e. HF steers fed LCP-LME) were the only restricted treatment group which showed greater LWG during compensatory growth (i.e. during Phase 2) than unrestricted HF steers fed high CP and ME at an approximately equivalent LW (during Phase 1). Also, limited ME intake did not seem to affect the concentration of T4 in BX steers and it only decreased the level in HF steers consuming the low CP diet. This could be an indication that the LCP-LME diet lead to a greater reduction in RMR in HF steers which may have favoured the higher LWG during compensatory growth.

The similarity in the pattern of DM intake of HCP-LME and LCP-LME during Phase 2 seems to indicate a similar factor regulating intake in both groups during re-alimentation. At the peak of intake in Phase 2, previously restricted (i.e. HCP-LME and LCP-LME) BX and HF cattle were consuming on average 33.5 and 33.9 g DM/kg LW.day respectively. This represents 11.9 and 13.9 g NDF/kg LW.day respectively which is comparable to the upper limit of daily NDF intake (12.5 ± 1 g NDF/kg LW.day (Mertens, 1994). Thus, ME intake and LWG during compensatory growth were most likely limited by the NDF content of the diet during re-alimentation. The NDF content (356 g/kg DM) of lucerne chaff offered during Phase 2 is lower than NDF content commonly reported in tropical grasses

during summer growth (Johnson et al., 2001; Vitor et al., 2009). Therefore, is likely that NDF content would have limited DMI and LWG to a greater extent than observed in the present experiment in a practical grazing condition. McLennan (2014) showed that the majority (77 to 100%) of compensatory growth effect is achieved in the first 100 days of the wet season. This result agrees with the present observation on the pattern of DM intake during re-alimentation. The cubic nature of this response curve is also in agreement to the previous description of LWG during compensatory growth described by Hornick et al. (2000).

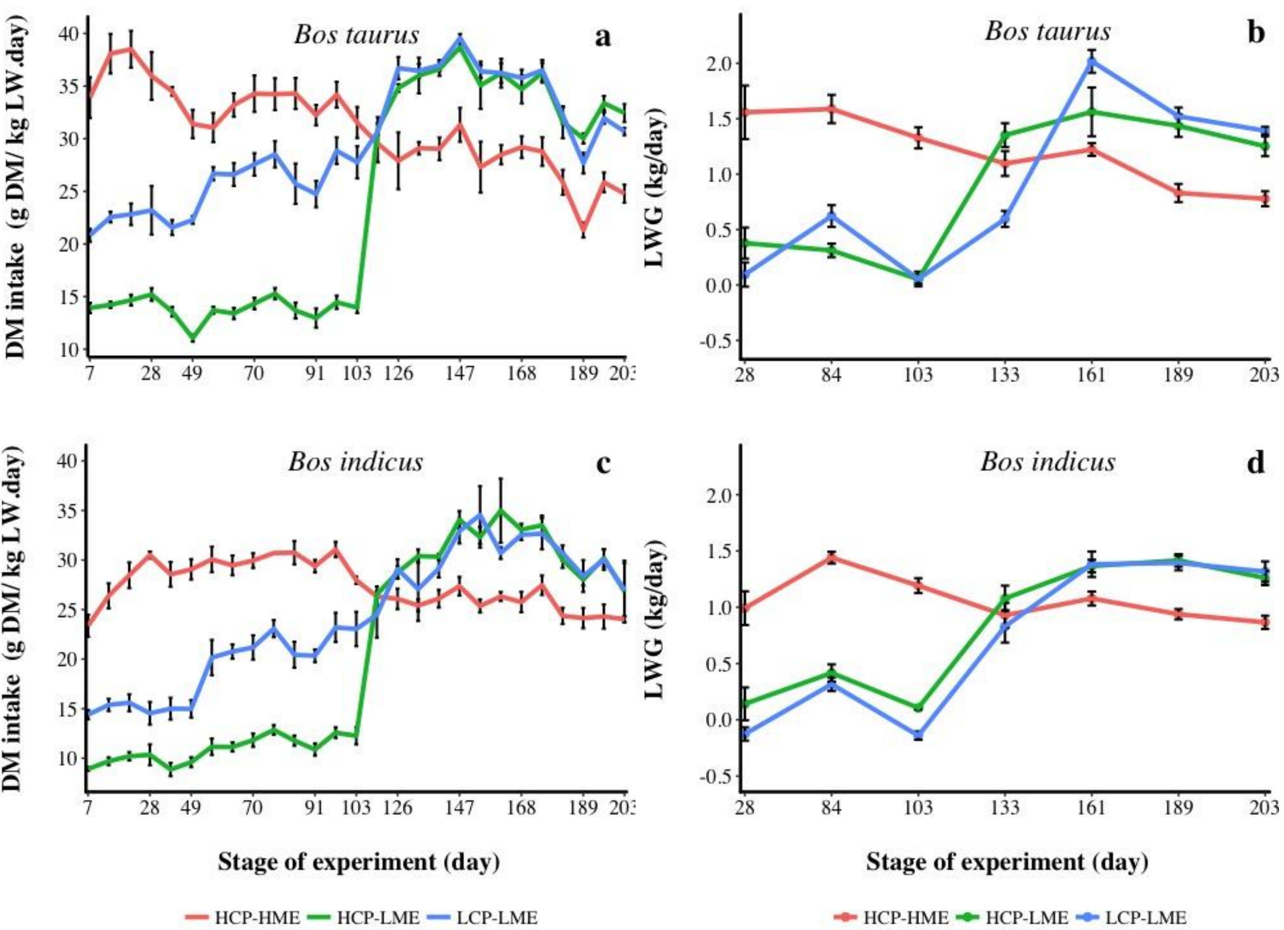


Figure 4-26 Dry matter (DM) intake and liveweight gain (LWG) *Bos taurus* (a and b) and *Bos indicus* (c and d) steers fed HCP-HME, HCP-LME or LCP-LME during experiment (mean ± SEM).

The effect of nutritional restriction and realimentation on organ and digestive tract size was not evaluated in the present experiment. However, studies which have analysed these parameters indicates that during nutritional restriction there is a significant reduction in organ mass (e.g. liver and spleen) and digestive tract (e.g. stomach and intestine) (Drouillard et al., 1991; Ryan et al., 1993b; Yambayamba et al., 1996b). During realimentation, Yambayamba et al. (1996b) observed an overcompensation of liver, spleen and stomach between the 50 and 78 days after the start of

realimentation. Despite the fact that the gastrointestinal tract and liver contribute less than 10% of the liveweight (Ferrell, 1988), it represents a great proportion or energy requirement. Moreover, the size of these organs are related with the capacity of the animal to process food and synthesize protein and may be the restricting factors limiting increase in voluntary food intake at early stages of compensatory growth. This hypothesis is in agreement with Ryan et al. (1993a) who suggested that only after the liver and digestive tract are replenished that compensating animals would be able to increase their feed intake above that of non-restricted cohorts. This would help to explain the steady increase in pattern of DMI and LWG observed at early stages of the realimentation phase observed in this thesis (Figure 4-26) and elsewhere (Hornick et al., 2000). In this scenario, the decrease in organs and muscles during nutritional restriction would represent a potential for protein deposition and lead to stimulus of voluntary intake (Radcliffe and Webster, 1976, 1979; Kyriazakis and Oldham, 1993; Webster, 1993; Cooper et al., 1994).

In this study, LWG and skeleton elongation rate (assessed as HHG) showed different patterns during nutritional restriction and re-alimentation. During nutritional restriction, HHG was proportionally less affected (reduced by 66% - average of HCP-LME and LCP-LME) than LWG (reduced by 89% - average of HCP-LME and LCP-LME) resulting in a disconnection between hip height and LW. Over the re-alimentation period (i.e. Phase 2), LWG (increased by 50% - average of HCP-LME and LCP-LME) was proportionally greater than the changes observed in HHG (increased by 18% - average of HCP-LME and LCP-LME) when compared to control group (i.e. HCP-HME). Moreover, the results provide evidences to support that *growth plate delayed senescence* hypothesis may also play a role explaining catch-up growth in cattle and increased intake was the main driver for compensatory growth. Taken together these results may suggest two separate mechanisms control LWG and HHG during nutritional restriction as well as during re-alimentation. However, it is unknown if the LWG during re-alimentation is somehow affected by changes in skeleton frame size.

The stepwise linear regressions analysis (Figure 4-27) of LWG and HHG on ME and CP intake showed ME intake to be the best predictor of LWG (R^2=0.92) while HHG was best explained by CP intake (R^2=0.82). These responses were both independent of genotype. The increased DM intake during the *ad libitum* phase seems to be the main mechanism that led to the faster growth rates following restriction. A similar conclusion was also reached by many other researchers investigating the compensatory growth phenomenon but up to the moment there is no information available to explain the long-term control of voluntary intake following periods of feed restriction (Ryan et al., 1993a; Keogh et al., 2015).

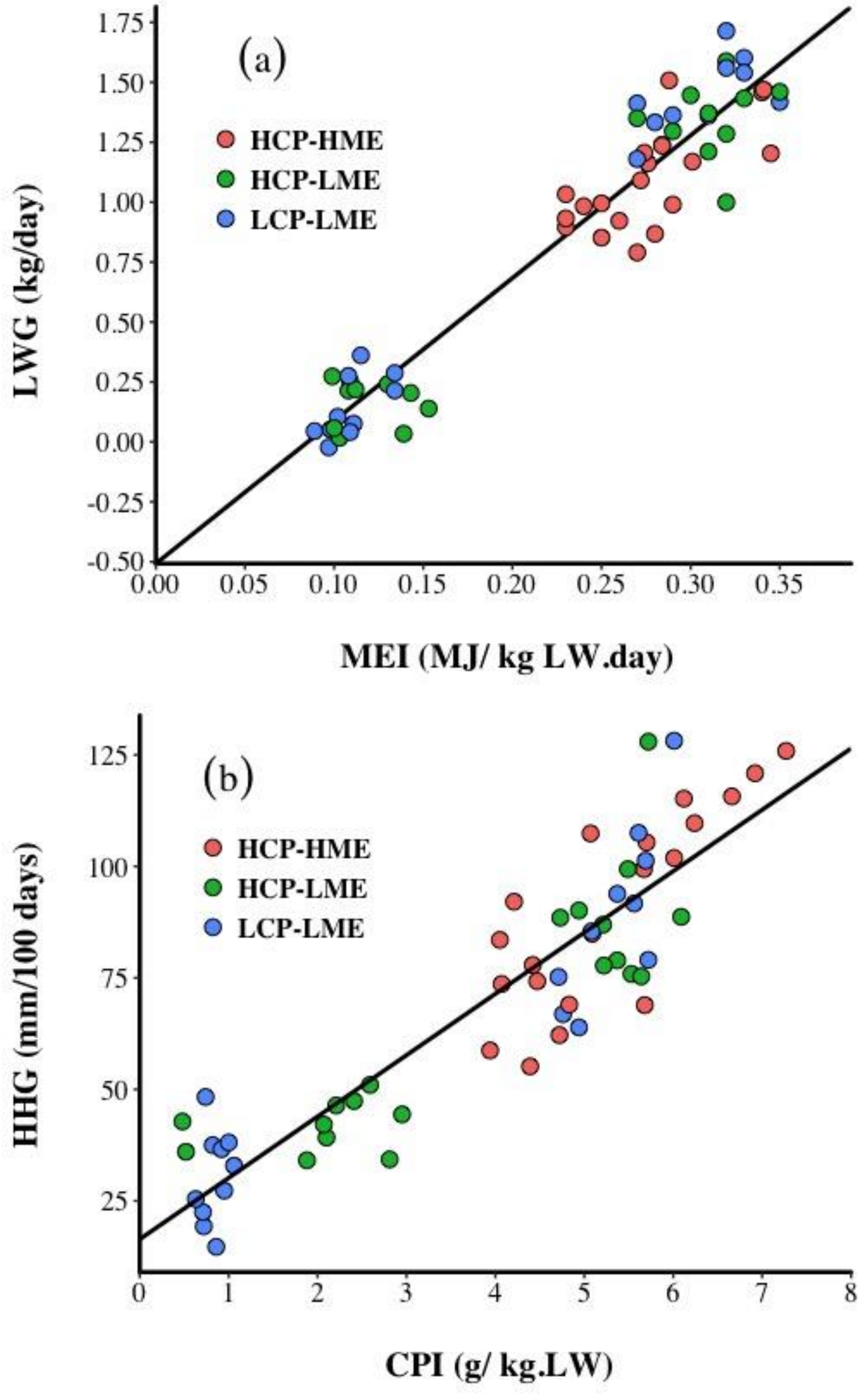


Figure 4-27 Effect of metabolizable energy intake (MEI) on liveweight gain (LWG; a; Y= -0.507 +5.95X, R^2=0.92, RSE=0.16, P<0.001) and the effect of crude protein intake (CPI) on hip height gain (HHG; b; Y= 16.41 + 13.75X, R^2=0.82, RSE=13, P<0.001) of steers fed HCP-HME, HCP-LME or LCP-LME during experiment. Each symbol represents the mean for individual steers in one out of the two phases of the experiment.

4.7 Summary and conclusion

This experiment demonstrated that steers with a high CP intake but low ME intake had greater HHG than steers with a low CP intake at an equivalent ME intake. The higher HHG in response to the additional CP intake was associated with a higher concentration of T3 in the plasma and a greater diameter of terminal hypertrophic chondrocyte in steers but no differences in the concentration of IGF-1 in the plasma were evident whilst the skeleton of steers with restricted ME intake continued to grow, static histomorphometry and the concentration of biomarkers (PYD and BAP) in the plasma suggested a loss of trabecular bone compared to steers with high ME intake undergoing higher rates of HHG. As expected, HF steers had greater ME intake, LWG and HHG than BX steers when CP and ME were not limiting.

It appears that whilst the difference in HH at the end of the restriction period due to CP intake was statistically different, the difference was probably not biologically large enough to have an effect on subsequent LWG during the recovery period. Contrary to the initial hypothesis, HF steers fed the LCP-LME diet were the only steers to demonstrate greater LWG during compensatory growth compared with unrestricted HF steers. This response was associated with a lower concentration of T4 in the plasma during nutritional restriction and no differences in DM intake during recovery which may indicate a further reduction in basal metabolic rate and maintenance requirements when compared to steers with a higher CP intake. Steers which have experienced a period of restricted ME intake display greater skeletal growth than unrestricted steers at the same age (i.e. catch-up growth) once offered ad *libitum* access to a non-limiting diet. The rate of skeletal growth of cattle during catch-up growth is independent of the CP intake during the restriction period. Therefore, high CP intake during ME restriction does not enhance compensatory nor catch-up growth in cattle.

Chapter 5. Feeding strategies for Early and Normally weaned replacement heifers in northern Australia.

5.1 Introduction

Early weaning of calves at the start of the dry season is one management strategy to increase weaning rates and reproduction rates in breeder herds in northern Australia (Tyler, 2012). In this environment, early weaned (EW) calves weigh between 100 and 150 kg and normally weaned (NW) calves weigh over 150 kg (Tyler, 2012). Despite the advantage of decreasing nutrient requirements of the dam and thus enhancing re-conception rates, early weaning introduces new challenges around nutritional management of the EW calves (McCosker et al., 1984). This is especially important for replacement heifers since LWG after weaning is one of the main factors affecting the onset of puberty. In northern Queensland, Holroyd et al. (1990) reported heifers EW in April had partially decreased the initial LW difference at weaning from NW heifers from 54.4 kg at weaning to 20.3 kg at 2.5 years old. Yet, average (mean of three years) pregnancy percentage of EW heifers was 69% while NW was 85%.

Post-weaning supplementation of EW heifers can accelerate growth rates and reduce the difference in LW relative to NW heifers (Moriel et al., 2014). In addition, an acceleration of the age of onset of puberty has been reported when feeding high-concentrate diets for 10 weeks to 3.5 months old beef crossbred heifers (½ Angus, ¼ Brahman, ¼ Hereford) (Cardoso et al., 2014). However, limited information is available to assess the long-term effects of post-weaning supplementation on replacement heifers in northern Australia. Compensatory and catch-up growth are expected to play a role in this scenario dictating mature LW and frame size of replacement heifers. In cattle, reduced LW caused by nutritional restriction at an early age (i.e. less than 6 months old) requires a much longer time to overcome the LW difference from unrestricted counterparts than when the restriction is imposed at an older age (Berge, 1991). However, the physiological reasons for such differences are not clear. Higher levels of ME and CP intake through supplementation increase the concentration of circulating GH and IGF-1 (Elsasser et al., 1989). Such somatotrophic hormones are known to affect bone endochondral ossification as well as bone remodelling (Mohan et al., 2003; Mohan and Kesavan, 2012). The way nutrition affects bone elongation and the process of bone turnover at the growth plate and trabecular bone in cattle at different stages of maturity (i.e. early and normally weaned) has not been previously explored. Supplementation with protein meals during dry season increases the ME and MCP production (McLennan, 2004). In Chapter 4, a linear increase in LW and HHG was observed when increasing metabolizable energy and crude protein intake of steers.

Therefore, supplementation during the first dry season is expected to enhance liveweight and hip height gain of replacement heifers. This is an important aspect to be understood because cattle frame size together with BCS will ultimately drive liveweight and reproductive performance. Moreover, the effect of supplementation on trabecular bone remodelling in early and normally weaned heifers has not been studied before. Biomarkers of bone metabolism have been previously used to indicate bone turnover in dairy cattle, however there is no information about the use of such markers in *Bos indicus* heifers. It has been shown in section 4.5.4 of this thesis that there are significant interactions between cattle genotype and nutrition. Therefore, in order to use bone biomarkers as a tool to indicate bone turnover it is necessary to know the range of concentration of such markers in the different cattle genotypes and nutritional status. The balance between bone formation and resorption has a direct effect on mineral reserves (Underwood, 1999). Thus an understanding of how supplementation might influence bone turnover dynamics may lead to better mineral supplementation strategies.

The aim of this experiment was to investigate the effect of different growth paths manipulated by levels of supplementation during the first dry season for early and normally weaned heifers and the long-term effects on growth (liveweight and hip height) and reproductive performance. In addition, the impact of nutrition on skeletal growth (as measured by hip height), histomorphometry parameters of the growth plate and trabecular bone as well as the plasma concentration of hormones, blood metabolites and bone biomarkers related to bone elongation and turnover were assessed.

5.2 Hypothesis

It was hypothesised that supplementation over the first dry season will increase liveweight gain as well as skeletal growth rates. During the first dry season, early weaned heifers are expected to require higher supplementation to achieve similar liveweight gain of normally weaned ones. Heifers fed high levels of supplement are also expected to have higher plasma concentration of IGF-1, T3 and insulin. Moreover, the increased rates of bone elongation due to supplementation are expected to be associated with morphological differences at the tuber coxae growth plate such as size of HZ and diameter of terminal hypertrophic chondrocytes. The concentration of plasma bone metabolism biomarkers are expected to reflect the structural differences in trabecular bone promoted by supplementation. Compensatory growth in liveweight and catch-up growth in hip height during the first wet season are expected to partially offset the differences obtained through supplementation during the first dry season. Early weaned heifers fed supplements over the first dry season may attain puberty earlier than non-supplemented heifers and therefore demonstrate higher pregnant percentage when mated at 2-years old.

5.3 Materials and Methods

The procedures realized during this work were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by The Charles Darwin University Animal Ethics Committee.

5.3.1. *Description experiment sites*

The pen experiment was conducted at Katherine Research Station (KRS; Katherine, NT, Australia) from 21/05/2014 until 30/01/2015 when all heifers were moved to Victoria River Research Station (VRRS; 16°7'S, 130°57'E, Katherine, NT, Australia) and remained there until the end of the experiment when reproduction rates were quantified. The climate in this region is classified as tropical by the major class classification system of Köppen with a markedly wet summer and a long dry winter. The historic mean rainfall and maximum and minimum temperatures are presented in Table 5-1 and Table 5-2 along with rainfall during the experiment for both KRS and VRRS. Sullivan and O'Rourke (1997a) described the three main soil types of VRRS as cracking clays, calcareous red earths and sandy red earths. The main native pastures species encountered on VRRS are *Chrysopogon fallax*, *Iseilema* spp., *Enneapogon* spp., *Heteropogon contortus* and *Sehima nervosum.*

Table 5-1 Climate description of Katherine Research Station[1].

	Historic maximum mean temp (°C) [1]	Historic minimum mean temp (°C)	Historic rainfall mean (mm) [2]	2014 rainfall (mm)	2015 rainfall (mm)
Jan	34.1	24.1	236.7	230.8	274.2
Feb	33.9	23.5	216.0	220.4	100
Mar	34.3	22.4	160.0	46.4	128.6
Apr	34	22.5	33.1	11.6	0.6
May	32.2	18.6	5.5	0	19.6
Jun	30	14.5	1.9	0	0
Jul	30.2	12.6	0.9	0.2	0
Aug	32.2	12.2	0.5	0	0
Sep	35.8	16.7	6.6	0	0
Oct	37.6	22.2	27.7	2.8	0.6
Nov	37.4	25.2	88.1	124.2	67.4
Dez	35.6	25.2	200.2	319	582.4
Annual	-	-	971.2	955.4	1173.4

[1] Data sourced from Bureau of Meteorology Website and refers to the data collected at Tindal RAAF station, which is approximately 10 km from Katherine Research Station.
[2] Historic maximum and minimum temperatures mean correspond to the period of 1985 to 2017.
[3] Historic mean rainfall correspond to the period of 1969 to 2017.

Table 5-2 Climate description of Victoria River Research Station[1].

	Historic maximum mean temp (°C) [1]	Historic minimum mean temp (°C)	Historic rainfall mean (mm) [2]	2015 rainfall (mm)	2016 rainfall (mm)
Jan	36.1	25.1	174.6	369.2	158.4
Feb	35.6	24.7	189.2	88.1	32.4
Mar	35.3	23.4	127	78.2	131.9
Apr	35.3	20.7	23.7	1.4	0.6
May	32.3	15.9	5.2	0	0
Jun	29.6	12.6	3.3	0	0
Jul	30.2	12.4	2.4	0	0
Aug	32.5	13.4	0.1	0	0
Sep	36.7	19.7	4.3	0	44.8
Oct	38.5	23.6	25.1	0	25
Nov	38.8	24.9	63.9	76.7	189.4
Dez	37.4	25.3	146.9	362.4	212.9
Annual	-	-	758	976	795.4

[1] Data sourced from Bureau of Meteorology Website
[2] Historic maximum and minimum temperatures mean correspond to the period of 1996 to 2012.
[3] Historic mean rainfall correspond to the period of 1969 to 2017.

5.3.2. *Experimental design, animals, diets and feeding*

One hundred and thirty-five Brahman crossbred heifers were selected from two commercial cattle stations (Kalala and Hayfield stations) in the Katherine region based on their LW at weaning and transferred to KRS. After arrival the animals grazed the same holding paddock (40 ha) for 30 days with access to mixed hay sabi grass (*Urochloa mosambicensis*) and Dolichos lablab (*Lablab purpureus*) in order to accustom the heifers to the management and reduce any previous nutritional effects. The chemical composition of the sabi grass hay was 894 g OM, 64 g CP, 644 g ash-free neutral detergent fibre (NDF), 1.8 g P and 3.2 Ca/kg dry matter (DM). They were supplemented with approximately 100 g/head.day of copra meal (935 g OM, 180 g CP, 506 g NDF, 4.7 g P and 0.7 g Ca/kg DM). During the adaptation period all heifers were treated for external parasites [Dectomax (Doramectin 10 mg/L), Pfizer; West Ryde, NSW, Australia)] and vaccinated against clostridial diseases (5 in 1 Websters, Virbac; Milperra, NSW, Australia) and botulism (Ultravac Botulium, Pfizer).

The experimental design was a 2x5 factorial composed of two weaning weights (WW) and five levels of post-weaning supplementation (S), with one level being a control group, and three replicates (pens) per WWxS combination (i.e. overall n=30 replicates). At the beginning of the experiment, heifers were weighed after a 15 h feed curfew with water available *ad libitum* and split into the following

WW groups: early-weaned (EW; 118 ± 6 kg LW) and normally-weaned (NW; 183 ± 6 kg LW). It is important to note that the age of these cattle are unknown due to the current management practices in cattle stations in northern Australia. The weight differences could be due to age or dam milk production. Generally in this environment differences in weaning weight are due to age differences and this is the general description adopted by the cattle industry (Tyler, 2012) in northern Australia as well as previous publications generated from studies in this environment (Holroyd et al., 1990; Sullivan et al., 1992; Sullivan and O'Rourke, 1997b).

Within each WW group heifers were ranked on LW and assigned to one of three heifer blocks (heavy, medium, light heifers). Heifer blocks were allocated to blocks of 10 adjacent pens at random. Weaning weight and supplement treatment combinations were randomly allocated to pens within each pen block, so that each WWxS combination was represented in each block of 10 pens. Within each heifer block heifers were randomly allocated to a pen, with n = 4 or n = 5 heifers/pen for EW and NW respectively.

The experiment was run over approximately 2 years with the experimental periods described as:

- 1st dry season (169 days; 18-June-2014 to 4-December-2014)
- 1st wet season (168 days; 5-December-2014 to 22-May-2015)
- 2nd dry season (160 days; 23-May-2015 to 30-October-2015)
- 2nd wet season (207 days; 31-October-2015 to 25-May-2016)

During the first dry season, all heifers were offered *ad libitum* access to sabi grass hay (897 g OM, 38 g CP, 612 g NDF, 1.6 g P and 2.8 g Ca/kg DM) and mineral block lick [MBL; (Rumevite: 30% Urea + P; Ridley Agriproducts, Toowoomba, QLD, Australia), 890 g CP, 20 g P and 70 g Ca/kg DM]. Hay was chopped to approximately 2 cm in length (NDE 1402; New Direction Equipment, Sioux Falls, SD, USA) prior to feeding and was fed in a separate trough to the supplement block. Each pen was also assigned to one out of five supplement levels 0, 1, 2.5, 5 and 10 g supplement/kg LW.day which were denoted T1, T2, T3, T4 and T5 respectively as demonstrated in Table 5-3. From the start of the 1st dry season until 28-August-2014, copra meal (copra) was fed as the supplement and from day 78 until the end of the first dry season a 50:50 mix (copra:corn; *as fed* weight basis) of copra meal and cracked corn [*Zea mays* L. ;(935 g OM, 78 g CP, 152 g NDF, 2.3 g P and 0.1 g Ca/kg DM)] was fed. Supplements were offered daily at 0730 h and residues were collected, weighed and sampled weekly. Sub-samples of supplement offered were collected weekly and bulked for every month. Hay sub-samples were collected daily and also bulked monthly. The amount of supplement offered (i.e. kg/pen.day) was adjusted weekly based on the mean LW of each pen at the start of each week.

Table 5-3 Description of post-weaning supplementation treatments

Supplement treatment	Supplementation level (g supplement/ kg LW.day)[1]	Mineral supplement
T1	0	MBL *ad libitum*
T2	1	MBL *ad libitum*
T3	2.5	MBL *ad libitum*
T4	5	MBL *ad libitum*
T5	10	MBL *ad libitum*

[1] Supplement composition was modified on experimental day 78 (i.e. 28/08/2014) as described on the text above.

On days 20-August-2014 and 08-October-2014 average hay intake was measured over 7 consecutive days. Average hay intake was determined by collecting and weighing the refusals at the end of each collection period, subtracting from the total offered and dividing by the number of heifers in the pen. Samples of hay offered and refusals were collected daily from each pen and pooled within collection period and pen. Sub-samples of each period and pen were then utilized for DM determination and chemical analysis.

At the end of pen feeding period at the end of the 1st dry season all heifers were transferred to an irrigated improved pasture (28 ha) composed of Mekong grass (*Brachiaria brizantha* cv. Mekong; 915 g OM, 54 g CP, 528 g NDF, 1.1 g P and 2.4 g Ca/kg DM) and Altum grass (*Panicum altum*; 889 g OM, 60g CP, 627 g NDF, 1.0 g P and 4.1 g Ca/kg DM). Heifers grazed the improved pasture paddock at KRS for 57 days and then were transferred to VRRS on 30-January-2015 where they remained until the end of the experiment. At VRRS all heifers were subjected to the same management and were offered *ad libitum* access to mineral loose lick [50% salt, 25% urea, 10% Kynophos (21 % P, 16% Ca and 1.5% Mg; Brisbane Export Corporation, Brisbane, QLD, Australia) and 15% ammonium sulphate (Gran-am; Incited Pivot, Brisbane, QLD, Australia)] during the second dry season and wet season mineral loose lick (50% salt, 35% Kynophos and 15% Gran-am) during the first and second wet season which are used routinely on the experiment station to supply N and minerals, especially P.

Heifers were exposed to bulls for mating from 05-January-2015 to 25-May-2016. Bulls were tested for fertility by standard bull breeding soundness before mating and heifers were vaccinated against bovine venereal campylobacteriosis (Vibrovax; Pfizer, West Ryde, NSW, Australia) at the beginning of the mating period.

5.3.3. *Liveweight, body measurements and body condition score*

At the beginning and end of the 1st dry season in pens heifers were weighed after feed curfew (15 h off feed but free access to water) but all the other LW measurements within this period were made on unfastened animals at the same time and day prior to feeding every 14 days. Liveweight gain was calculated by both the difference in LW between start and end of the period and also by regression of change in LW over time. During the subsequent grazing periods all LW measurements were made after 15 h feed curfew but with free access to water. The LW recorded at the end of the second dry season was used to generate the relationship between Pre-mating LW (PM.LW) and pregnancy rate.

Hip height was measured using a tape suspended from a support structure in the crush that was able to move back and forwards, in the horizontal direction to adapt for different body lengths. The measurement was taken at the highest point of the sacrum between the tuber coxae bones. Hip height was then calculated by the difference from the distance to the ground and each individual animal measurement. Hip width (HW) was accessed as the distance between the most distant points of the tuber coxae on either sides of the animal. It was measured using an adapted caliper in order to decrease any possible interference caused by changes in body condition score that could occur when using a measuring a tape.

Cumulative LW, HH and HW were calculated by subtracting the initial from the final measurement of each phase. Liveweight gain, HHG and hip width gain (HWG) over the first dry and wet seasons were calculated by regressing each measurement over time. For the second dry and wet seasons the rates were calculated by dividing the cumulative change of each measurement by the number of days within each period.

Body condition score (BCS) was measured every month (1st dry and wet season) and start and end of the period (2nd dry and wet season) using a 1 to 5 scale where 1 is emaciated and 5 is obese (Ayres et al., 2009).

5.3.4. *Eye muscle area and fat depth at P8 and P13 sites.*

Eye muscle area (EMA; cm^2) was measured using a cross sectional image of *longissimus dorsi* between the 12th and 13th rib. Subcutaneous fat depth was measured at rump (P8; mm) and between the 12th and 13th ribs (P13; mm) sites (Robinson et al., 1992). All measurements were conducted using an ultrasound (Aloka SSD-500, Corometrics Medical Systems; Wallington, CT, USA). EMA was

estimated based on the muscle depth (EM.d) and width (EM.w) using the Equation 5-1 described by Ferreira et al. (2012):

Equation 5-1: $EMA = (\frac{EM.d}{2}\ x\ \frac{EM.w}{2}) \times \pi$

5.3.5. *Blood samples*

Four heifers from each pen were selected for blood collection throughout the experiment. The selection was balanced for origin of animals from the source of station. Blood samples were collected following the procedures described in Chapter 3 and were performed at the start of 1st dry season, end of 1st dry season, early in 1st wet season at KRS) and end of 1st wet season at VRRS. The plasma samples were analysed to determine the concentration of IGF-1, T3 and leptin, as well as CTX-1 and BAP.

5.3.6. *Bone samples and histomorphometry*

Within the group selected for blood samples, another sub-group (n = 2/pen) of one heifer per station source in each pen was chosen for bone biopsy (total n=6/WWxS treatment combination). Biopsies were collected at the end of 1st dry season and beginning of the 1st wet season (13/01/2015) whilst still at KRS. The first bone collection was obtained from the left tuber coxae and the second from the right tuber coxae. The surgical procedure performed in order to collect the biopsies as well as the handling of samples after collection and measurement of histomorphometry bone parameters are described in Chapter 3.

5.3.7. *Ovary scanning and pregnancy test*

All heifers weighing over 250 kg at the end of the 2nd dry season were ovary scanned using an ultrasound (HS-2200, Honda Electric, Inc.; Toyohashi, Japan) with a 7.5 MHz linear interoperative 38mm probe by trans-rectal examination and classified according to the presence of the *corpus luteum* (CL) or not. The process was repeated 10 days later in order to detect possible heifers that were cycling but did not have a CL developed by the occasion of the first scanning. All ovary scanning and pregnancy testing were conducted by the same experienced operator. All heifers recorded with a CL were considered to have reached puberty.

At the end of the second wet season all heifers were pregnancy tested by rectal palpation and ultrasonography. The pregnancy test was conducted by a single experienced operator. All heifers were classified as 0 or 1 for negative and positive pregnancy status respectively.

5.3.8. *Laboratory analysis*

Chemical analyses of feed offered (i.e. hay, supplement and pasture samples) were performed according to the procedures described in Chapter 3. The concentration of metabolites, hormones and bone markers in plasma were determined in samples collected at the dates described in section 5.3.5 of this Chapter following the procedures described in Chapter 3.

5.4 Statistical analysis

All statistical analyses were conducted using the open-source software R version (R Core Team, 2013). For all parameters except pregnancy rate the pen was used as the experimental unit. Growth rate measurements, EMA, P8 and P13 fat depth, histomorphometric parameters, and the concentration of metabolites, hormones and bone markers in the plasma were carried out using the linear mixed models procedure of the package “nlme” (Pinheiro et al., 2014). Weaning weight group, S and their interaction were included in the model as fixed effects and pen within block as a random factor. The initial measurement of each phase was included as a covariate for the growth rate measurement models. Cumulative measurements were analysed using a generalized linear model with WW group, S and their interaction as main effects. The block effect was tested and dropped from the model, as it was not significant (P>0.05) for any variable. Prior to analysis all data were checked for normality and homoscedasticity and if necessary data were transformed according to the box-cox procedure (Osborne, 2010).

A regression analysis was performed to test the effect of supplement intake and type (i.e. copra and copra:corn) on LWG and hay intake. The type of supplement was dropped from the model when not significant (P>0.05). A sequence of models starting with degree=3 was fitted to test the polynomial level needed to describe the response. Then non-significant terms were sequentially removed to reduce the degree of polynomial. The same procedure was adopted to test the effect of first dry season LWG on first wet season LWG and the cumulative LWG of the 1st dry season on the cumulative LWG of the whole experimental period. The effect of WW group was tested but removed from the model since it was not significant.

The effect of WW, S and their interaction on pregnancy rates was tested using a generalized liner model with binomial variance and a logit "link". The p-values for main factors were generated using the chi-square test. A similar model using the PM.LW recorded at the end of the 2nd dry season and WW group as an explanatory variable was built to investigate the relationship of LW and pregnancy rates. As the WW group was not significant (P=0.71) it was excluded from the model and means from both groups were pooled.

5.5 Results

5.5.1. *Liveweight gain and hay intake response to level of supplementation*

Heifers with access only to MBL (T1 treatment group) maintained LW over the first dry season (Table 5-4 and Figure 5-1). Increasing the intake of copra or copra:corn lead to a quadratic increase in LWG of heifers. The maximum intake of the copra supplement was 5.4 g DM/kg LW.day in one of the T5 pens whilst mean copra intake was 3.9 and 3.3 g DM/kg LW.day for the T5 level of the NW and EW groups respectively. For the remainder of the first dry season (the copra:corn mix was utilised as the supplement in an attempt to increase supplement and ME intake. Intake of the copra:corn supplement supplement increased to 7.2 and 6.6 g DM/ kg LW.day for the T5 level of the NW and EW groups respectively.

Hay intakes for T1 heifers (mean of NW and EW) were 23.4 ± 0.52 and 21.6 ± 0.64 g DM/kg LW.day during the periods when copra and copra:corn were fed as supplements to the other groups (Figure 5-2). There was a significant (P<0.001) linear decrease in hay intake as the intake of supplement increased. Supplement intakes were lower than the expected but still effective to generate a divergence in LW and HH growth paths during the 1st dry season (Figure 5-3).

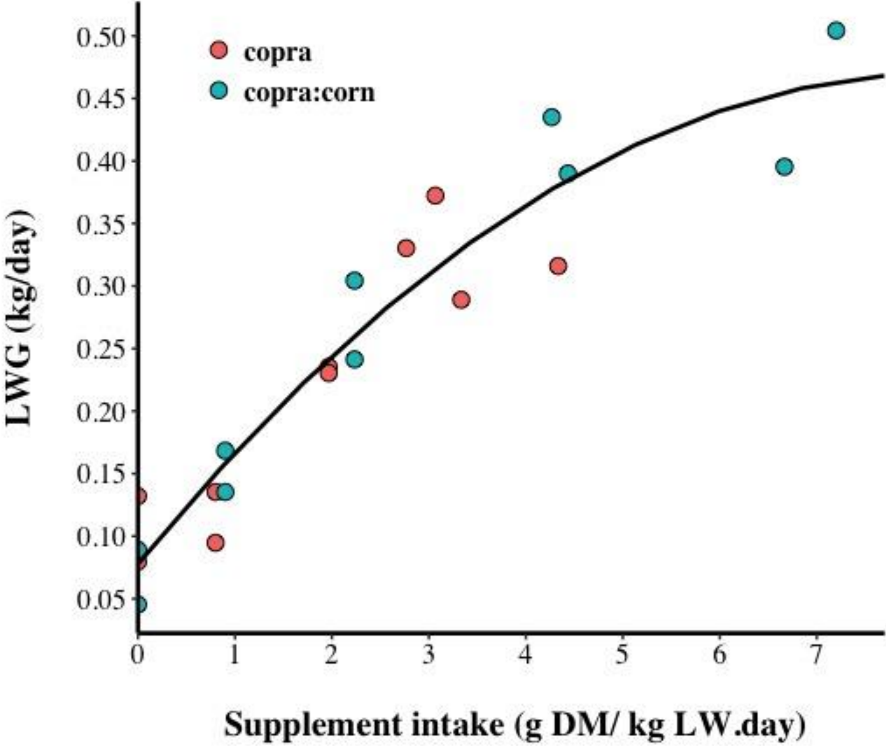


Figure 5-1 Effect of copra and copra:corn supplement intake on liveweight gain (LWG) of crossbred Brahman heifers fed low quality sabi grass hay *ad libitum*. Copra alone was supplied over the first 72 days and then changed to a copra:corn mix for the remaining 97 days. Details of the composition of the supplements are given in text. The regression first included the type of supplement as a factor but this was removed from the model as it was not significant and a single equation was generated including both supplements across the entire experimental period ($Y= 0.077743 + 0.094519X - 0.005690X^2$; $R^2=0.81$, RSE=0.0062, $P<0.001$)

Table 5-4 Liveweight gain (LWG; g/day), hip height gain (HHG; mm/100 days), hip width gain (HWG, mm/100 days) and body condition score (BCS) recorded at the end of each phase of early (EW) and normally (NW) weaned[1] crossbred Brahman heifers fed different levels of supplement[2] over the 1st dry season[3,4].

		Supplementation treatment (S)					Weaning weight group (WW)			*P* value		
		T1	**T2**	**T3**	**T4**	**T5**	**EW**	**NW**	SEM	S	WW	S x WW
1st dry season	LWG	116^{a}	188^{a}	300^{b}	389^{bc}	423^{c}	264	303	46	<0.0001	0.09	0.18
	HHG	54^{a}	58^{ab}	65^{bc}	70^{c}	73^{c}	67	61	3.8	<0.0005	<0.01	0.68
	HWG	9.9^{a}	10^{a}	17^{ab}	23^{b}	24^{b}	16	17	1.9	<0.0001	0.54	0.37
	BCS[5]	2.8^{a}	3.0^{ab}	3.1^{b}	3.4^{c}	3.5^{c}	3.0	3.8	0.08	<0.0001	<0.0001	0.12
1st wet season	LWG	412^{b}	418^{b}	391^{b}	359^{ab}	290^{a}	375	372	35	<0.0005	0.86	0.41
	HHG	27	29	30	29	26	34	22	3.5	0.35	<0.0001	0.56
	HWG	26	30	29	29	23	29	26	1.7	0.09	0.15	0.58
	BCS	2.9	3.0	3.0	3.0	3.1	2.9	3.1	0.1	0.13	<0.0001	0.63
2nd dry season	LWG	27	-8	-37	-2	-16	17	-32	37	0.16	<0.005	0.77
	HHG	27	29	25	28	23	27	26	4.2	0.48	0.43	0.62
	HWG	19	19	16	19	22	19	19	1.2	0.06	0.46	0.74
	BCS	2.7	2.7	2.7	2.8	2.6	2.6	2.8	0.09	0.19	<0.0001	0.72
2nd wet season	LWG	492	519	535	500	487	500	513	30	0.17	0.33	0.61
	HHG	27^{b}	21^{ab}	25^{ab}	19^{a}	23^{ab}	29	17	2.5	<0.01	<0.0001	0.82
	HWG	15^{ab}	18^{b}	15^{ab}	12^{a}	12^{a}	17	13	1.6	0.03	<0.01	0.65
	BCS	3.0	3.0	3.0	3.0	3.1	3.0	3.1	0.05	0.37	<0.005	0.06

[1,2] See materials and methods for description of weaning weight groups and supplement treatments.

[3] Data are least squares means with standard error of the mean (SEM).

[4] Means within a row with different superscript differ (*P*<0.05).

[5] BCS recorded at the end of each phase.

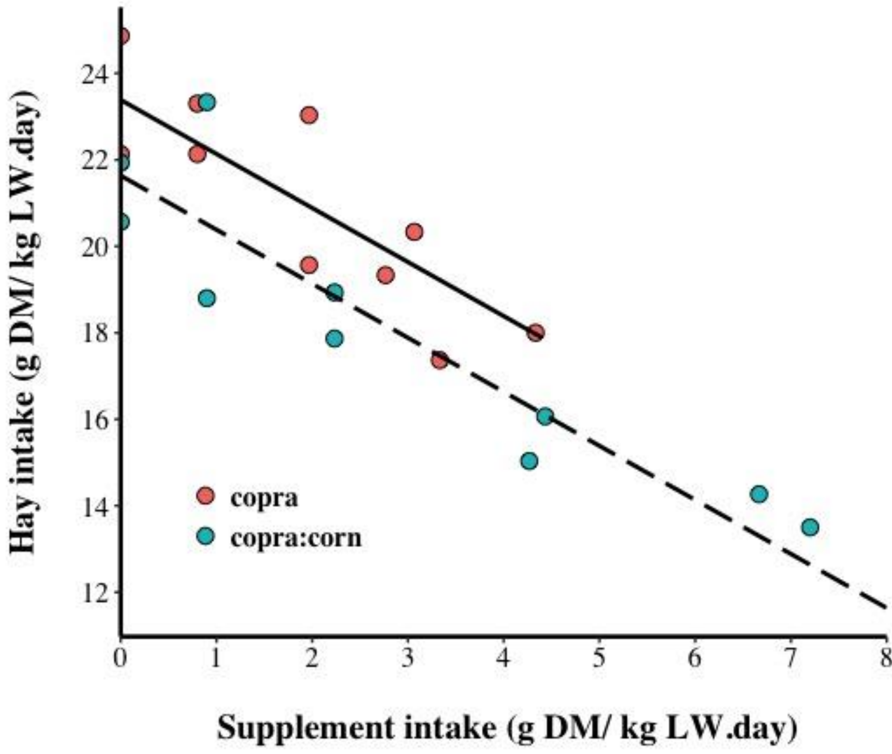
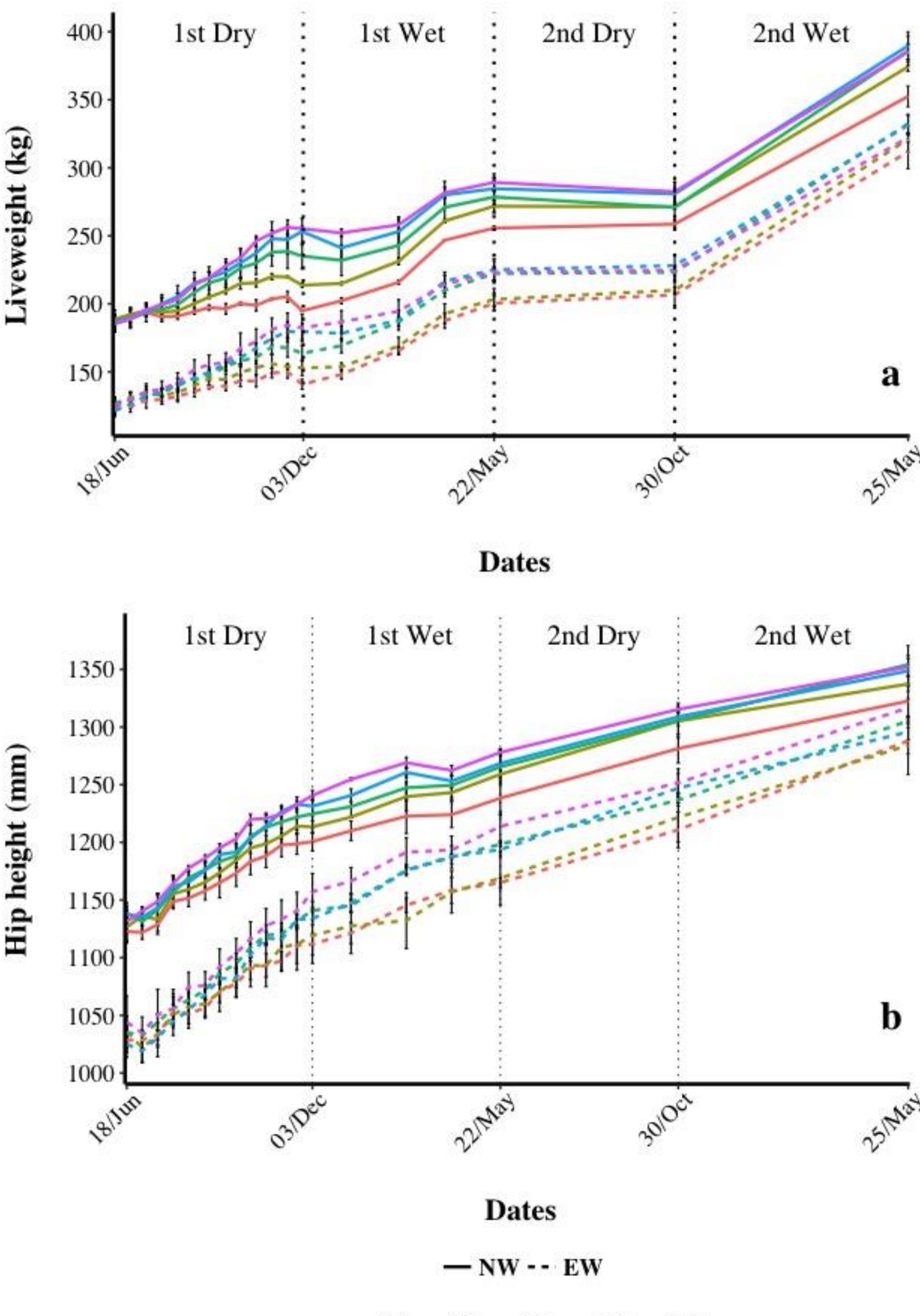


Figure 5-2 Effect of copra (solid line; Y1) and copra:corn (dashed line; Y2) intake on sabi grass hay intake of crossbred Brahman heifers. Copra alone was supplied over the first 72 days and was changed to a copra:corn mix for the remaining 97 days. Details of the composition of the supplements are given in text. The supplement type (i.e. copra and copra:corn) was significant ($P<0.01$) but not the interaction between supplement type and supplement intake ($P=0.32$), thus two lines were fitted with a different intercept for each supplement type (Y1=23.385 -1.2497X, $R^2=0.61$, RSE=2.41, $P<0.001$; Y2=21.63-1.2497X, $R^2=0.61$, RSE=2.41, $P<0.001$).



1st Dry
1st Wet
2nd Dry
2nd Wet
Liveweight (kg)
400
350
300
250
200
150
a
18/Jun
03/Dec
22/May
30/Oct
25/May
Dates
1st Dry
1st Wet
2nd Dry
2nd Wet
Hip height (mm)
1350
1300
1250
1200
1150
1100
1050
1000
b
18/Jun
03/Dec
22/May
30/Oct
25/May
Dates
NW
EW
T1
T2
T3
T4
T5

Figure 5-3 Liveweight (a) and hip height (b) (mean ± SEM) of early (EW) and normally (NW) weaned Brahman crossbred heifers fed five different supplement treatments (T1 to T5, description in text) during the 1st dry season after weaning and when grazed together over the next 18 months.

5.5.2. *Cumulative and rate of liveweight, hip height and hip width gain, and body condition score.*

During the first dry season, growth and rate of growth of all parameters assessed increased in response to increasing supplement intake (Table 5-5). Whilst there was no difference in LWG between EW and NW heifers, HHG was higher in EW heifers compared with NW heifers and EW were in lower BCS at the end of the period (P<0.001).

Heifers with higher supplement intake during the first dry season gained less weight during the following wet season (Table 5-5). A significant negative linear relationship existed between LWG during first dry season and LWG during the first wet season (Figure 5-4; R^2=0.31, P=0.004). At the end of the first wet season T1 heifers had recovered 44% of the LW difference from T5 that existed at the end of the first dry season. Liveweight was the only parameter that showed any degree of compensation during this period. Overall there was a greater increase in HH by EW heifers compared to NW during the first wet season.

Heifers maintained LW over the second dry season independently of supplement intake during the first dry season. EW heifers had a statistically significant (P<0.01) but biologically insignificant increase in cumulative LW during this period while NW LW (2 *vs* -3 kg). At the end of the second dry season BCS of NW heifers was higher than EW heifers despite the higher LWG achieved by the latter group during the period. During the second wet season, heifers that had the highest supplement intake during the first dry season showed lower HH and HW growth rates and cumulative growth when compared to heifers with low or no supplement intake. EW heifers also had higher HH (29 *vs* 17 mm/100 days, P<0.01) and HW (17 *vs* 13 mm/100 days, P<0.05) gain than NW heifers during the wet season but nodifferences in LWG were observed (500 *vs* 513 g/day, P=0.33). The analysis of cumulative LW, HH and HW over the entire experimental period showed that supplementation over the first dry season had a significant effect only on LW. Control heifers with access to MBL only (T1 treatment group) gained less LW than heifers offered the T3 and T4 across the entire experimental period (173 *vs* 197 and 201 kg) with no further differences between the other supplement groups (Table 5-4). Supplement intake in the first dry season had no effect on cumulative growth of any other parameter across the entire experiment. The cumulative growth of EW heifers was greater than NW heifers across the entire experimental period for all parameters, however the EW heifers remained smaller and lighter than NW heifers at the end of the experimental period.

Table 5-5 Cumulative liveweight (CLW; kg), hip height (CHH; cm), hip width (CHW; cm) of early (EW) and normally (NW) weaned[1] crossbred Brahman heifers fed different levels of supplement[2] over the 1st dry season[3,4] recorded at the end of each phase of the experiment

		Supplementation treatment (S)					Weaning weight group (WW)			*P* value		
		T1	**T2**	**T3**	**T4**	**T5**	**EW**	**NW**	SEM	S	WW	S x WW
1st dry season	CLW	8^{a}	20^{b}	38^{c}	57^{d}	58^{d}	35	38	2.3	<0.0001	0.28	0.05
	CHH	7.8^{a}	8.2^{ab}	9.5^{ab}	10.6^{bc}	11.5^{c}	10.0	8.8	0.92	<0.0001	0.006	0.76
	CHW	1.5^{a}	1.6^{ab}	2.5^{b}	2.7^{b}	3.7^{b}	2.4	2.3	0.2	<0.0001	0.58	0.71
1st wet season	CLW	60^{b}	54^{b}	51^{b}	38^{a}	37^{a}	51	45	2.1	<0.0001	0.01	0.01
	CHH	4.5	5.0	4.6	4.9	4.6	5.5	3.9	0.94	0.94	<0.0005	0.73
	CHW	4.6	4.8	4.8	4.6	3.7	4.7	4.3	0.3	0.18	0.17	0.52
2nd dry season	CLW	4	1	-3	-0.5	-2	2	-3	1.9	0.12	<0.01	0.80
	CHH	4.4	4.7	4.1	4.6	3.7	4.4	4.1	0.85	0.45	0.42	0.59
	CHW	3.1	3.1	2.7	3.0	3.6	3.2	3.0	0.2	0.05	0.45	0.72
2nd wet season	CLW	100	106	109	104	100	104	104	2.3	0.10	0.96	0.29
	CHH	5.7^{b}	4.5^{ab}	5.2^{ab}	3.9^{a}	4.9^{ab}	6.2	3.6	0.78	0.02	<0.0001	0.88
	CHW	3.3	3.8	3.2	2.6	2.6	3.5	2.7	0.3	0.05	0.01	0.57
Total[5]	CLW	173^{a}	185^{ab}	197^{b}	201^{b}	194^{ab}	195	185	4.4	0.005	0.04	0.58
	CHH	22.8	22.9	23.9	24.1	25.1	26.6	20.9	1.71	0.29	<0.0001	0.82
	CHW	12.6	13.5	13.4	13.2	13.7	14.0	12.6	0.5	0.19	<0.0001	0.04

[1,2] See materials and methods for description of weaning weight groups and supplement treatments.

[3] Data are least squares means with standard error of the mean (SEM).

[4] Means within a row with different superscript differ ($P<0.05$).

[5] Total represents the entire experimental period.

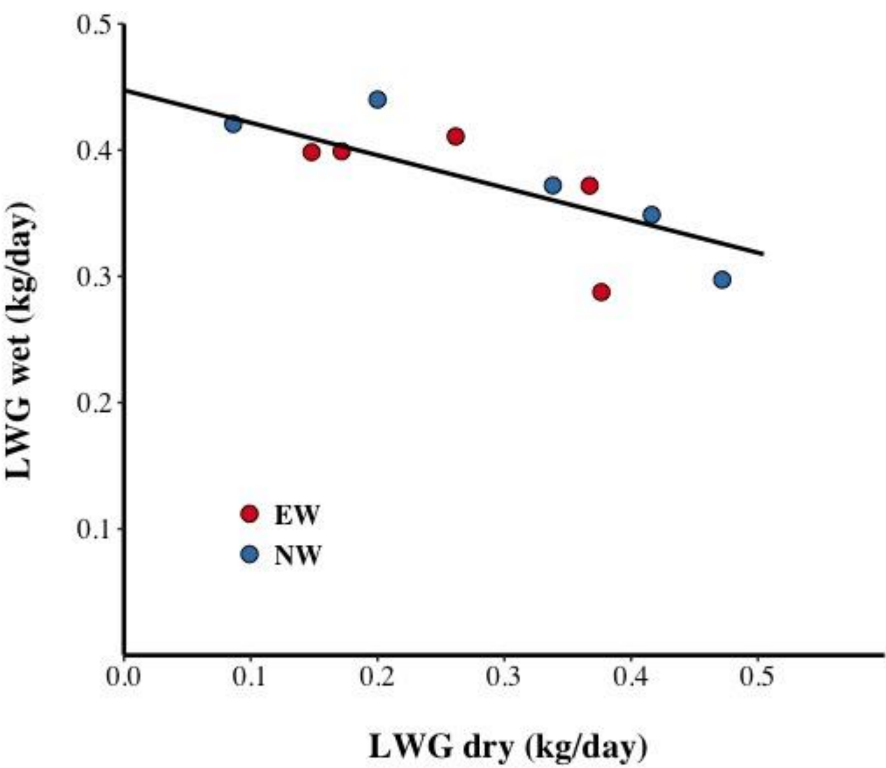


Figure 5-4 Relationship between liveweight gain (LWG) during the 1st dry season (LWG dry) and LWG over the subsequent wet season (LWG wet) of early (EW) and normally (NW) weaned crossbred Brahman heifers. Initial model included weaning weight group as a factor which was not significant and removed from the final model (Y= 0.44296 -0.2649X, R^2=0.32, RSE=0.05, P=0.004).

5.5.3. *Eye muscle area, body condition score and fat depth at P8 and P13 sites.*

Eye muscle area was initially larger in NW compared to EW heifers (Figure 5-5). During the 1st wet season EW heifers had a greater increase in EMA after which there was no difference in EMA between WW groups. Heifers with the highest supplement intake had a larger EMA than heifers fed T1 (MBL) at the end of the first dry and wet seasons. The BCS of supplemented heifers increased during the first dry season (Figure 5-6). The difference in BCS between supplemented and non-supplemented heifers decreased during the 1st wet season and was no longer evident by the end of this phase. EW heifers showed significantly lower BCS than NW for most of the experimental period although by the end of the 2nd wet season there were no longer differences between these groups.

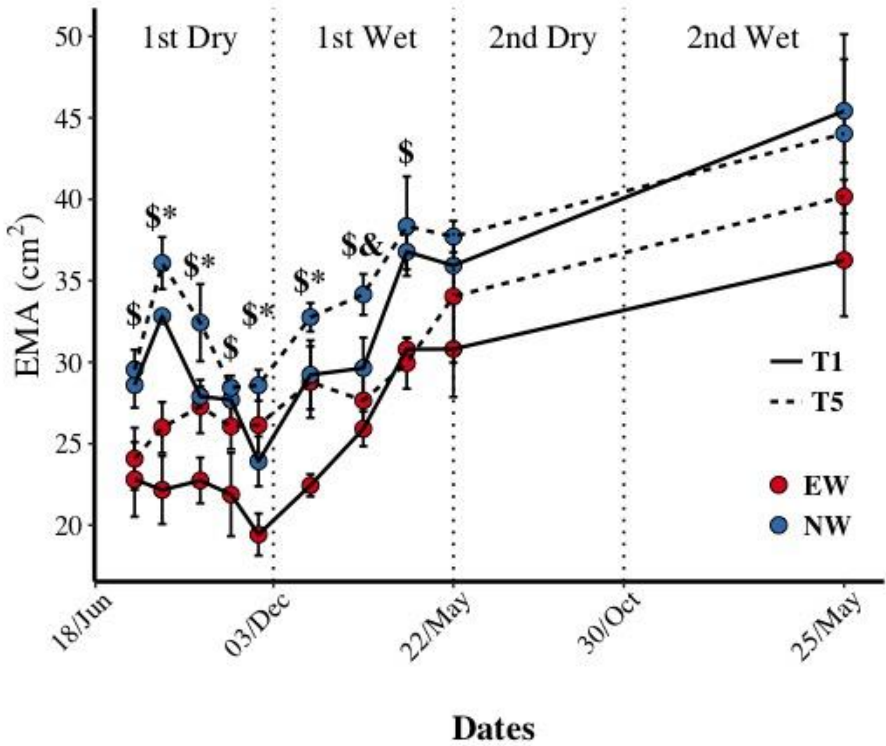


Figure 5-5 Eye muscle area (cm^2) (EMA; mean ± SEM) of Early (EW) and Normally (NW) weaned beef heifers fed T1 or T5 supplement treatments[1] during 1st dry season. Symbols represent significant weaning weight (WW) or supplement effect within dates (Tukey's HSD; $: WW *P*<0.05; *: Supplement *P*<0.05; &: Supplement *P*<0.10).

[1]Supplement treatments are described in the materials and methods section.

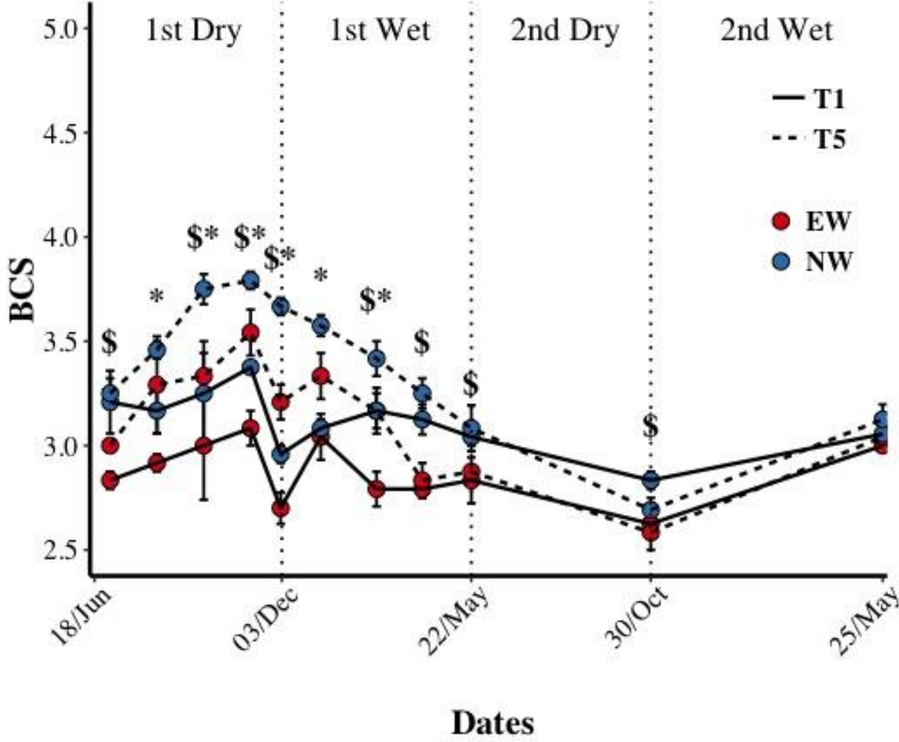
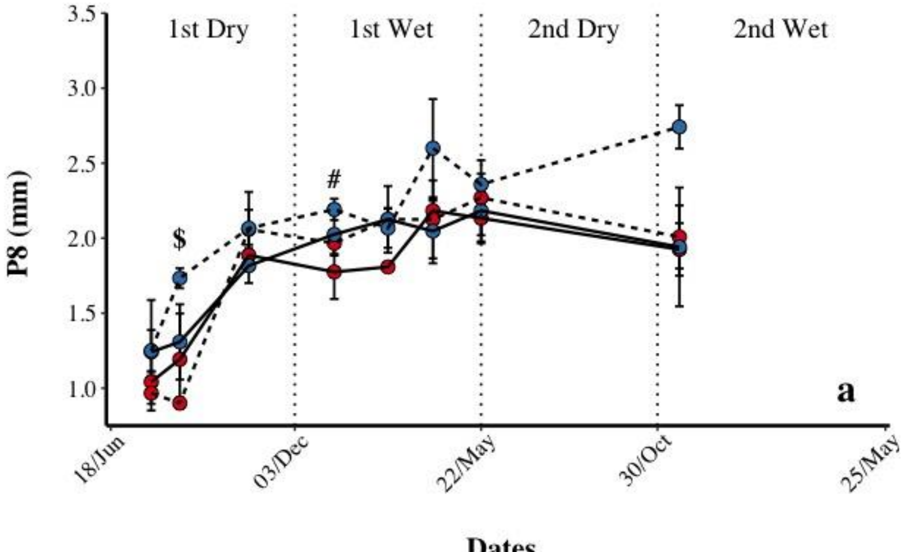


Figure 5-6 Body condition score (1 to 5 scale) (BCS; mean ± SEM) of Early (EW) and Normally (NW) weaned beef heifers fed T1 or T5 supplement treatment[1] during the 1st dry season. Symbols

represent significant weaning weight (WW) or supplement (Sup) effect within dates (Tukey's HSD; $: WW P<0.05; *: Sup P<0.05).

[1]Supplement treatments are described in the materials and methods section.

Fat depth at the P8 and P13 sites showed a great increase in T1 and T5 groups during the 1st dry season in pens and a smaller increase over the 1st wet season (Figure 5-7). Heifers fed the highest level of supplement (i.e T5) had significantly more fat at P13 and P8 at the beginning of the 1st dry and wet seasons. By the end of the 1st wet season, a significant interaction between supplement treatments and weaning weight groups show that T1 EW heifers had significant less fat stored at the P13 site than the other groups. During the second dry season T5 NW seemed to increase fat depth at both P8 and P13 sites although this difference was not statistically significant (P>0.05).



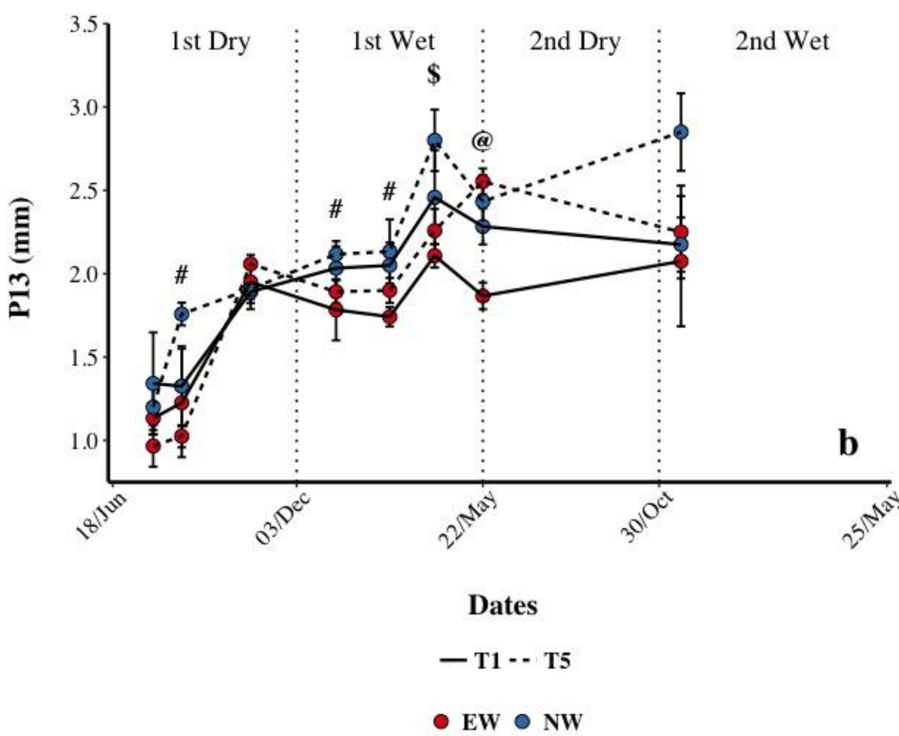


Figure 5-7 Fat depth at P8 (a) and P13 (b) sites (BCS; mean ± SEM) of Early (EW) and Normally (NW) weaned beef heifers fed T1 or T5 supplement treatment[1] during the 1st dry season. Symbols represent significant effect of weaning weight (WW), supplement (Sup) or the interaction between WW and Sup within dates (Tukey's HSD; $: WW *P*<0.05; #: Sup *P*<0.10; @: WW x Sup *P*<0.05).

[1] Supplement treatments are described in the materials and methods section.

5.5.4. *Plasma metabolites, hormones and bone metabolism markers*

The concentration of IGF-1 was higher in the plasma of NW heifers compared with EW heifers prior to commencement of the experiment (*P*=0.01) with no differences in the concentration of metabolites, other hormones or bone markers observed at this time (Tables 5-5 and 5-6). The concentration of PUN, insulin, IGF-1, T3 and BAP in the plasma increased in response to supplementation during the first dry season (Table 5-7). The concentration of leptin and CTX-1 were unaffected by supplement intake. The concentration of all metabolites, hormones and bone markers at the end of the first dry season was unaffected by WW. Similarly there were no significant interactions between supplementation and weaning weight (Table 5-6 and Table 5-7) at the end of the first dry season.

At the start of the 1st wet season heifers fed supplements during the previous dry season had higher IGF-1 and lower NEFA concentration in the plasma compared to unsupplemented heifers (Table 5-6 and Table 5-7). The concentration of IGF-1, P, total protein, PUN and NEFA were higher in the plasma of NW compared to EW heifers at the start of the 1st wet season. However by the end of the

1st wet season the effects of WW or supplementation were only apparent on the concentration of IGF-1 and Ca in the plasma of heifers.

Table 5-6 The concentration of calcium (Ca; mmol/L), phosphorus (P; mmol/L) total protein (g/L), plasma urea nitrogen (PUN; mmol/L) and non-esterified fatty acids (NEFA; mEq/L) in the plasma of Early (EW) and Normally (EW) weaned crossbred Brahman heifers fed two different supplement treatments[1] during the 1st dry season.[2,3]

		EW		NW			*P* value		
		T1	**T5**	**T1**	**T5**	SEM	Sup	WW	Sup x WW
Start 1st Dry	Ca	1.7	1.7	2.0	1.9	0.1	0.97	0.09	0.54
	P	2.3	2.3	2.5	2.4	0.1	0.84	0.30	0.66
	Total protein	63	64	67	64	2.8	0.95	0.40	0.60
	PUN	2.6	2.9	2.2	2.3	0.2	0.54	0.10	0.75
	NEFA	0.5	0.5	0.5	0.4	0.1	0.60	0.98	0.67
End 1st Dry	Ca	1.6	1.8	1.8	1.9	0.1	0.07	0.11	0.98
	P	2.0	2.3	2.3	2.2	0.07	0.23	0.20	0.11
	Total protein	58	60	60	62	1.6	0.24	0.32	0.67
	PUN	1.1	1.4	1.2	1.9	0.1	0.02	0.08	0.21
	NEFA	0.3	0.3	0.4	0.4	0.07	0.85	0.32	0.98
Start 1st Wet	Ca	2.1	2.2	2.3	2.3	0.08	0.54	0.17	0.80
	P	2.2	2.2	2.6	2.3	0.1	0.14	0.03	0.18
	Total protein	69	68	72	73	1.6	0.83	0.01	0.37
	PUN	2.2	1.9	2.2	2.5	0.1	0.95	0.03	0.07
	NEFA	0.48	0.35	0.55	0.50	0.03	0.03	0.02	0.30
End 1st Wet	Ca	2.5	2.5	2.4	2.3	0.06	0.63	0.04	0.44
	P	1.3	1.3	1.6	1.2	0.1	0.18	0.63	0.15
	Total protein	75	75	74	76	1.0	0.35	0.63	0.21
	PUN	7.8	7.3	6.9	6.9	0.3	0.53	0.07	0.45
	NEFA	1.4	1.0	1.1	1.4	0.08	0.85	0.43	0.06

[1] See materials and methods for description of weaning weight groups and supplement treatments.

[2] Data are least squares means with standard error of the mean (SEM).

[3] Start and end of 1st dry and start and end of 1st wet refer to the following collection dates: 18/06/2014, 02/12/2014, 06/01/2015 and 22/05/2015

Table 5-7 The concentration of insulin (IU/mL), insulin-like growth factor-1 (IGF-1; ng/mL), triiodothyronine (T3; nmol/L), leptin (ng/mL), bone-specific alkaline phosphatase (BAP; U/L) and C-terminal telopeptides of type I collagen (CTX-1; ng/mL) in the plasma of Early (EW) and Normally (EW) weaned (weaning weight, WW) crossbred Brahman heifers fed two different supplement (S) treatments[1] during the 1st dry season.[2,3]

		EW		NW			*P* value		
		T1	**T5**	**T1**	**T5**	SEM	S	WW	S x WW
Start 1st Dry	Insulin	7.1	8.6	8.8	9.6	1.1	0.19	0.13	0.69
	IGF1	5.8	11.2	25.7	24.5	5.2	0.69	0.01	0.55
	T3	0.7	1.0	1.1	0.9	0.1	0.86	0.29	0.17
	Leptin	5.4	6.8	6.4	7.0	1.1	0.36	0.57	0.68
End 1st Dry	Insulin	7.3	9.8	8.6	15.9	1.7	0.05	0.19	0.56
	IGF1	9.2	53.7	18.3	100.8	19.3	0.001	0.14	0.66
	T3	1.0	1.8	0.9	1.48	0.2	0.04	0.33	0.84
	BAP	35	46	25	35	5.0	0.05	0.08	0.99
	CTX-1	1.7	1.8	1.8	1.3	0.2	0.21	0.17	0.13
	Leptin	5.5	6.2	4.9	4.7	0.7	0.94	0.22	0.56
Start 1st Wet	Insulin	8.8	7.9	8.3	9.6	1.3	0.68	0.41	0.31
	IGF1	12.5	32.9	34.4	39.8	7.7	0.03	0.04	0.12
	T3	0.9	0.9	0.9	0.7	0.1	0.29	0.20	0.28
	BAP	28	31	42	31	4.5	0.47	0.07	0.10
	CTX-1	1.9	1.1	1.6	1.9	0.2	0.16	0.25	0.06
	Leptin	7.0	5.4	4.3	6.4	1.5	0.87	0.52	0.21
End 1st Wet	Insulin	7.6	10.2	10.3	8.5	1.3	0.78	0.59	0.14
	IGF1	25.7	48.2	54.7	66.7	14.0	0.01	0.005	0.23
	T3	1.8	1.8	2.0	1.5	0.2	0.38	0.75	0.37
	Leptin	11.1	9.8	12.3	12.3	1.5	0.53	0.30	0.73

[1] See materials and methods for description of weaning weight groups and supplement treatments.

[2] Data are least squares means with standard error of the mean (SEM).

[3] Start and end of 1st dry and start and end of 1st wet refer to the following collection dates: 18/06/2014, 02/12/2014, 06/01/205 and 22/05/2015.

5.5.5. *Growth plate and trabecular bone histology*

Heifers that had high supplement intake had larger PZ, HZ and HC diameter than un-supplemented heifers (Table 5-8). The height of the PZ of the growth plate showed a significant (P=0.01) interaction between supplementation and WW, with the increased the height of the PZ apparent in EW but not NW heifers. The height of the HZ and the diameter of HC were increased by supplementation in both WW groups. After 35 days into the grazing phase (start 1st wet) when level of nutrition had improved, None of the morphological differences in bone evident at the end of the first dry season persisted when measured again early in the first wet season.

Table 5-8 Height of proliferative (PZ; µm) and hypertrophic zone (HZ; µm), number of hypertrophic chondrocytes (HC) per column (n/column), diameter terminal HC (µm), bone volume (BS.TV; %), trabecular separation (Tb.Th; µm), trabecular thickness (Th.Sp; µm) and bone surface area (mm^2) of Early (EW) and Normally (EW) weaned crossbred Brahman heifers fed two different supplement (S) treatments[1] during the 1st dry season.[2,3]

		EW		NW			*P* value		
		T1	**T5**	**T1**	**T5**	SEM	S	WW	S x WW
End 1st dry	PZ	184 [a]	221 [b]	221 [b]	218 [b]	8.4	0.03	0.03	0.01
	HZ	180	220	195	207	9.8	0.03	0.89	0.19
	HC number	8.3	9.0	8.8	8.9	0.3	0.27	0.62	0.36
	HC diameter	26.3	29.1	24.4	28.2	1.2	0.004	0.11	0.54
	Bv.Tv	20.2	20.9	21.2	21.1	0.1	0.83	0.70	0.78
	Tb.Th	149	158	165	149	5.7	0.62	0.44	0.05
	Tb.Sp	561	540	609	511	42	0.23	0.76	0.41
	BS	41	44	43	43	2.1	0.74	0.90	0.75
Start 1st wet	PZ	195	214	225	208	8.3	0.90	0.14	0.05
	HZ	194	221	210	191	9.4	0.68	0.49	0.05
	HC number	8.9	8.2	7.7	7.6	0.5	0.15	0.56	0.61
	HZ diameter	25.0	26.6	25.6	24.9	0.8	0.52	0.42	0.12
	Bv.Tv	15.8	19.0	18.3	17.7	0.8	0.19	0.53	0.07
	Tb.Th	129	129	145	137	6.4	0.53	0.11	0.56
	Tb.Sp	644	574	644	524	41.7	0.09	0.78	0.39
	BS	32	39	38	37	1.8	0.21	0.44	0.07

[1] See materials and methods for description of weaning weight groups and supplement treatments.

[2] Data are least squares means with standard error of the mean (SEM).

[3] End of 1st dry and start of 1st wet refer to the following collection dates: 02/12/2014 and 06/01/2015.

5.5.6. *Reproduction*

At the end of the 2nd dry season 35% of the NW and none of the EW heifers were classified as pubertal (presence of a CL on ovaries) (Table 5-9). Weaning weight but not supplementation had a significant effect on the percentage of heifers exhibiting puberty at the end of the second dry season ($P<0.0001$) and the percentage of heifers pregnant at the end of the 2nd wet season ($P<0.001$) (Table 5-8).

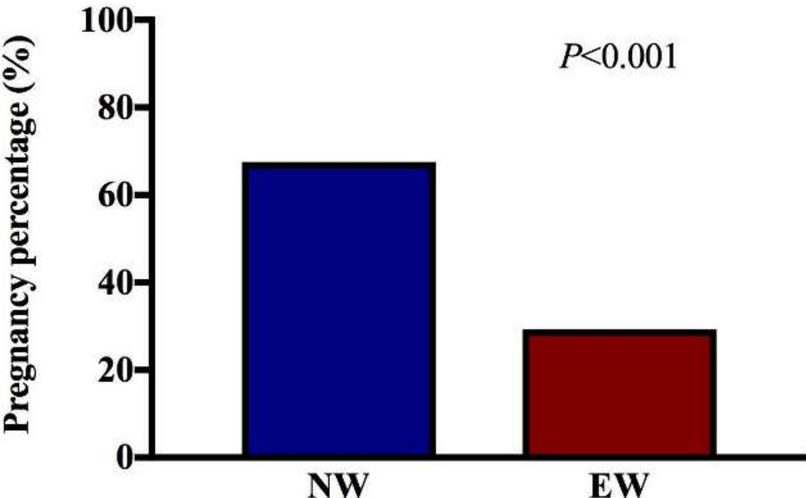


Figure 5-8 Pregnancy percentage of Early (EW) and Normally (NW) weaned Brahman heifers. Standard deviation of pregnancy percentage for NW and EW groups are 5.4 and 5.9 % respectively.

Table 5-9 Reproductive performance of Early (EW) and Normally (NW) weaned crossbred Brahman heifers fed different supplement treatment[1] over the 1st dry season and determined at end of the 2nd dry (puberty) and wet (pregnancy) seasons.[2]

Weaning weight group	Supplement treatment	Weaning weight (kg)	Pubertal percentage (%) [3]	Pre-mating LW Oct (kg)	Cumulative LW during mating (kg)	Pregnancy percentage (%) [3]
NW	T1	185.6 ± 5.3 **a**	13.3	258.6 ± 3.0 **ab**	93.9	53.3
	T2	188.7 ± 3.2 **a**	42.9	271.3 ± 1.6 **a**	103.0	71.4
	T3	187.4 ± 8.0 **a**	20.0	270.9 ± 14.0 **a**	115.1	46.7
	T4	185.8 ± 6.0 **a**	40.0	280.9 ± 8.5 **a**	108.8	86.7
	T5	185.8 ± 6.5 **a**	60.0	282.4 ± 7.02 **a**	102.8	80.0
EW	T1	123.0 ± 6.0 **b**	0.0	206.5 ± 9.5 **c**	105.3	25.0
	T2	126.6 ± 4.5 **b**	0.0	210.0 ± 2.3 **c**	111.1	45.5
	T3	126.5 ±5.0 **b**	0.0	223.2 ± 4.8 **bc**	109.1	25.0
	T4	121.4 ± 2.4 **b**	0.0	228.1 ± 7.8 **bc**	103.3	36.4
	T5	124.4 ± 5.6 **b**	0.0	224.6 ± 3.3 **bc**	97.6	16.7

[1] See materials and methods for description of weaning weight groups and supplement treatments.

[2] Data of weaning weight and Pre-mating LW are least squares means, with standard error of the mean (SEM). Mean within column with different letters differ at Tukey (*P*<0.05).

[3] Pubertal status was determined by ovary scanning realized at the end of the 2nd dry season and pregnancy rate was determined by rectal palpation at the end fo the 2nd wet season. See materials and methods for description of the methodology.

NW heifers from the T3 supplement group had a lower percentage of heifers exhibiting puberty and pregnant than what would be expected based on the average PM.LW. The high variation in PM.LW of T3 group could have affected the percentage of heifers exhibiting puberty. Pre-mating LW recorded at the end of October had a significant (P<0.0001) effect on pregnancy percentage (Figure 5-9). The initial model also included HH and WW which were not significant and were subsequently removedresulting in the following equation:

Equation 5-2: $Pregnancy\ percentage\ (\%) = \frac{e^{\theta}}{(1+e^{\theta})}$

Equation 5-3: $\theta = -7.33539 + 0.02937PM.LW$

Where Pregnancy percentage is the estimation of heifers classified as pregnant based on PM.LW described in Equation 5-3 and Pre-Mating LW (PM.LW) is the feed curfew LW recorded on at the end of second dry season.

On average, pregnant heifers were 30 kg heavier (263.3 vs 233.7 kg) and 3.2 cm (128.7 *vs.* 125.5 cm) taller than non-pregnant heifers at the time of PM.LW. The density plot of the PM.LW by pregnancy status is presented in Figure 5-10.

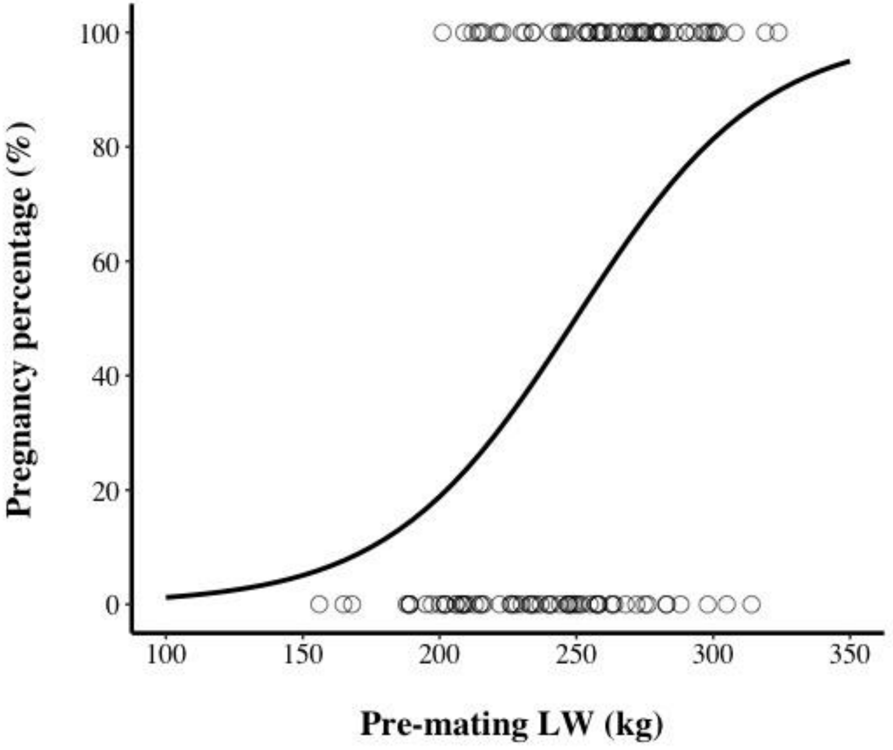


Figure 5-9 Relationship between curfew Pre-mating LW (October/November) and pregnancy percentage of crossbred Brahman heifers in northern Australia. Initially weaning weight group was included as a factor and since it was not significant a single model was created using data from early and normally weaned heifers.

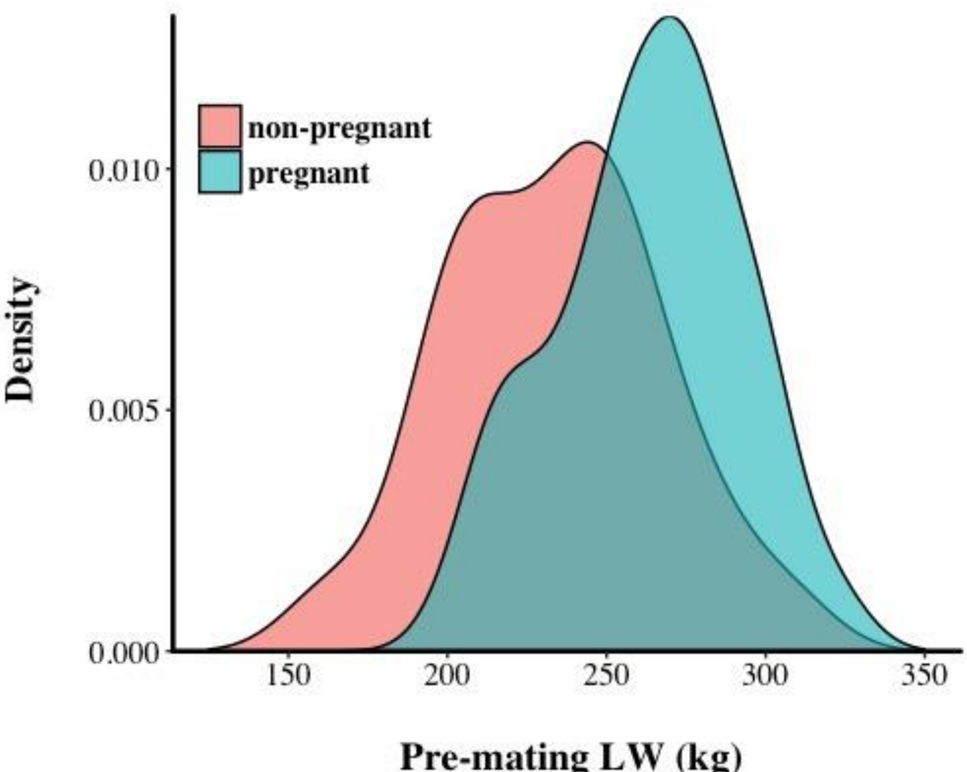


Figure 5-10 Pre-mating liveweight (LW) distribution of crossbred Brahman heifers classified by pregnancy status.

5.6 Discussion

The current experiment examined the effect of weaning weight and weaning supplementation strategies on long term growth and reproduction of *Bos indicus* heifers. The results of this experiment show that dry season supplementation imposed after weaning accelerates LWG and skeletal growth rate in heifers. The heifers with the highest growth rates had higher concentration of IGF-1, T3 and insulin in the plasma during the supplementation period which may be available to exert increased stimulatory effect on cellular activity at the growth plate. Skeletal growth rates of EW heifers appeared to be more responsive to supplementation than NW heifers, however no difference in hormone concentration in the plasma were evident between these groups. Supplementation and WW had no effect on histomorphometry parameters of the tuber coxae trabecular bone or on the concentration of CTX-1 in the plasma. However, supplemented heifers showed a tendency to a greater concentration of BAP, a marker of bone formation. Non-supplemented heifers demonstrated compensatory growth in LW over the first wet season while evidence of catch-up growth in the skeleton was observed over the second wet season. Despite the increase in skeletal growth and LWG in supplemented heifers over the first dry season, supplementation did not lead to an increase in pregnancy percentage when heifers were mated at approximately 2-years of age. Heifers weaned at 180 kg (NW) had a significantly higher pregnancy rate than EW heifers independent of supplement intake over the first dry season. This outlines the long term consequence of WW and first dry season supplementation on pregnancy, a practical objective of this experiment. However the underlying reasons behind the long term consequence of WW and supplement intake during the first dry season are explored further below in relation to LW, BCS and skeletal frame size.

5.6.1. *The use of copra meal for weaners supplementation*

Voluntary intake of copra meal was less than expected when fed with and without addition of cracked corn (3.5 and 6.9 g DM/kg LW.day for copra and copra:corn respectively). McLennan (2004) reported voluntary intakes of copra meal ranging from 7.5 to 9.0 g DM/kg LW.day when feeding Brahman crossbred weaners of 220 kg average LW. Marsetyo et al. (2012) also observed an intake of total supplement as high as 8.3 g DM/kg LW.day when feeding Bali cattle with an equal proportional mix of rice bran and copra meal. The reasons for the lower than expected supplement intakes in the current experiment are unknown. During the experiment no obvious problems with the

supplement were noticed. Ehrlich et al. (1990) utilized copra meal as a supplement to grazing Holstein-Friesian cows and described it as unpalatable to a degree. The voluntary intake of the highest level of copra meal offered (6 kg/head) was only around half or even less than that of the expected and anticipated intake in that study. The variation in acceptance of this product by cattle should be taken into consideration when selecting a protein meal supplement. The highest level of supplement intake achieved during this experiment, without increasing refusals, were obtained with 2.5 and 5 g DM/kg LW.day for copra and copra:corn respectively. However, the range of supplement intake achieved in the first dry season resulted in differences in LW and HH which was the objective of the experiment and design of the nutritional treatments.

5.6.2. *Effect of first dry season supplementation and weaning weight on subsequent liveweight gain*

The pattern of LWG following a period of nutrient restriction has been described by Hornick et al. (2000) as a cubic response. The authors showed that LWG increases substantially during the first month of re-alimentation and decreases over the following 4 months This same pattern was evident in the previous experiment (Chapter 4) and this response was highly correlated with ME intake during the re-alimentation phase. These observations are in agreement with the results of the present study. Un-supplemented heifers showed a greater LWG and EMA increase over the 1st wet season than supplemented heifers although in this study DM intake was not measured during the grazing phase.

Approximately 53% (average of T1, T2 and T3 including both weaning weight groups) of the LW difference achieved feeding supplements during the first dry was lost in the subsequent wet season during compensatory growth and by the start of the 2nd wet season this had reduced further to 66% and at the end of the experiment the average recovery index was 94% of the initial weight difference. This negative linear relationship between first dry and wet season LWG was independent of WW and only existed for LW. Overall, EW heifers had higher cumulative increases in LW, HH and HW than NW heifers over the experimental period. There was no evidence of any long-term negative effect on growth of EW heifers when fed only a mineral urea lick (the T1 treatment) during the first dry season. However, EW heifers entered the mating period at a much lower LW (272.8 *vs* 218.5 kg; P<0.001) and BCS (2.8 *vs* 2.6 units; P<0.001) than NW heifers and this was associated with a significantly lower pregnancy rate when compared to NW heifers (67.6 *vs* 29.7 %; P<0.001). Thus the difference in WW does not impair the growth of the animal but the heifer still needs to reach a target LW and BCS if it has to have a high probability of mating successfully and so extra inputs are required over the growth path to reach these targets by a defined date. So the issues around early weaning are the better re-conception rates of cows, which have calves weaned early and the higher input required to

recoup the lower LW of the early weaned heifer if she is to be mated successfully at 2-years of age. There appears no reason to supplement EW heifers with true protein or to reach a target LWG with any supplement form (e.g. grain, protein meals etc) for welfare and subsequent growth rate of the heifer other than that a target LW and BCS is required for successful pregnancy and this may not be achieved without some form of supplement depending on the starting LW of the heifer at weaning. This becomes an economic decision rather than a biological decision. Figure 5-11 best shows this relationship where the overall cumulative LW over 2 years is plotted against the different 1st dry season cumulative LW for different WW groups. This shows that the much of the difference can be made up but, depending on the starting LW, this may not be enough to reach target LW for successful mating or in the case of steers to reach target LW for live export by a certain time.

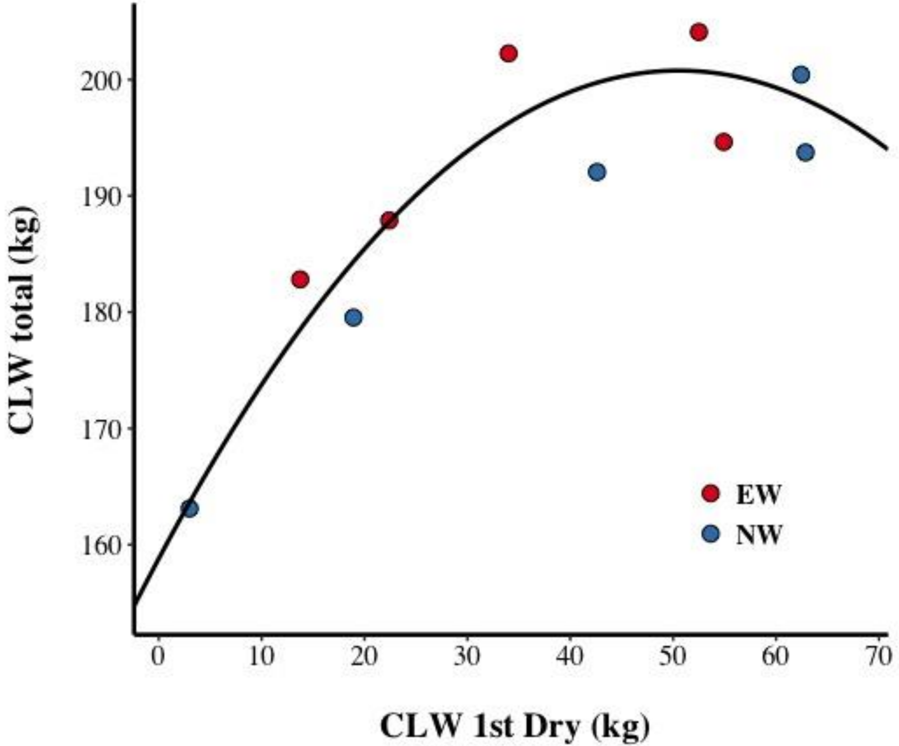


Figure 5-11 Relationship between cumulative liveweight (CLW) during the 1st dry season and CLW over the total experiment length (~ 2 years) of early (EW) and normally (NW) weaned crossbred Brahman heifers. Initial model include weaning weight group as a factor which was not significant and subsequently removed from the final model ($Y= 158.8 + 1.660067X -0.016418X^2$, $R^2=0.31$, RSE=10.1, $P<0.001$).

5.6.3. *Factors affecting skeletal growth rates*

As a consequence of low voluntary intake of the supplements, LWG of the groups receiving the highest amounts of supplement were also lower than expected. Nevertheless, the supplement treatments were successful in promoting a separation of LW growth paths (Figure 5-3). The

divergences of HH growth trajectories between the groups were less pronounced (Figure 5-3). It appears that heifers in this study showed the highest HHG reported for cattle close to the state of LW maintenance (54 mm/100 days, the T1 treatment of a MBL) ever reported. McLennan (2014) found values of 35-39 mm/100 days for steers grazing dry season pastures in northern Australia and receiving a loose mineral mix supplements with urea and sulphur. In the previous study (Chapter 4) Brahman crossbred steers grew at 31 mm/100 days when fed Mitchell grass (3.8 g CP/kg DM) in pens. The possible reasons for this difference are discussed later.

It is likely that most of the divergence in skeletal elongation rate observed here when compared across experiments from this thesis and other work in this laboratory (Chapter 4, (McLennan, 2014) is due to differences in growth plate maturation and in nutritional status of the heifers. In this case, assuming that the comparison is made between cattle with same genetic potential and growth history (i.e. not restricted during gestation or prior to weaning) growth plate maturity seems to be the first factor setting the limit on skeleton growth rate. It was previously believed that age was the determinant of the potential skeletal growth rate. This was assumed due to the fact that mammals in general display significant decrease in bone elongation from birth up to the closure of the epiphyseal growth plate, as it grows older. However, recent studies have demonstrated that at least part of the limit of bone elongation rate is set by morphological and physiological changes in the growth plate itself (Lui and Baron, 2011). In general terms, it implies that a younger animal with a less mature growth plate has a greater potential to have a faster bone elongation when compared to an older animal with a more mature growth plate. Therefore comparisons of bone elongation rates across different experiments are difficult since different levels of growth plate maturation set different growth rate potentials.

Nutrition is also a major factor to determine bone growth rate for a given body size (i.e. for a given level of maturity). In the previous study (Chapter 4), it was shown that CP intake explained 82% of the variation in the rate of HHG. In the present study, during the first dry season the amount of supplement offered and WW group had a significant effect on HHG (Figure 5-12). The growth rates of both WW groups were very similar for T1 and T2 but EW heifers had a greater HHG than NW when offered higher amounts of supplement (T3, T4 and T5) even though quantitatively small (67 *vs* 61 mm/100 days for EW and NW respectively; $P<0.001$). These results seem to indicate the action of the two factors mentioned previously (i.e. body size/growth plate maturation and nutrition) however the statistical analysis did not show a significant interaction between these factors. It is likely that a larger difference in HHG between WW groups would be observed when increasing the intake of CP and ME but unfortunately this was not achieved in the current experiment even though that was the original intent.

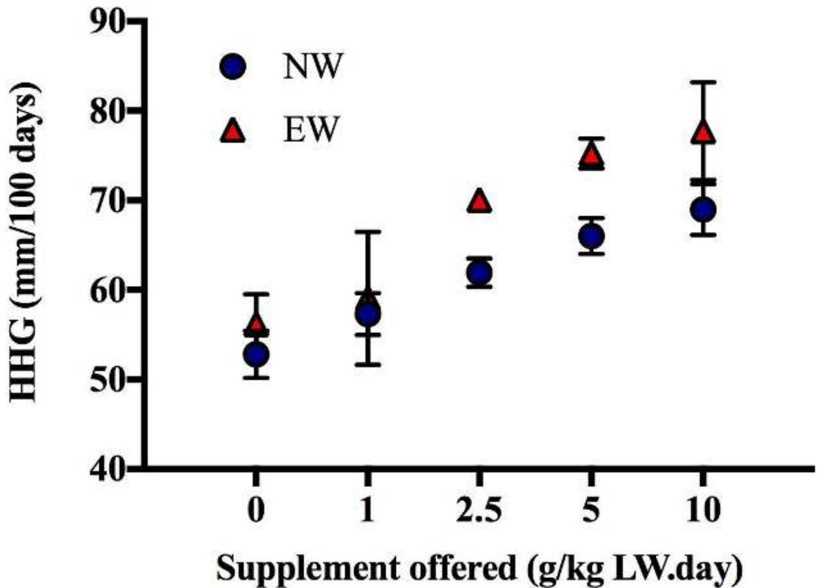


Figure 5-12 Effect of different dry season supplementation on hip height gain (HHG) of Early (EW) and Normally weaned (NW) heifers. Data are means of each treatment with error bars representing standard error of the mean. A significant effect was detected for weaning weight group ($P<0.01$) and supplement treatment ($P<0.001$) but not for the interaction of these two factors ($P=0.68$).

5.6.4. *Longitudinal growth and hormones concentration*

The observed increases in LWG and measurements of growth rate of various LW and skeletal parameters when feeding supplements were accompanied by changes in hormone concentration as well as morphological changes at the growth plate. Supplemented heifers had higher plasma concentrations of insulin, IGF-1 and T3 hormone as well as thicker PZ and HZ and larger terminal hypertrophic chondrocytes (Table 5-7 and Table 5-8). Similarly, Pando et al. (2014) reported severe reductions in the concentration of circulating IGF-1 (reduced to about 20% of concentration in control animals) during caloric restrictions associated with a smaller epiphyseal growth plate. Moreover, IGF-1 null mice have a smaller HZ and terminal hypertrophic chondrocytes as well as slower bone elongation rate when compared to wild-type mice. Nutritional effects on endocrine and morphological parameters of the growth plate have been previously reported in rabbits (Heinrichs et al., 1997) and rodents (Rossi et al., 2001; Cornelia et al., 2003; Gat-Yablonski et al., 2008; Pando et al., 2014). Longitudinal bone growth at the growth plate is regulated by a complex system of endocrine signals (Nilsson et al., 2005). Many hormones can individually regulate the growth plate locally or in concert with others metabolic signals (Van der Eerden et al., 2003), although changes in the concentration of growth regulating hormones (e.g. GH and IGF-1) are not able to explain the observed changes in long bone growth rate observed over a lifetime (Lui and Baron, 2011). For instance, the uncoupling of hormone concentration and bone elongation in the long term can be

observed comparing the IGF-1 concentration between EW and NW across the different seasons of this experiment with the skeletal growth rates assessed by body measurements. The IGF-1 concentration was significantly higher in NW heifers in 3 out of 4 collections (e.g. start of 1st dry season, start and end of 1st wet season) compared to EW, however the increase in HH was significantly greater in EW than NW across all these time points. This observation is in agreement with the previous assumption that growth plate maturation is the first factor setting limits to the rate of growth plate endochondral ossification.

Overall the concentration of IGF-1 in the plasma of supplemented and non-supplemented heifers in this experiment were lower than usual values reported by other authors in cattle under restricted nutrition. The concentration of IGF-1 in this experiment ranged from 5.8 ng/mL in EW at the start of the experiment up to 100.8 ng/mL in NW heifers from the T5 supplementation group at the end of the 1st dry season. Hayden et al. (1993) reported a concentration of 150 ng/mL of IGF-1 in restricted Chianina crossbreed steers (276 kg) growing at 530 g/day. Yambayamba et al. (1996a) described a slightly lower (106 ng/mL) concentration of IGF-1 than Hayden et al. (1993) in restricted crossbreed Hereford heifers (227 kg) at LW maintenance. On the other hand, Moriel et al. (2012) described IGF-1 concentrations as low as 64 ng/mL in crossbreed Brahman X British heifers (241 kg) growing at 190 g/day. Similarly, Quigley and Poppi (2013a) reported that plasma concentration of IGF-1 in Brahman crossbred steers (138 kg) ranged from 14.5 to 46.7 ng/mL . The IGF-1 concentrations in the present experiment were positively correlated with liveweight ($r=0.60$, $P<0.001$) which helps to explain the higher concentration of IGF-1 in supplemented compared to un-supplemented heifers in the following seasons post-supplementation as well as the initial difference (i.e. before imposition of nutritional treatments) in concentration between EW and NW heifers. The lower than usual concentration of IGF-1 reported in this experiment probably reflects the poor nutrition and lower growth rates of cattle grazing tropical pastures in northern Australia. Nevertheless, the concentrations of IGF-1 in heifers during supplementation are in agreement (53.7 and 100.8 ng/mL for EW and NW respectively) with values previously described for cattle (Quigley and Poppi, 2013a; Moriel et al., 2014) of comparable LW and LWG.

5.6.5. *The effect of supplementation on the trabecular bone structure, hormones and bone biomarkers plasma concentrations.*

In humans and rodents, it is known that caloric restriction affects balance of bone remodelling resulting in a decrease in bone formation and occasionally also an increase in bone resorption (Soyka et al., 1999; Hamrick et al., 2008). In order to detect physiological changes in bone turnover,

circulating bone metabolism biomarkers can be measured as an indicator of these processes (Seibel, 2007). Bone-alkaline phosphatase concentration, as a marker of bone formation, showed a tendency to be higher (P=0.056) in heifers offered the highest level of supplement than un-supplemented heifers. In contrast, CTX-1, as a marker of bone resorption, was unaffected by supplementation. The highest level of supplementation (i.e. T5) led to a greater rate of HH and HW growth, therefore more bone was being mineralized in order to keep pace with the increased elongation rate which helps to explain the slightly higher concentration of BAP in these heifers despite similar trabecular bone structure between T1 and T5 supplement groups.

The concentration of bone metabolism biomarkers in cattle are normally higher in young growing cattle than in mature cattle due to the fast growing skeleton at this time which requires an accelerated process of bone mineralization and resorption (DeLaurier et al., 2004; Sato et al., 2013). In addition, the variability in concentration of bone biomarkers in young animals is greater than mature cattle. This pattern has been demonstrated in cattle with BAP (Sato et al., 2013) and DPD (DeLaurier et al., 2004). The concentration of BAP in this experiment ranged from 25 U/L in non-supplemented NW up to 46 U/L in EW supplemented heifers during supplementation. In Holstein heifers aged between 11 days and 1 year old, Sato et al. (2013) described concentrations of BAP varying from approximately 75 to 440 U/L. Kurosaki et al. (2007) reported a concentration of BAP of 10 U/L in mature Holstein cows 40 days prior to parturition while first calf heifers had approximately 25 U/L. In the previous chapter of this thesis (Chapter 4), fast growing steers had 80 U/L of BAP in plasma while steers fed energy restricted treatments had approximately 20 U/L independent of CP intake during the energy restriction. There are no published reports in the literature on the concentration of CTX-1 in young growing cattle but *Bos indicus* cross heifers and steers have plasma CTX-1 concentrations from about 1 to 3 ng/mL (Anderson, unpublished observations). In mature Swedish Red and White cows, CTX-1 in plasma ranged from 0.4 ng/mL during the non-lactating period up to 4.4 ng/mL during the first weeks of lactation (Ekelund et al., 2006). The results of the present experiment are within the range of concentration previous reported for BAP and CTX-1 in the plasma of cattle. Taken together these results seem to indicate that when offered a high quality diet fast growing young cattle have the potential to increase bone remodeling leading to higher concentrations of bone formation and resorption markers. However, young cattle fed poor quality diets have bone biomarker concentrations similar to mature animals with low bone turnover activity, such as mature dry cows. In this case, part of the variation in concentration of BAP and DPD observed by Sato et al. (2013) and DeLaurier et al. (2004) could be related to nutritional status leading to a greater variation in young animals.

5.6.6. *The long-term effect of mineral block lick with urea or copra meal supplementation during the first dry season on the frame size of early and normally weaned replacement heifers.*

The underlying hypothesis in this thesis is that skeletal elongation rate determines growth rate of the animal through the close relationship between muscle growth and the passive stretch mechanism of the length of the bones to which they are attached (Hooper, 1978; Young and Sykes, 1987). It has also been hypothesized for this thesis that compensatory growth is linked to the divergence of the LW-for-HH value of animals undergoing restricted nutrition compared to normal fed controls following a standard growth curve. In this hypothesis, those animals who diverge a long way from the standard LW-for-HH curve will show greater compensatory growth in LW so that animals move back towards the normal trajectory of LW-for-HH. These are animals that are taller and of lower LW or have low BCS at a particular LW illustrating the potential to put on tissue, in particular muscle, and hence LW quite rapidly.

Cattle commonly grow faster in terms of LW (i.e. compensatory growth) and body dimensional measurements (i.e. catch-up growth) following a period of growth restriction when compared to the same age unrestricted cohorts (Lawrence and Pearce, 1964a). However in this thesis, whilst compensatory growth in LW was observed, there was no evidence of catch-up growth over the first wet season. Interestingly, animals fed lower amounts of supplement during the pen phase had a significantly higher HHG (27 *vs.* 19 mm/100 days for T1 and T4; $P<0.05$) and HWG (18 *vs.* 12 mm/100 days for T2 and T5; $P<0.05$) during the second wet season. This was observed approximately a year and half after the imposition of the supplement treatments. Early-weaned heifers also showed greater cumulative increases in body measures (10 kg, 5.7 and 14 cm for LW, HH and HW; $P<0.05$) than NW during the whole experimental period (Table 5-5). These observations may indicate that the nutritional stress imposed at early weaning would not impair these heifers to achieving, at an older chronological age, a similar mature frame size of unrestricted and normally weaned cohorts. In addition, it shows that catch-up growth may occur not only just after the suppression of the restrictive factor but at later stages of development leading to a longer growth phase due to a later closure of the epiphyseal growth plate. Moreover, it provides further evidences to the suggestion made on Chapter 4, that the biological mechanisms regulating LW and skeletal growth recovery (i.e. compensatory and catch-up growth) following a period of nutritional restriction are not the same.

Studies with humans who suffered nutritional restriction during childhood and subsequently received adequate nutrition have indicated similar patterns of skeletal growth recovery (i.e. catch-up growth). For instance, Steckel (1987) reported the data on height, age and sex recorded from slaves taken to USA. The results show that height of the children on arrival in USA was comparable to children from

tribes of New Guinea Highlands but during adolescence they demonstrated catch-up growth with heights above the 25th centile for males and 35th centile for females of the general American population. Moreover, the author also reported that females only attained their final height as old as 25 years. In his review, Golden (1994) suggested that the reason for this impressive catch-up growth in the slave population was due to the regular allocation of food, notably pork (about ½ lb. per day), while in America despite the horrendous working conditions. Incomplete catch-up has been reported by several studies with rodents using a design of 40% of food restriction for 10 days and *ad libitum* access to feed for 10 or 26 days during the re-alimentation period. However, by the end of the 26-day recovery the epiphyseal growth plate (includes resting, proliferative and hypertrophic zones) of rodents submitted to catch-up growth showed a smaller reduction in thickness when compared to control animals (Gat-Yablonski et al., 2008; Pando et al., 2014). Therefore, the possibility that previously restricted rodents demonstrate a greater catch-up in skeletal size than that observed at 26 days or even complete catch-up (i.e. same body size as controls) at older stages of life should not be discounted.

In the present study, by the end of the first dry season heifers fed T5 treatment were 4 and 4.6 cm taller (assessed by hip height) than NW and EW non-supplemented heifers respectively. This difference was reduced to 2.7 and 2.8 cm, for the same mentioned groups, by the end of the experimental period. Unfortunately, no bone biopsy was collected at the end of the experimental period to provide information on the histomorphometric parameters of the growth plate at this point in time. However, the growth path of hip height (Figure 5-3) as well as cumulative changes in body dimensions (Table 5-5) during the second wet season suggests that none of the heifer groups had achieved their mature size by the end of the experimental period. For humans, an individual is classified as stunted when the height is more than two standard deviations below the mean height of the reference population (UNICEF-WHO-WB, 2016). Appling this same concept to the present study, the difference in hip height between non-supplemented and supplemented heifers would need to be greater than 3.4 cm (average between early and normally weaned) in order to be considerate stunted. Therefore, it can be concluded that EW and NW replacement heifers fed a mineral block lick (MBL) with urea during the first dry season do not display any difference in mature size when compared to heifers offered high levels (i.e. 10 g of supplement/kg LW.day) of protein meal supplementation at 2 years old.

5.6.7. *Reproductive parameters*

Increasing branding rates is a major challenge for tropical cattle breeding operations. Early weaning is a tool used to substantially increase pregnancy percentage especially in harsher environments such as northern Australia (Laster et al., 1973; Schlink et al., 1988; Arthington and Kalmbacher, 2003) by reducing the nutrient demands of the cow. The question is whether a higher proportion of pregnant cows is more advantageous than weaning heavier calves. The information from this experiment will enable such economic calculations to be done based on historical records of weaning or branding percentages and the annual LWG which are achieved for a particular class of country.

The model developed in this work (Equation 5-2 and Equation 5-3) to predict percentage of pregnancy based on PM.LW is a useful tool that has practical application. It suggests that two-year-old heifers need to weigh approximately 300 kg at PM.LW in October/November to achieve 80% of pregnancy percentage, regardless of weaning weight. Schatz and Hearnden (2016) developed a similar model using data collected at the same location of this experiment. The authors proposed a target LW (curfew) of 253 kg measured in October/November would result in 80% conception rates for two-year-old heifers. Similarly, working with 2-years-old Brahman cross heifers Doogan et al. (1991) proposed ~270 kg as target LW at the start of mating (January) in order to achieve 80% confirmed pregnancy rate. Liveweight has a very significant effect on pregnancy but many other factors can affect this relationship (e.g. bull performance, diseases, genetics and environment). The pregnancy percentages found in this experiment are lower than what would be expected based on the PM.LW recorded in October/November in the previously mentioned studies. However, the pregnancy percentages of this study are comparable to the results obtained by Schatz and McCosker (2016) when evaluating 2-year-old Brahman cross heifers at the same year and location. The reasons for the lower than expected reproductive performance for this year and location are not clear but the low numbers used in this study may have contribute to this variation.

5.7 Conclusions

Early weaning did not reduce the long-term performance of heifers in terms of LWG or skeletal elongation of HH or HW. Supplementation increased bone elongation rate and this was associated with a thicker hypertrophic zone and greater diameter of terminal hypertrophic chondrocytes in both weaning weights groups. However, EW heifers also exhibited a thicker proliferative zone when supplemented. This provides the first evidence of how changes in skeletal growth come about under Brahman cross heifers undergoing different growth trajectories and weaned at different weights and presumably physiological age. As expected, compensatory growth in weight was exhibited in the wet season following differential dry season growth rates achieved by variable supplement levels. On the other hand, evidence of catch-up growth in the skeleton was observed only in the second wet season which was approximately 1.5 years after the imposition of the different supplementation treatments. Pregnancy rate at two years of age, the second wet season, was closely aligned to the LW achieved by the weaning weight groups such that EW animals had difficulty achieving the target weight and pregnancy rate. This requirement for significant supplement input is dependent on class of country and duration of the wet seasons but in the region where this study was done there is difficulty achieving these target weights within that 2-year time frame.

Higher levels of supplement intake during the first dry season increased LW gain and skeletal bone elongation rates. Compensatory growth during the wet season reduced ~50% of the LW difference attained through dry season supplementation. This effect partially reduces the benefits of dry season supplementation, but supplemented groups still achieved numerically higher percentages of heifers reaching puberty and pregnant heifers. Pre-mating LW was the main factor influencing pregnancy and a minimum of ~300 kg in October is predicted to achieve 80% of pregnancy percentage in this scenario. Regardless of the level of supplement offered during the 1st dry season, it would take another year for EW to attain the LW necessary for achieving satisfactory percentage pregnancy levels.

Early-weaned heifers can be fed a simple N supplement during the first dry season without detrimental effects on long-term mature frame size and there is no need for a protein meal to achieve a low level of LW gain for any welfare or long term performance and frame size of these heifers, although pre-mating LW and BCS targets still must be achieved to ensure good reproduction rates. Early and normally weaned heifers show the same response in LW gain and HH gain to increases in supplement intake.

Chapter 6. Relationship between the tuber coxae growth plate and trabecular bone histomorphometric measurements with nutritional parameters, hip height gain, hormonal and bone markers concentration in cattle.

6.1 Introduction

Endochondral ossification is the process whereby the skeleton grows in length. The rate of skeletal elongation is a factor of the rate of chondrocyte proliferation, terminal size of hypertrophic chondrocyte and rate of cartilage matrix deposition (Hunziker, 1994). Breur et al. (1991) demonstrated that the volume of hypertrophic chondrocytes of rats and pigs were highly correlated with the rate of longitudinal bone growth (rats r=0.98, pigs r=0.83). Endochondral ossification is modulated by a variety of signalling substances (e.g. growth factors, hormones, epigenetic mechanisms, transcriptor factors and microRNAs) which may stimulate or reduce the pace of its occurrence (Gat-Yablonski et al., 2013). In cattle, little is known about the nutritional and endocrine effects on the morphological aspects of the growth plate. In addition, it is not known if there is any relationship between growth plate components (i.e. proliferative and hypertrophic height and diameter of terminal hypertrophic chondrocyte) and bone elongation such as demonstrated in rats and pigs (Breur et al., 1991).

Bone remodelling is also affected by a large spectrum of factors such as hormones, growth factors, 1,25-Dihydroxyvitamin D_3 and minerals (Seibel, 2007). The exact mechanisms by which nutrition can regulate endochondral ossification and bone turnover are still unknown but it is believed that it occurs by multiple levels of regulation (Gat-Yablonski et al., 2013). In humans, there is a consistent body of evidence linking the decreases in IGF-1 during nutritional restriction with increases in bone loss by increased bone resorption (Grinspoon et al., 1996; Misra et al., 2009). In cattle, there is no information available about the association of endocrine changes and trabecular bone parameters during nutritional restriction. Bone markers are a well-developed technique utilized to assess bone formation and resorption in medicine (Hannon and Eastell, 2006). This is an important tool that provides a non-invasive method to assess bone-remodelling activity. Some earlier publications have reported the use of bone markers in bovines but all of these focused on dairy cattle (Liesegang et al., 1998; Kim et al., 2010; Elizondo Salazar et al., 2013). Dairy and beef cattle have had different genetic selection processes and this may be related to the capacity of bone formation and resorption. For instance, dairy cattle require a much greater capacity of bone resorption during the lactation period in order to accommodate the calcium requirements for milk production. Moreover, the concentration

of bone markers can be affected by other factors such as their metabolic clearance in the animal which is also affected by the physiological state (Swaminathan, 2001). In addition, none of the previous work has investigated an association between the concentration of bone markers in the circulation and morphological changes in bone. The examination of the relationship between the concentration of such biomarkers and changes in bone structure is essential for the validation of this technique (Delmas et al., 2000).

This study had several objectives. Firstly, it examined the relationship between nutritional and endocrine parameters with growth plate histomorphometry in cattle. Secondly, it investigated the relationship between the morphological changes in different zones of the growth plate with the rate of bone elongation measured as HHG. Thirdly, the correlation between the concentration of hormones and bone markers in the plasma with structural trabecular bone measurements were examined.

6.2 Materials and methods

All data utilized in the present chapter were obtained from the studies described in Chapter 4 (Exp.1) and Chapter 5 (Exp.2). The methodology utilized to collect and process the bone biopsies, hormones and bone markers assays were previously described in Chapter 3. In Experiment 1 the first bone biopsy was collected prior to the imposition of the nutritional treatments and it was excluded from the present analysis. Metabolizable energy and CP intake, T4, adiponectin, OCN, PYD and tDPD were only measured in Exp.1, whereas C-terminal Telopeptides of Type I Collagen CTX-1 was only measured in Exp.2. The HH measurements were taken using a measuring stick in Exp.1 and a suspended measure tape installed on a support structure of the crush in Exp.2. Regardless of the technique used, HH was measured at the highest point of the sacrum between the tuber coxae bones and the two methods were not considered different. Hip height gain was calculated by regressing hip height measurements over time (approximately at 14 day intervals) for a period of approximately 40 days prior to each biopsy.

6.2.1. *Statistical analysis*

All statistical analyses were conducted using the open-source software R (R Core Team, 2013). The data were previously checked for the presence of outliers and to meet the assumptions of normality, linearity and homoscedasticity. When necessary the data were transformed according to the box-cox procedure (Osborne, 2010). Pearson's correlation coefficient (r), confidence interval (CI) at $\alpha=0.05$ for the correlation coefficient and P-value were calculated for all the investigated variables. The

comparisons were made separately for each experiment in order to compare the variation in results between the two studies. The variables which presented high correlation (r>0.7) were investigated further by a multiple regression analysis. The initial models include cattle genotype as an explanatory variable and it was dropped from the model when not significant (*P*>0.05).

6.3 Results

6.3.1. *Growth plate histomorphometric parameters and concentration of hormones.*

The relationships between growth plate histomorphometric parameters and concentrations of various hormones are presented in Table 6-1. The results show that height of the proliferative zone was positively related to IGF-1 concentrations in both experiments, albeit in Exp.1 was only approaching significance (P<0.07). However the concentration of all other hormones were not related to proliferative zone height. IGF-1 concentrations also showed a significant positive correlation with hypertrophic zone height and diameter of terminal hypertrophic chondrocytes in both studies. The height of the hypertrophic zone was also associated with higher plasma insulin (both experiments) and total T3 (only Exp. 1).

Table 6-1 Pearson's correlation between growth plate histomorphometric measurements and leptin, insulin-like growth factor-1 (IGF-1), Thyroxine (T4) and triiodothyronine (T3) concentration in cattle.

	Exp.1 (n=60)			*Exp.2 (n=96)*		
	Pearson coefficient	95% CI	*P*-Value	Pearson coefficient	95% CI	*P*-Value
Proliferative zone height (μm)						
Leptin	0.051	-0.367; 0.406	0.911	-0.190	-0.400; 0.050	0.120
IGF-1	0.262	-0.01; 0.504	0.065	0.247	-0.01; 0.452	0.034
Insulin	0.216	-0.068; 0.469	0.124	0.029	-0.201; 0.258	0.802
T4	-0.088	-0.360; 0.197	0.544	-	-	-
T3	0.306	-0.100; 0.626	0.135	0.156	-0.075; 0.373	0.185
Hypertrophic zone height (μm)						
Leptin	0.145	-0.141; 0.409	0.319	0.227	-0.012; 0.441	0.062
IGF-1	0.606	0.391; 0.758	<0.001	0.422	0.212; 0.594	<0.001
Insulin	0.374	0.100; 0.595	0.007	0.112	-0.120; 0.333	0.344
T4	-0.051	-0.331; 0.235	0.726	-	-	-

T3	0.671	0.376; 0.842	<0.001	0.069	-0.163; 0.294	0.558
Diameter of terminal hypertrophic chondrocyte (µm)						
Leptin	0.178	-0.096; 0.428	0.201	-0.012	-0.246; 0.223	0.919
IGF-1	0.560	0.342; 0.721	<0.001	0.247	0.023; 0.447	0.031
Insulin	0.414	0.159; 0.617	0.001	0.180	-0.047; 0.389	0.119
T4	0.106	-0.171; 0.368	0.454	-	-	-
T3	0.570	0.234; 0.784	0.002	0.143	-0.085; 0.357	0.143

6.3.2. *Growth plate histomorphometric parameters and metabolizable energy and crude protein intake*

Since intake of metabolizable energy and crude protein was only measured in Exp.1 the results presented in this section do not include Exp.2. The results show a significant positive correlation between metabolizable energy and crude protein intake, PZ and HZ height as well as with the diameter of terminal HC (Table 6-2). The height of the HZ showed a strong association with ME intake (r=0.75) thus the relationship between these variables was further investigated by a regression analysis (Figure 1). The relationship between HZ and ME intake was shown to be significantly affected by genotype. The results demonstrate that *Bos taurus* have a thicker hypertrophic zone than *Bos indicus* cattle when consuming the same amount of ME.

Table 6-2 Pearson's correlation between growth plate histomorphometric measurements and intake of metabolizable energy and crude protein in cattle.

	Exp.1 (n=90)		
	Pearson coefficient	95% CI	*P*-Value
Metabolizable energy intake (MJ/kg LW.day)			
Proliferative zone height	0.542	0.311; 0.713	<0.001
Hypertrophic zone height	0.759	0.607; 0.857	<0.001
Diameter of terminal hypertrophic chondrocyte	0.457	0.213; 0.647	<0.001
Crude protein intake (g CP/kg LW.day)			
Proliferative zone height	0.511	0.273; 0.691	<0.001
Hypertrophic zone height	0.687	0.505; 0.810	<0.001
Diameter of terminal hypertrophic chondrocyte	0.511	0.272; 0.691	<0.001

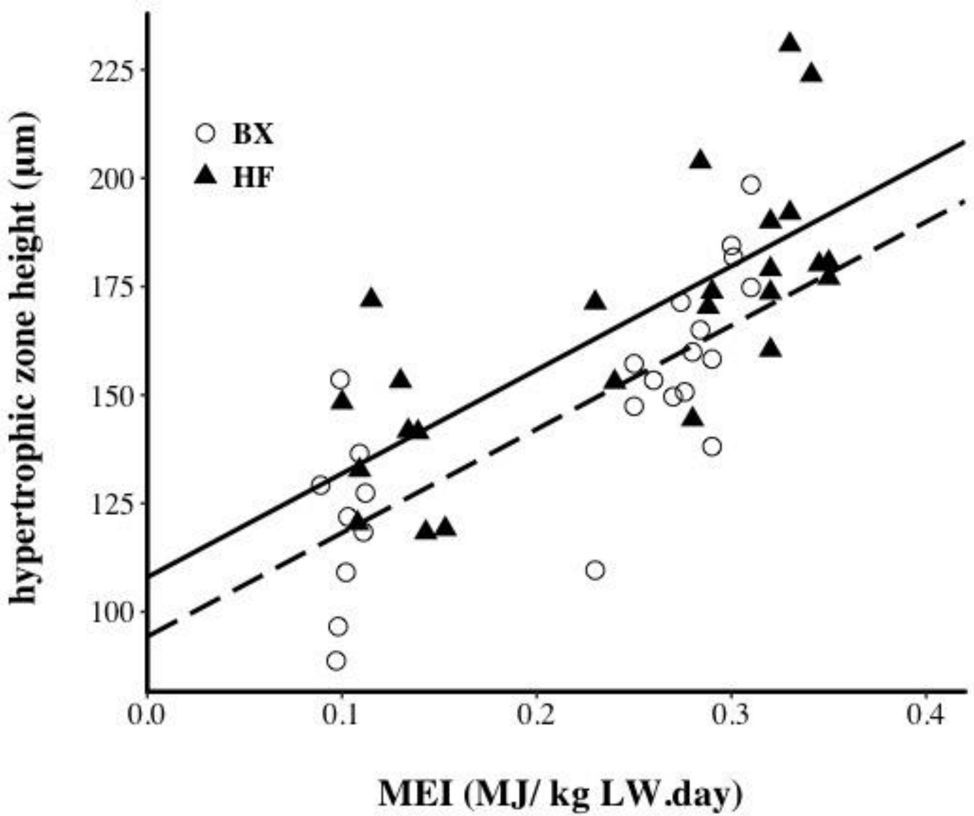


Figure 6-1 Relationship between metabolizable energy intake (MEI) and height of hypertrophic zone of *Bos indicus* (BX; open circles; dashed line, Y= 94.2 + 239.1X, R^2=0.62, RSE=19.1, P<0.001) and *Bos taurus* (HF; filled triangles; continuous line, Y= 107.9 + 239.1X, R^2=0.62, RSE=19.1, P<0.001) cattle. The regression analysis showed a significant effect (P<0.01) for genotype but not significant interaction between genotype and MEI (P=0.80) so two lines with different intercept but the same slope are plotted representing each breed type.

6.3.3. *Relationship between growth plate histomorphometric parameters and hip height gain*

Hip height gain (mm/100 days) showed a positive correlation with proliferative (r=0.63; P<0.001) and hypertrophic zone height (r=0.75; P<0.001) and diameter of terminal hypertrophic chondrocytes (r=0.48; P<0.001) in Exp.1. However, in Exp.2, hip height gain was only significantly associated with the diameter of terminal hypertrophic chondrocytes (r=0.31; P<0.001). The strongest correlation (r=0.75; P<0.001) was found between hip height gain and height of the hypertrophic zone in Exp.1 so a regression analysis was conducted to investigate this relationship (Figure 6-2). The results of the regression analysis confirmed a significant association between height of hypertrophic zone and hip height gain. The effect of genotype was investigated and the results showed that the cattle breed type did not significantly affect the relationship.

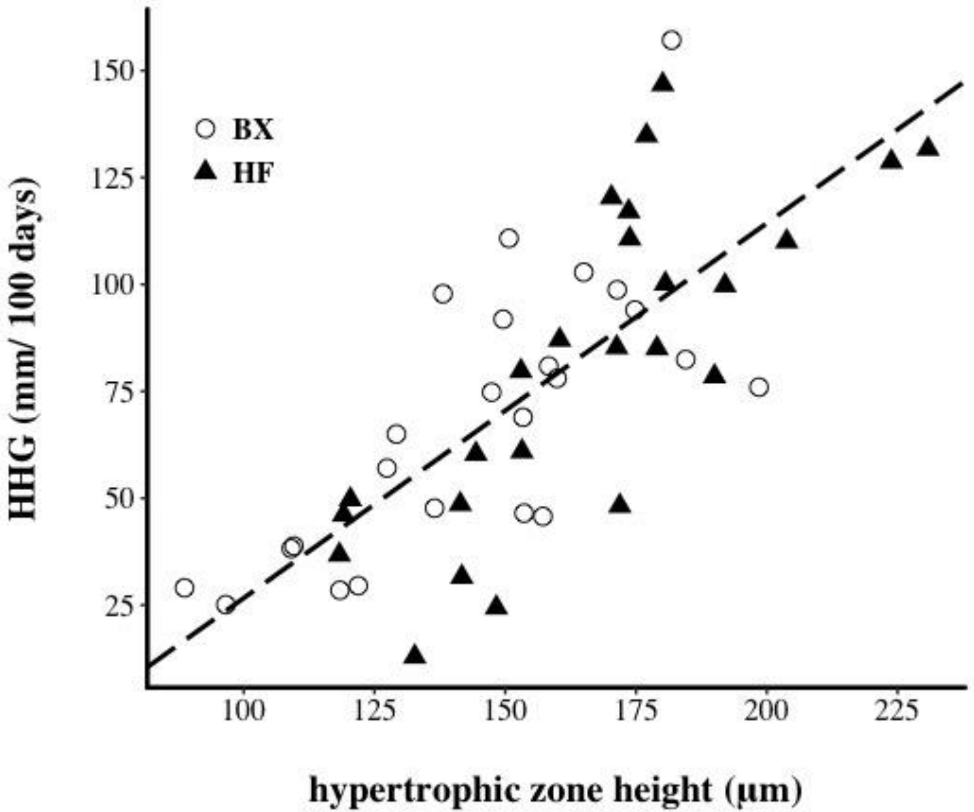


Figure 6-2 Relationship between height of hypertrophic zone (µm) and hip height gain (HHG) (mm/100 days) of *Bos indicus* (BX; open circles) and *Bos taurus* (HF; filled triangles) cattle. The regression analysis did not show a significant effect (*P*=0.32) for genotype so a single dashed line is plotted representing both breed types (Y= -61.04 + 0.876X; R^2=0.56, RSE=23.6, *P*<0.001).

6.3.4. *Trabecular bone histomorphometric measurements and hormone concentrations*

The correlation between trabecular bone histomorphometric measurements and the plasma concentration of various hormones is presented in Table 6-3. In Exp. 2, none of the measured hormones showed a significant association with trabecular bone histomorphometric measurements. In Exp.1, IGF-1, insulin and total T3 showed moderate positive correlations with bone volume and trabecular thickness. Leptin, T4 and adiponectin were not correlated with bone volume nor trabecular thickness. The relationship between T3 concentration and trabecular bone was independent of cattle genotype (Figure 6-3)

Table 6-3 Pearson's correlation between between trabecular bone histomorphometric measurements and concentration of leptin, insulin-like growth factor-1 (IGF-1), Thyroxine (T4), triiodothyronine (T3) and adiponectin in cattle.

	Exp.1			*Exp.2*		
	Pearson coefficient	95% CI	*P*-Value	Pearson coefficient	95% CI	*P*-Value
Bone volume (%)						
Leptin	-0.047	-0.316; 0.228	0.737	-0.123	-0.33; 0.102	0.282
IGF-1	0.615	0.411; 0.760	<0.001	0.091	-0.12; 0.300	0.406
Insulin	0.549	0.323; 0.716	<0.001	0.041	-0.174; 0.253	0.705
T4	0.007	-0.268; 0.282	0.959	-	-	-
T3	0.697	0.408; 0.858	<0.001	0.010	-0.204; 0.224	0.924
Adiponectin	0.142	-0.135; 0.399	0.313	-	-	-
Trabecular thickness (μm)						
Leptin	0.047	-0.228; 0.316	0.739	-0.124	-0.337; 0.100	0.277
IGF-1	0.659	0.470; 0.790	<0.001	0.020	-0.194; 0.233	0.853
Insulin	0.516	0.281; 0.693	<0.001	-0.017	-0.231; 0.197	0.873
T4	0.072	-0.207; 0.341	0.613	-	-	-
T3	0.722	0.450; 0.871	<0.001	0.053	-0.162; 0.265	0.626
Adiponectin	0.147	-0.130; 0.403	0.297	-	-	-

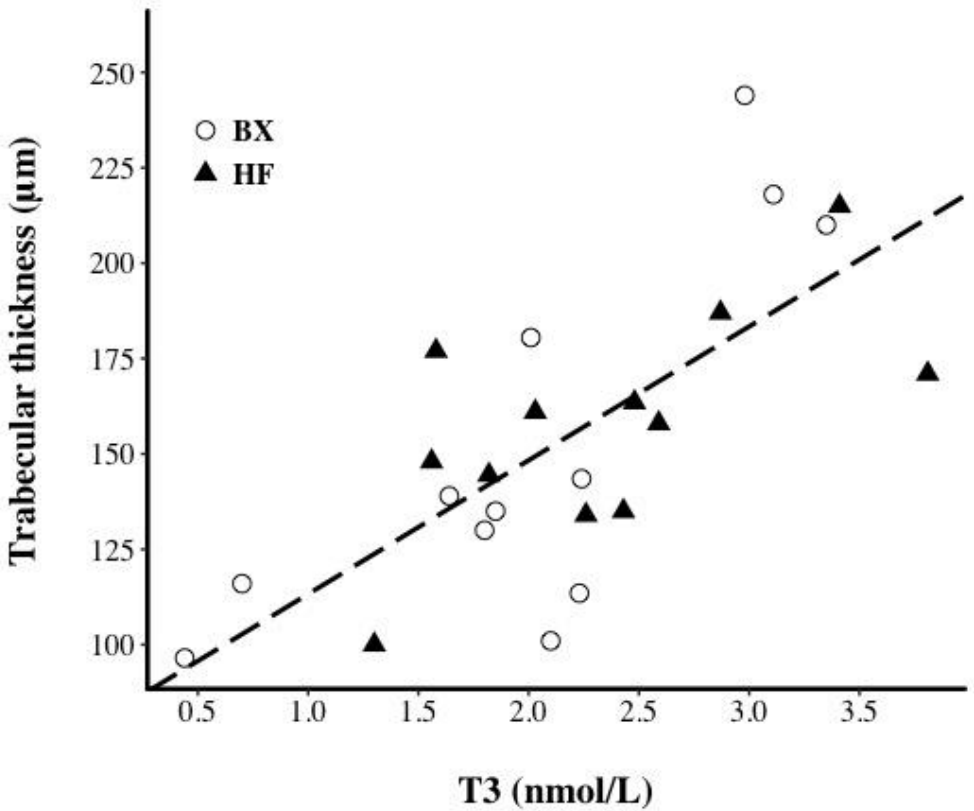


Figure 6-3 Relationship between the concentration of triiodothyronine (T3) (nmol/L) and trabecular thickness (µm) of *Bos indicus* (BX; open circles) and *Bos taurus* (HF; filled triangles) cattle. The regression analysis did not show a significant effect (P=0.61) for genotype so a single dashed line is plotted representing both breed types (Y= 78.2+ 35.0X; R^2=0.52, RSE=27.9, P<0.001).

6.3.5. *Trabecular bone measurements and concentration of bone markers.*

Bone-specific alkaline phosphatase concentration showed a moderate positive correlation with bone volume and trabecular thickness in steers in Exp.1 (Table 4-2). However, in Exp.2 BAP concentration was negatively associated with these same trabecular bone parameters in heifers. PYD concentration was negative correlated with bone volume and trabecular thickness in Exp.1. The concentration of osteocalcin, tDPD and CTX-1 were not correlated with any trabecular bone measurement. In order to interrogate the difference in the relationship of BAP and trabecular bone parameters between the two studies, the datasets from Exp.1 and 2 were merged and reanalyzed, and the results showed a positive correlation of BAP with bone volume (r=0.41, P<0.001) and trabecular thickness (r=0.77, P<0.001). In addition, the nutritionally restricted treatments from Exp.1 (i.e. HCP-LME and LCP-LME) were also analyzed separately (i.e. excluding the group fed the high CP and high ME (HCP-HME) diet) and the results showed no significant association between BAP and bone volume (-0.08, P=0.753) and a tendency for a negative correlation with trabecular thickness (-0.450, P=0.09).

Table 6-4 Pearson's correlation between trabecular bone histomorphometric measurements and the concentration of bone-specific alkaline phosphatase (BAP), osteocalcin (OCN), pyridinoline crosslinks (PYD), total deoxypyridinoline Crosslinks (tDPD) and C-terminal Telopeptides of Type I in cattle (CTX-1).

	Exp.1			*Exp.2*		
	Pearson coefficient	95% CI	*P*-Value	Pearson coefficient	95% CI	*P*-Value
Bone volume (%)						
BAP	0.595	0.385; 0.747	<0.001	-0.249	-0.440; -0.036	0.022
OCN	-0.166	-0.419; 0.111	0.239	-	-	-
PYD	-0.302	-0.531; -0.03	0.02	-	-	-
tDPD	0.183	-0.09; 0.434	0.192	-	-	-
CTX-1	-	-	-	-0.021	-0.234; 0.194	0.849
Trabecular thickness (μm)						
BAP	0.578	0.363; 0.735	<0.001	-0.280	-0.466; -0.070	0.009
OCN	-0.202	-0.450; 0.074	0.150	-	-	-
PYD	-0.361	-0.577; -0.097	0.008	-	-	-
tDPD	0.068	-0.207; 0.335	0.627	-	-	-
CTX-1	-	-	-	-0.088	-0.296; 0.128	0.425

6.4 Discussion

In the present study, there were associations between nutritional factors, hip height gain, hormones and concentration of bone markers with growth plate and trabecular bone histomorphometric parameters which are indicators of endochondral ossification and bone remodelling. The nutritional factors (i.e. ME intake and CP intake) were more highly correlated with the height of proliferative and hypertrophic zones and diameter of terminal hypertrophic chondrocytes than any of the hormones measured. The plasma concentration of the hormone IGF-1 showed significant association with all tuber coxae growth plate parameters but the strongest correlation was with the height of the hypertrophic zone. In both experiments, the diameter of terminal hypertrophic chondrocytes showed a significant positive linear relationship with hip height gain. Bone remodelling at the tuber coxae trabecular bone assessed by the structural histomorphometric measurements were associated with IGF-1, insulin and T3 concentration. Trabecular bone volume and thickness were not correlated with the concentration of bone-specific alkaline phosphatase during nutritional restriction. However, trabecular bone parameters showed a significant positive correlation when fast growing cattle were included in the analysis. In addition, pyridinoline showed a significant negative association with bone volume and trabecular thickness. Taken together these results support the hypothesis that the diameter of terminal hypertrophic cells play an important role driving skeletal elongation rate and it is directly related to level of nutrition. Moreover, they validate the use of bone-alkaline phosphatase and pyridinoline as good predictors of tuber coxae trabecular bone volume and trabecular thickness in growing cattle.

Bone elongation occurs by the endochondral ossification process at the growth plate of long bones. The morphological structure of the growth plate is composed of three different zones which represent the stages of differentiation of chondrocytes, namely the resting, proliferative and hypertrophic zone. The exact contribution of each stage of chondrocyte differentiation during the several stages of bone development throughout life is not known. Wilsman et al. (2008) suggested that the regulation of rate of bone elongation growth is a function of the number of cells produced through proliferation multiplied by the volume of terminal hypertrophic chondrocytes. Moreover, the authors reported that cell proliferation and hypertrophy have differential contributions to bone growth rates depending on the stage of animal development.

The somatotropic axis (i.e growth hormone and IGF-1) is a major regulator of postnatal growth. The great proportion (~99%) of IGF-1 in the blood is bound to IGF binding proteins (IGFBP-1 to IGFBP-6) which protects it from proteolytic degradation (Mohan and Baylink, 2002). The most abundant

(70-80 %) binding protein is IGFBP3 in a ternary complex with an acid labile subunit (ALS) which is also produced by the liver when stimulated by growth hormone (GH) (Baxter and Martin, 1989; Yakar et al., 2002). Here in the current experiments, the concentration of IGF-1 was correlated with the height of proliferative and hypertrophic zones as well as the diameter of terminal hypertrophic chondrocytes. Moreover, the association of the concentration of IGF-1 was stronger with changes in the hypertrophic zone than in the proliferative zone. This result is in agreement with Wang et al. (1999) who showed that IGF-1 null mice had a 35% reduction in rate of bone elongation, 36% reduction in height of the hypertrophic zone and 30% reduction in the diameter of terminal hypertrophic chondrocytes. In addition, the reduction in bone growth rate in this experiment was independent of changes in the size of proliferative zone and number of proliferative chondrocytes. In an *in vitro* study, Mushtaq et al. (2004) also showed that the infusion of IGF-1 increased (3-fold) the height of the hypertrophic zone without affecting the size of the proliferative zone or the number of BrdU-positive cells which are an indicator of cell proliferation.

The contribution of each phase of chondrocyte differentiation to skeletal growth has been described as a function of multiple factors (Wilsman et al., 1996) while others suggests a more direct effect of cell volume (Breur et al., 1991; Cooper et al., 2013). In this matter, Wilsman et al. (1996) concluded that the relative contribution of cell division, matrix synthesis and chondrocyte hypertrophy to growth changes when comparing between fast and slow growing growth plates. However, is widely accepted that chondrocyte enlargement at the hypertrophic zone is the main determinant of growth rates (Breur et al., 1992; Hunziker, 1994; Wilsman et al., 2008). Breur et al. (1991) studied the effect of the volume of the terminal hypertrophic chondrocytes from the distal and proximal radius bones as well as distal and proximal tibia of pigs and rats on rates of bone elongation. A very high correlation (rats r=0.98 and pigs r=0.83) was found between the volume of hypertrophic cells and bone elongation growth rates. This relationship was independent of the location of the growth plate in the animal and also of the animal's age. However, both rats and pigs were not subjected to any sort of growth limitation such as nutritional restriction. Interestingly, for a given hypertrophic cell volume the growth rate response was different between the two species. Pigs showed faster rates of bone elongation than rats when compared at the same hypertrophic cells volume. These results are in agreement with the findings presented in this chapter. The diameter of the terminal hypertrophic chondrocytes showed a consistent positive relationship with hip height gain across both studies. However, in the present study the height of the hypertrophic zone showed a stronger correlation with hip height gain than the diameter of terminal hypertrophic chondrocytes. Interestingly, metabolizable energy intake affected the height of hypertrophic zone differently in *Bos indicus* and *Bos taurus* steers. Thus one could extrapolate that metabolizable energy intake would result in different hip height gain in *Bos indicus*

and *Bos taurus* cattle. However, this hypothesis was tested in Chapter 4 and no differences in terms of liveweight and hip height gain in response to metabolizable energy intake were found between the two genotypes.

In a recent study, Cooper et al. (2013) suggested that during hypertrophic enlargement chondrocytes undergo three different phases. In phase 1, there is an increase of approximately 3-fold from 600 femtoliters (fl) to 2,000 fl that is achieved by an increase in dry mass production and fluid uptake. The second phase a 4-fold increase from 2,000 to 8,000 fl is achieved by a greater increase in volume than in mass. Finally in phase 3 there is another increase of 2-fold of proportional increases in dry mass and volume. In addition, the authors found that chondrocytes of IGF-1 null mice undergo normally through phase 1 and 2 but do not progress to phase 3 failing then to double their volume. The authors concluded that phase 3 of hypertrophic chondrocytes hypertrophy and its association with IGF-1 concentration is the main driver for variations in skeletal elongation rate. Interestingly, in Chapter 4 cattle fed a high protein diet during energy restriction showed significantly larger terminal hypertrophic diameters and hip height gain when compared to steers fed a low energy and protein diet without demonstrating significant differences in IGF-1 concentration. These changes however were associated with significantly higher plasma concentrations of T3 hormone with no difference in IGF-1 concentration in cattle fed higher levels of protein when compared to animals fed low protein diet during energy restriction. It is known that thyroid hormone deficiency leads to reductions in endochondral ossification (Shao et al., 2007), although, there is no well-documented direct effect of this hormone acting on the chondrocyte hypertrophic process such as observed with IGF-1. Moreover, studies in different tissues have shown that expression of IGF-1R mRNA is stimulated by T3 concentration in a dose dependent manner in human osteoblastic cells (Pepene et al., 2001) as well as in the anterior pituitary gland (Matsuo et al., 1990). In *vitro* studies have also demonstrated that T3 stimulates local IGF-1 and IGFBP-2 production by osteoblastic cells which is also known to have a direct effect on endochondral ossification (Schmid et al., 1992). Transgenic mice with inactivated osteoblastic production of IGF-1 show a reduction of 14 and 15 % of body and femur length without a reduction in concentration of IGF-1 in the circulation (Govoni et al., 2007a). Taken together these findings suggest a possible synergistic action of T3 concentration enhancing the action of IGF-1 by either increasing the expression of IGF-1 receptors in terminal hypertrophic chondrocytes or by stimulating local production of IGF-1 by osteoblasts and thus leading to a higher rate of bone elongation in cattle fed a high protein diet during energy restriction.

In mature healthy bone, the process of bone turnover (or remodelling) of the trabecular bone is a tightly regulated mechanism of resorption and formation that ensures no major net changes in bone

mass or mechanical strength after each remodelling cycle (Feng and McDonald, 2011). However, the balance between formation and resorption can be affected by several factors (e.g. hormones, growth factors and nutrition) leading to changes in bone density and volume, thickness and number of trabeculae and consequently mechanical strength. Here we found that increases in trabecular volume and thickness were positively correlated with increases in plasma IGF-1, insulin and T3 in Exp.1. Yet, none of these hormones, nor leptin, showed significant association with the same (i.e. bone volume and trabecular thickness) histomorphometric trabecular bone parameters in Exp.2. The lack of correlation between leptin concentration and the trabecular bone parameters is in agreement with Rauch et al. (1998) who analysed the relationship between the concentration of this hormone and trabecular bone mineral density, bone area and cortical bone area in mature women. Conversely, Thomas et al. (2001) found a positive correlation between leptin concentration and bone mineral density in pre (r=0.30, $P<0.001$) and post-menopausal (r=0.42, $P<0.001$) women. Leptin is mostly produced by white fat mass and the concentration in the body is increased in obesity showing a strong quadratic relationship (r=0.85, $P<0.001$) with percentage of body fat (Considine et al., 1996). Thus confounding factors, such as weight-bearing mechanical effects, could lead to misinterpretations of the isolated effect of leptin on the remodelling process. Nevertheless, a study using ovariectomized rats and leptin treatments suggests that leptin treatment is effective at reducing trabecular bone loss, trabecular architectural changes and periosteal bone formation (Burguera et al., 2001). In Exp. 1 and 2, the concentration of leptin did not differ between the nutritionally restricted and non-restricted treatments. This result was not expected since the concentration of circulating leptin is regulated by body fat content, nutrient intake and insulin concentration (Houseknecht et al., 1998). In sheep, Chilliard et al. (2005) showed that the relationship between plasma leptin concentration and body fatness was not linear and no increment in leptin concentration were observed up to a threshold of 20% of lipids in the body which is similar to the pattern described in humans by Considine et al. (1996). In cattle, Delavaud et al. (2002) also described a similar quadratic relationship between the mean adipocyte volume and the concentration of leptin in the plasma. It is possible that the lack of difference in leptin concentration between the restricted and non-restricted cattle in Exp 1 and 2 was due to the overall initial low percentage of body fatness in these cattle due to their age and weight. In agreement with this rationale, Daniel et al. (2002) demonstrated that fasting reduced by two fold the concentration of plasma leptin in fat ewes when compared to the reduction observed in lean ewes.

Osteopenia (mild reduction in bone mass) is a complication of reduced food intake in anorexia nervosa that leads to a decrease in bone mineral density. The reduction is bone density has been associated with lower bone formation and normal bone resorption (Soyka et al., 1999), but other studies have also reported higher bone resorption allied to lower bone formation (Grinspoon et al.,

1996) when compared to healthy patients. Similar to the effects of nutritional restriction in cattle, this illness is associated with hypothyroidism, delayed puberty, IGF-1 deficiency, osteopenia and hypoglycemia (Munoz and Argente, 2002). Grinspoon et al. (1996) treated osteopenic adult women with rhIGF-1 and observed a dose dependent response on bone turnover assessed by bone marker concentrations. At the dose of 100 μg twice daily the authors reported an increase in bone formation and resorption while a dose of 30 μg twice daily only increased formation. A more recent but similar study with adolescent women have confirmed the previous results of Grinspoon et al. (1996) and concluded that rhIGF-1 administration increases the plasma concentration of markers of bone formation (Misra et al., 2009). This result supports the findings of the present work reinforcing the evidence of the effect of nutritional restriction on IGF-1 concentration and its impact on the bone remodelling process. Importantly, the nutritional restrictions imposed in both studies reported here were also associated with a reduction in concentration of T3 hormone which is also observed during anorexia nervosa (Miyai et al., 1975).

The action of thyroid hormones on the remodelling process is not completely understood. Generally in humans, hyperthyroidism shortens the remodelling cycle leading to increased resorption and reduction in bone mass whereas hypothyroidism leads to an increase in bone mass caused by a reduction in osteoclast activity and bone resorption (Bassett and Williams, 2016). In an *in vitro* study, Britto et al. (1994) demonstrated that T4 was 100-fold less potent than T3 in stimulating osteoclast activity. The addition of T3 induced osteoblasts to indirectly enhance bone resorption by osteoblast cells. In the experiments of this thesis there was a linear increase in bone volume and trabecular thickness with increments of T3 while there was no correlation with T4 concentration. The lack of relationship between T4 concentration and trabecular bone volume and trabecular thickness is in agreement with the hypothesis that T3 exerts the main thyroid effect on the remodelling process. In addition, the results show an association between the increase in trabecular bone accretion with a higher concentration of T3 which seems to contradict the previous reported studies (Britto et al., 1994; Bassett et al., 2008). However, the results presented here are in agreement with changes observed in trabecular bone morphology and endocrinal parameters described in humans suffering from anorexia nervosa and rodents fed restricted diets (Spaulding et al., 1976; Sohal and Weindruch, 1996; Devlin et al., 2010; Zuckerman-Levin et al., 2014). Thus, these results seem to indicate that during nutritional restriction osteoclast activity (i.e. bone resorption) is somehow less responsive to changes in T3 concentration. In fact, the opposite trend can be observed in Exp.1 when cattle fed a high protein diet during energy restriction (i.e. HCP-LME) had a higher concentration of T3 and a lower concentration of the bone resorption marker pyridinoline than cattle fed a low protein and low energy diet (i.e. LCP-LME). In addition, the percentage of change in bone volume and trabecular

bone surface at the end of the restricted period in cattle fed the high protein diet was less affected than animals fed the diet with the low protein content. The reasons for such differences are difficult to clarify with the current information on trabecular bone metabolism in cattle.

Adiponectin is an adipocyte produced tissue-specific protein hormone that stimulates osteoclast formation by enhancing RANKL and decreasing osteoprotegerin (OPG) mRNA expression (Luo et al., 2006). RANKL is a protein expressed by osteoblast cells that stimulates osteoclast formation and function whereas OPG is a natural inhibitor of RANKL activity and plays a role regulating bone resorption (Boyce and Xing, 2008). Interestingly, Lee et al. (2007) demonstrated that osteocalcin produced by osteoblast cells regulates the expression of adiponectin. Moreover, Lee and colleagues also observed that osteocalcin can improve glucose tolerance thus playing a role in maintaining glucose homeostasis and energy metabolism. In humans, the relationship of adiponectin concentration in the blood and bone mineral density shows conflicting results, some studies have reported significant negative correlation with bone mineral density (Peng et al., 2008) while others found no association (Gonnelli et al., 2008). During bone formation, osteocalcin is secreted by osteoblasts and most of it is incorporated into the bone matrix while the remainder enters into the circulation and thus the concentration can be assessed as a predictor of bone formation (Hauschka and Wians, 1989). However, it has been reported that fragments of osteocalcin are released during bone resorption, which led some authors to suggest the use of this marker as an indication of bone turnover as opposed to being a specific marker for bone formation (Hannon and Eastell, 2006). In cattle, during lactation there is an increase in calcium and inorganic phosphorus mobilization from bone tissue in order to maintain milk production. The increased demand of minerals can lead to a metabolic disorder (i.e. periparturient paresis commonly known as milk fever), which is more commonly encountered in highly productive dairy cows (Yarrington et al., 1976). Elizondo Salazar et al. (2013) showed that osteocalcin concentration in dairy cows reduced after parturition and consistently increased from day 10 to 20 and reduced again from day 28 to 42. The changes in osteocalcin concentration were consistent with the pattern of change of phosphorus balance and showed an opposite trend of pyridinoline. In the present work, there was no association between osteocalcin or adiponectin with trabecular bone parameters despite the dramatic changes observed during metabolizable energy restriction in Exp.1. However, there was a negative correlation between pyridinoline and bone volume as well as with trabecular thickness. Interestingly, a significant positive correlation between bone-specific alkaline phosphatase and trabecular bone hystomorphometric parameters was found in Exp.1 while a negative relationship, for the same parameters, was detected in Exp.2. A significant negative correlation between bone-specific alkaline phosphatase and trabecular bone parameters was not expected.

There are a few differences between Exp.1 and 2 that may explain the divergence in association between bone-specific alkaline phosphatase and trabecular bone parameters between the two experiments. Firstly, in Exp.1 the rate of hip height gain in the non-restricted treatment (i.e. HCP-HME) was much greater (104.7 *vs* 70 mm/ 100 days) than the non-restricted treatment in Exp.2 (T5). Secondly, the concentration of bone-specific alkaline phosphatase of non-restricted cattle (40.5 U/L; average of early and normally weaned groups) in Exp.2 was only half of the concentration of non-restricted treatment in Exp.1 (80.7 U/L; average of *Bos indicus* and *Bos taurus* cattle fed HCP-HME). Thirdly, bone volume and trabecular thickness of cattle fed T5 treatment (i.e. treatment offered 10 g of supplement/kg LW.day) (21% and 153 µm) in Exp.2 were lower than in cattle fed HCP-HME (30.6% and 201 µm, bone volume and trabecular thickness respectively) in Exp.1 and more comparable to values observed in steers fed restricted diets (20.4% and 135.6 µm, bone volume and trabecular thickness respectively). These results suggest that the trabecular bone structure of cattle fed the highest level of nutrition (i.e. T5) in Exp.2 was more comparable to the nutritionally restricted treatments of Exp.1 (i.e. HCP-LME and LCP-LME).

PYD and DPD are the two major cross-links that stabilize the connection between two adjacent collagen molecules (Hannon and Eastell, 2006). They are both released from bone during the resorption process or collagen breakdown and the free fraction can be measured by enzyme immunoassay using monoclonal antibodies in serum or urine (Swaminathan, 2001). The increase in concentration of these markers has been shown to be associated with decreases in bone mineral density in humans suffering from osteoporosis (Krupski et al., 2016). Delmas et al. (1991) evaluated the use of PYD and DPD determination in the urine in order to detect bone resorption in osteoporotic patients. The validation of bone markers was assessed by comparing the concentration in the urine with histomorphometric measurements of osteoclast surface ($r=0.35$, $P<0.05$ for PYD; $r=0.46$, $P<0.01$, for DPD). In dairy cows, Liesegang et al. (1998) evaluated the urinary excretion of DPD in cows with and without symptoms of periparturient paresis from the parturition until two weeks after calving. The authors reported an increase in concentration of DPD from parturition reaching a peak on day 9 and then decreasing. No difference in DPD concentration was found between cows exhibiting symptoms of periparturient paresis and non-symptomatic cows. In the present work there was no correlation between tDPD and the trabecular bone measurements but there was a significant negative association with PYD concentration. This result shows the first direct association of bone marker concentrations with parameters of trabecular bone structure in cattle.

6.5 Conclusion

It is important to notice that the results presented in this chapter demonstrate associations and do not necessarily imply causality. Nevertheless, possible causal relationships were discussed based on previous work obtained from the literature as well as results exhibited in the previous chapters of this thesis. The exact mechanisms by which level of nutrition exerts its effect on endochondral ossification are still unknown, but there appears to be a possible synergistic action of IGF-1 and T3 at the hypertrophic zone which may be the reason for increased hip height gain in cattle fed high protein diets during energy restriction even though quantitatively the increase does not reach the high values achieved with high intakes of energy and protein. This study reinforces the hypothesis that the main determinant of bone elongation is the size/volume of the terminal hypertrophic chondrocyte. Nutritional restriction in cattle seems to be associated with an increase in bone resorption and a decrease in bone formation leading to overall lower bone volume, trabecular thickness and bone surface. Bone-specific alkaline phosphatase and pyridinoline concentration in the serum show significant correlation with trabecular bone parameters such as bone volume and trabecular thickness and are valid tools to assess tuber coxae trabecular bone metabolism in cattle.

Chapter 7. The relationship between liveweight and hip height in *Bos indicus* cattle and the implication for compensatory growth.

7.1 Introduction

The physiological adaptation of the skeleton and muscles to nutritional restriction differs greatly, as well as the responses observed once the level of nutrition is increased. Bones cannot decrease in physical dimensions, in particular length, during nutritional restriction, but may decrease in weight and mineral composition. On the contrary, many examples have shown increases in bone length, despite a slower rate of elongation, even in situations of liveweight loss and maintenance (McLennan, 2014). Muscles decrease in diameter and weight during severe nutritional restriction and show an increased protein accumulation once the level of nutrition is increased (Lehnert et al., 2006). In addition, gastrointestinal tract and organs mass in proportion to empty body weight reduce during nutritional restriction and overcompensate during compensatory growth in order to adapt to higher levels of intake (Yambayamba et al., 1996b). The divergent physiological adaptation of these tissues (i.e. muscle, gastrointestinal tract and organs) during nutritional restriction leads to a decrease in the liveweight to height relationship. The divergence in this relationship tends to be restored once the level of nutrition is increased and animals exhibit enhanced growth or compensatory growth in liveweight (Berg and Butterfield, 1968). Ashworth (1969) noted that increased food intake was associated with fast weight gain of infants treated for protein-calorie malnutrition. Moreover, it was observed that increased intake and consequently weight gain, decreased once the patients reached their normal weight-for-height relationship. A similar observation was made by Allden (1968) when investigating the effect of nutritional stress on sheep. The low weight-for-height relationship may represent a potential for protein deposition and an intake driver during compensation (Webster, 1993).

Liveweight gains observed in cattle at grazing systems in northern Australia are characterized by a great variation throughout the year. In a literature review, Winter et al. (1991) found that approximately half (48%) of the publications reported gains over 0.6 kg/day during the wet season while the same proportion described animals in maintenance during the dry season. However in more recent works annual gains of 142 and 168 kg /head have been reported which demonstrates that higher gains can be achieved by adoption of annual forage crops and improving grazing management (Bowen et al., 2015; Bowen et al., 2016).

To find a "normal" liveweight-for-hip height relationship for agricultural animals is a challenging task since the common objective in production animals is to increase carcass weight, which could lead to an overestimation of liveweight for a given height as animals are fed very high levels of nutrition. In the latter situation, if animals are fed very high levels of nutrition it may be that whilst the muscle weight to bone length relationship follows the "normal" pattern, liveweight may not as excess energy is deposited as fat at younger ages than is usually observed. In addition, many factors such as sex, breed and use of exogenous hormones are likely to affect the relationship. The overall concept suggested in this thesis is that skeletal elongation drives muscle growth in part through the passive stretch mechanism (Hooper, 1978; Holly et al., 1980; Young and Sykes, 1987). Furthermore skeletal elongation rate will slow as animals approach mature size (i.e. height) (Berg and Butterfield, 1968) and this is associated with increased rate of fat deposition and decreased net protein deposition as animals approach maturity. Presumably at maturity when skeletal size has reached the maximum size and does not increase any further with age then mature muscle weight has also reached a maximum although there may be much smaller increases in other protein depots. This appears to be the case if one follows the weight of muscle, fat and bone over the growth curve of sheep and cattle to maturity (Berg and Butterfield, 1968). In the present chapter deviations from the muscle weight to bone length relationship are practically viewed as deviations from the liveweight to hip height relationship. It follows that when cattle are given restricted levels of nutrition for long periods of time a divergence in this relationship occurs leading to a lower liveweight for a given height. Once the level of nutrition is increased after the restricted period, enhanced muscle growth (and hence liveweight gain) will occur as the muscle attempts to get back to its allometric relationship with the stretch parameter of bone length. Catch up growth in bones (to use the distinctive term used in this thesis for faster growth in bones) seems to be a separate process that is intrinsically dependent on morphological and physiological changes at the growth plate of long bones. Hence skeletal elongation growth and muscle growth both respond to changes in the level of nutrition but respond differently in dimensional and weight changes and perhaps respond differently once level of nutrition is increased after a period of nutritional restriction. In other words, this hypothesis suggests that catch-up growth is independent of animal's liveweight whereas compensatory growth is a function of the liveweight to hip height relationship.

The opportunity was taken to develop a relationship to describe the liveweight-for-hip height (LW-for-HH) across a range of scenarios using data collected in this laboratory from a series of experiments. It was hypothesised that during periods of nutritional restriction, animals that showed a greater deviation from the "normal" LW-for-HH relationship have a higher potential for compensatory growth in weight and this would be seen in enhanced liveweight gain. This hypothesis

was then tested using the LW-for-HH relationship developed and data of animals undergoing compensatory growth of Chapter 4 as well as that of McLennan (2014).

7.2 Materials and methods

7.2.1. *LW-for-HH relationship*

Liveweight (LW) and hip height (HH) data (n=4201) from six different experiments and a group of mature fistulated *Bos indicus* cattle (n=4) were combined in order to generate a liveweight-for-hip height relationship. Most animals were sourced from the same genotype and herd (Queensland Department of Primary Industries and Fisheries, Swans Lagoon Research Station and Spyglass Research Station) selected at weaning (approximately 180 kg LW) but studies occurred in different years with different weaner groups. The studies selected included 21 treatments and these were obtained from Chapter 4 and 5 of this thesis, Quigley and Poppi (2013b), McLennan (2014) and two studies from R. Antari (unpublished). Mean liveweight and hip height of each experimental treatment within a time point were utilized in the regression analysis. The HH measurements were taken either by using a suspended measure tape installed on a support structure of the crush or using a measuring stick. Independent of the technique used, HH was measured at the highest point of the sacrum between the tuber coxae bones and the two methods were not considered different. Liveweight was measured prior to feeding in the morning without fasting. However, part of liveweight data obtained from Chapter 5 was measured after a feed curfew of 15 h and this was corrected using the percentage difference [5.2 ± 2.8; mean ± standard deviation (SD) %] between un-fasted and fasted liveweight obtained in two different measurements during this experiment. The dataset was classified by sex, use of hormone and nutrition level and these factors were included into the model. A brief description of each study design and treatments is provided in the following sequence.

7.2.1.1 <u>Experiment 1</u>

Exp.1 is composed of data obtained from Chapter 4 of this thesis using the high crude protein (CP) and high metabolizable energy (ME) (HCP-HME) treatment and *Bos indicus* cattle. Treatments of high CP and low ME (ME) (HCP-LME) and low CP and low ME (LCP-LME) were classified as nutritionally restricted and were not included in the subset utilized for the model development of “normal” growth path. Steers fed HCP-HME grew at an average of 1.0 kg/day during the experimental period.

7.2.1.2 Experiment 2

Exp.2 is composed of data obtained from Chapter 4 of this thesis. Treatments T1, T2 and T3 were classified as nutritionally restricted and were not included in the subset utilized for the model development of "normal" growth path. The T3, T4 and T5 treatments were higher levels of supplement and animals achieved liveweight gains of 0.6 kg/day throughout the experiment.

7.2.1.3 Experiment 3

Exp.3 data was obtained from Quigley and Poppi (2013b). The experiment consisted of thirty *Bos indicus* crossbred steers [227.8 ± 1.9 kg LW; mean ± standard error of the mean (SEM)] which were blocked on LW and randomly allocated to pens and one of five dietary P treatments in a completely randomized block design. Steers were fed pelleted diets that varied in P content and provided approximately 0.9 g (P-1), 1.3 g (P-2), 1.8 g (P-3), 2.1 g (P-4) and 2.5 g (P-5) P/kg DM, and were 60-65% DM digestibility with a CP content of 100-120 g/kg DM. Steers were fed treatment diets for 24 weeks (Phase 1). Steers were then fed the P-1 pellet *ad libitum* (previous days intake + 1 kg), with monosodium phosphate/Biofos P offered at 6 or 7 g/kg DM, respectively, to supply a dietary P content of approximately 2.5 g P/kg DM intake for 17 weeks (Phase 2). Treatments P-1, P-2 and P-3 were classified as nutritionally restricted and were not included in the subset utilized for the model development of "normal" growth path. All other periods promoted higher live weight gains of approximately 0.5 kg/day and were used in the data.

7.2.1.4 Experiment 4

Data for Exp.4 was sourced from McLennan (2014). This study was composed of two drafts (i.e. same treatments repeated in two years) of crossbred *Bos indicus* steers [212.4 ± 22.69 and 208.8 ± 18.09; mean ± SD for draft 1 and 2, respectively]. The experimental design was a randomized block of 5 treatments each with 3 replicates and 10 steers per replicate. The treatments consisted of a combination of nutritional interventions (i.e. supplementation) applied during the dry season in order to generate distinct growth paths. All animals grazed mainly native tropical pastures during the whole experimental period. Within the original blocking, half of the steers in each group were implanted with hormonal growth promotants (E2; Compudose; Elanco, Eli Lilly Australia Pty Ltd) throughout the experiment. The steers were slaughtered at the end of the experiment and this varied between 27-30 months of age. Un-supplemented treatments were classified as nutritionally restricted and were

not included in the subset utilized for the model development of "normal" growth path. All other treatments had liveweight gains of approximately 0.5 kg/day throughout the experiment.

7.2.1.5 Experiment 5

Exp.5 consisted of data provided by R. Antari (unpublished data). The experimental design was a factorial design including 3 nutritional and 2 exogenous hormonal treatments with 5 animal replicates/treatment. Steers were allocated to individual pens and each animal was considered the experimental unit. The nutritional treatments [high crude protein and high metabolizable energy (HCP-HME), high CP and low ME (HCP-LME) and low CP and low ME (LCP-LME) applied in this study are the same as utilized during Phase 1 of Chapter 4 (Exp. 1 in the description above). Hormonal treatments were composed of bovine somatotrophic hormone (bST; Posilac; Elanco; Indianapolis, IN) and saline control. The experiment was carried out for 14 weeks. Treatments HCP-LME and LCP-LME were classified as nutritionally restricted and were not included in the subset utilized for the model development of "normal" growth path. The other treatments achieved liveweight gains of approximately 1.0 kg/day during the experimental period.

7.2.1.6 Experiment 6

Data from Exp.6 was also provided by R. Antari (unpublished). This experiment had a complete random design consisting of three treatments, two of which were exogenous hormone implants; control, E2 (Compudose; Elanco, Eli Lilly Australia Pty Ltd) and TBA (Synovex Plus; Fort Dodge Animal Health; Fort Dodge, IA). Each treatment consisted of 8 crossbred *Bos indicus* steers per treatment and all animals were offered *ad libitum* access to a pellet (949 g OM, 162 g CP, 254 g NDF, 8.4 g Ca and 3.1 P g/kg DM) from a self-feeder throughout the experiment (446 days). The liveweight gains of steers in the control, E2 and TBA groups throughout the experiment were 0.85, 0.97 and 0.97 kg/day, respectively.

7.2.1.7 Fistulated steers (FS)

The fistulated steers (n=4) group was composed of mature crossbred *Bos indicus* steers grazing at the University of Queensland – Gatton campus. These animals were sourced from the same group of steers utilized in Chapter 4 (Exp.1 above) but were 4 years old and had been growing continuously at pasture to reach a weight of 790 kg at the time of measurement.

7.2.2. *Effect of LW-for-HH on compensatory growth*

The model selected to describe liveweight-for-hip height was then utilized to compare the effect of the difference between the liveweight predicted by the model to the observed liveweight during the compensatory growth phase of steers when level of nutrition was increased after a period of restriction. For this analysis, data from Exp.1 and 5 were utilized. The observed mean HH at the end of each restricted phase was used to predict the expected LW according to the selected model of the normal growth path and the liveweight gap (Equation 7-1) between the predicted LW to the observed LW was utilized to investigate the relationship between LW gap and liveweight gain (LWG) observed during compensatory growth or the period of better nutrition after a period of feed restriction. The data from un-supplemented treatments groups in Exp. 4 were named as Group 1, 2, 3, and 4 for Draft 1 – Wet season 1, Draft 1 - Wet season 2, Draft 2 – Wet season 1 and Draft 2 - Wet season 2, respectively. Data from Exp.1 did not include *Bos taurus* steers (i.e. only data from *Bos indicus* steers was utilized) and it is referred as Group 5.

Equation 7-1: $LW\ gap = LW.Pred - LW.Obs$

Where LW.Pred is the liveweight predicted by Model.5 using the measured hip height at the end of the nutritional restriction and LW.Obs is the liveweight measured for this given hip height value.

1.1.1 Statistical analysis

The statistical analyses were conducted using the open-source software R (R Core Team, 2013). Since the dataset utilized to generate the liveweight-for-hip height is composed of a variety of experiments that included nutritional restriction as treatments, a subset (n=243) of the main dataset selecting only treatments that were fed *ad libitum,* or high levels of nutrition, was utilized to generate the model. This subset was composed of the mean liveweight and hip height of each treatment within a defined time point. The group of fistulated steers were considered as a single treatment to represent the mature size as they were 4 years of age at the time of measurement and 790 kg liveweight. The relationship between liveweight-for-hip height was developed by a regression analysis where sex and type of exogenous of hormone used were fitted as factors. The model selection was based on the Akaike information criterion (AIC) (Akaike, 1974). The relationship between LWG and LW gap was also investigated by a regression analysis and the Group (as described above) was included as a factor. A sequence of models using the polynomial function starting with degree=2 was fitted to test the polynomial level needed to describe the response. Then non-significant terms were sequentially removed to reduce the degree of polynomial level.

7.3 Results

Table 7-1 shows a description of the data subset utilized to create the model to describe LW-for-HH relationship stratified by the different studies and the graphic representation of the data is provided in Figure 7-1. Exp.2 was the only study that contained females in the dataset and it was also the study that showed minimum LW and HH recorded. Mature fistulated steers provided the maximum means for LW and HH. A description of the models created to generate the LW-for-HH relationship is provided in Table 2. The coefficient of determination (R^2) and residual standard error (RSE) varied from 89.7 to 96.8 % and 45.5 to 25.4 kg, respectively. Sex and the type of hormonal implant had a significant effect on the model prediction. The lowest Akaike information criterion (AIC) was obtained with Model 5, which also showed the highest coefficient of determination and lowest RSE, and this was chosen as the model to examine the effect of LW-for-HH on compensatory growth.

Table 7-1 Description of liveweight and hip height measurements of *Bos indicus* cattle fed *ad libitum* diets.

	n of observations	sex	Liveweight (kg)			Hip height (cm)		
			mean	min	max	mean	min	max
Exp.1	14	steers	295.7	178.2	380.0	121.0	111.8	127.8
Exp.2	88	heifers	226.5	121.8	410.8	118.8	102.2	135.3
Exp.3	2	steers	383.3	374.0	392.6	129.7	127.7	131.7
Exp.4	16	steers	397.6	208.5	665.0	128.6	111.0	145.4
Exp.5	32	steers	248.8	191.0	320.2	119.2	114.0	124.8
Exp.6	90	steers	422.7	164.5	618.7	129.6	109.1	141.9
FS	1	steers	789.5	-	-	153.8	-	-

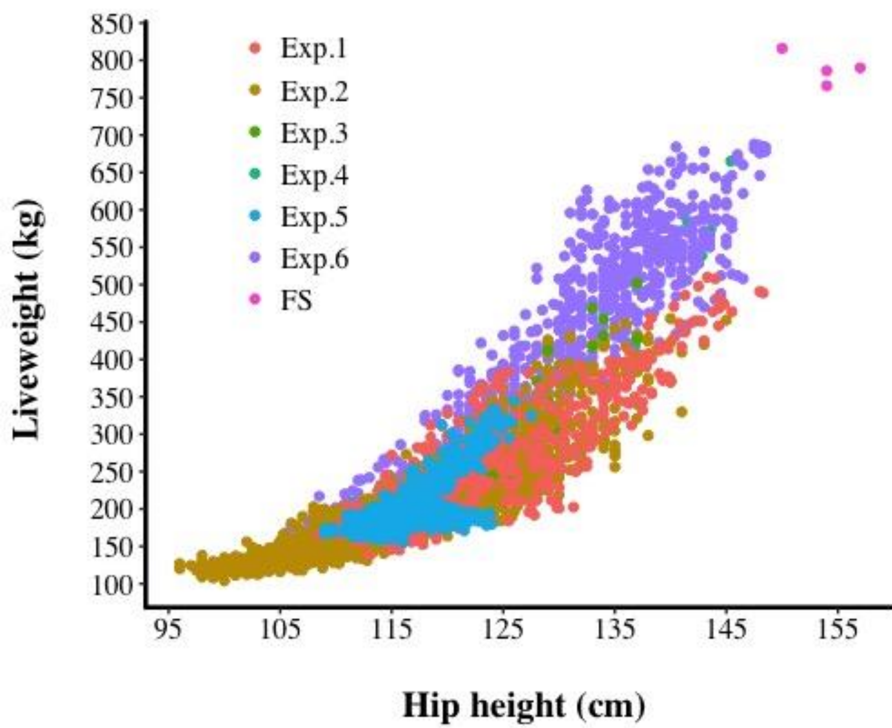


Figure 7-1 Liveweight (kg) and hip height (cm) data (n=4201) from 6 different experiments[1] and a group of mature fistulated *Bos indicus* cattle. Each dot represents a measurement of a single animal at different points of its growth path.

[1]A brief description of each experiment design and treatment groups is provided in the materials and methods section.

The graphic representation of Model 5 is demonstrated in Figure 7-2 with LW and HH data from Exp.1 and 5 during imposition of nutritional restrictions. The pattern displayed in Figure 7-2 showed a deviation from the normal LW-for-HH relationship during nutritional restriction. Steers fed restricted diets showed an increase in HH without changes in LW whereas steers fed diets *ad libitum* had simultaneous increases in HH and LW maintaining a close relationship to that predicted from Model 5.

Table 7-2 Regression coefficients, Akaike information criterion (AIC), coefficient of determination (R^2) and residual standard error (RSE) of regression models developed to describe the LW-for-HH relationship.

	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Intercept	-1292.1 (*P*<0.0001)	-1214.3 (*P*<0.0001)	2069.8 (*P*<.0001)	2172.2 (*P*<0.0001)	1896.2 (*P*<0.0001)	2068.5 (*P*<0.0001)
HH	13.0 (*P*<0.0001)	12.1 (*P*<0.0001)	-41.2 (*P*<0.0001)	-42.5 (*P*<0.0001)	-37.9 (*P*<0.0001)	-40.5 (*P*<0.0001)
HH^2	-	-	0.217 (*P*<0.0001)	0.219 (*P*<0.0001)	0.199 (*P*<0.0001)	0.211 (*P*<0.0001)
Sex						
steer	-	52.2 (*P*<0.0001)	-	52.7 (*P*<0.0001)	39.6 (*P*<0.0001)	-
heifer	-	0	-	0	0	-
Hormone implant						
None	-	-	-	-	5.4 (ns)	-18.1 (*P*<0.05)
TBA	-	-	-	-	66.4 (*P*<0.0001)	62.9 (*P*<0.0001)
E2	-	-	-	-	19.0 (*P*<0.001)	15.1 (*P*<0.05)
bST	-	-	-	-	0	0
AIC	2549.8	2477.2	2332.7	2332.7	2209.8	2297.6
R^2 (%)	89.7	92.4	93.1	95.8	97.5	96.4
RSE	45.5	39.7	37.4	29.0	22.4	26.9

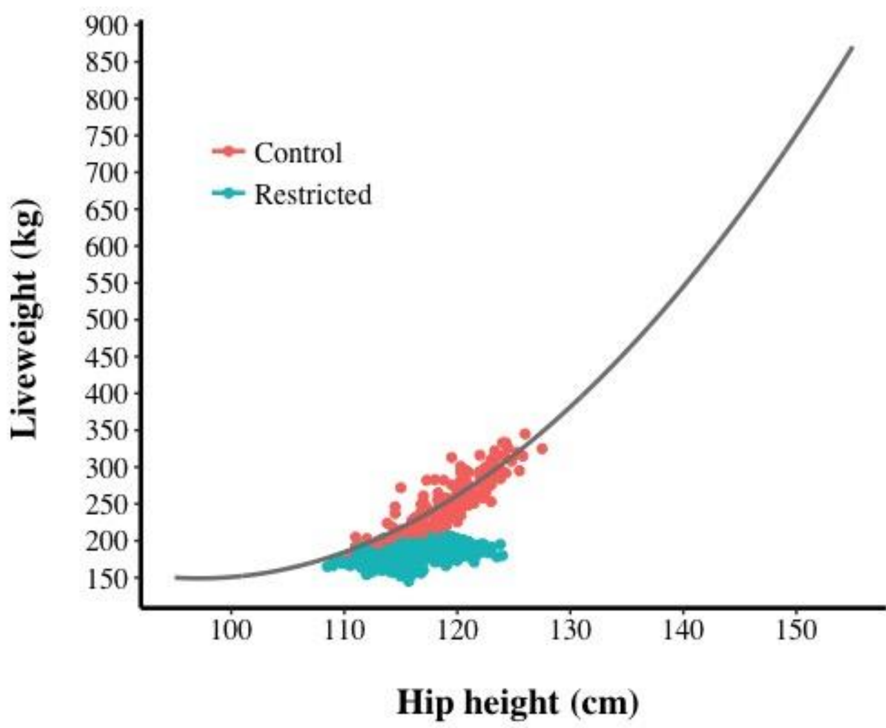


Figure 7-2 Liveweight-for-hip height relationship of Model 5 (equation for the model is provided in Table 2) is plotted alongside liveweight (LW) and hip height (HH) data from Exp. 1 and 5 during nutritional restriction. The nutritional treatments HCP-LME and LCP-LME were classified as restricted (green dots) level of nutrition and treatment HCP-HME as control high level of nutrition (red dots). Full description of the nutritional treatments is provided in Chapter 4 with a brief description above in section 7.2.1.1. Each dot on the graph represents a combination of a single HH and LW measurement per animal and time point.

Using the parameters generated by Model 5 the expected LW-for-HH was calculated using the HH measurements of the end of the nutritionally restricted phases of Exp. 1 and 4. The difference between the predicted LW and the observed LW was called the LW gap (Equation 7-1) and this parameter was utilized to investigate the relationship with LWG during compensatory growth. The greater the LW gap the greater the expected LWG during compensatory growth. The graphic representation of this analysis is shown in Figure 7-2 and the regression equations provided in Table 7-2. There was no significant interaction (*P*=0.36) between LW gap and Groups so a single slope was utilized for the different Groups. The results show that during nutritional restriction cattle deviate from the liveweight-for-hip height relationship leading to greater increases in hip height than in liveweight. The greater the difference between the expected liveweight to the observed liveweight at the end of a restricted period the higher is the potential for compensatory growth in weight.

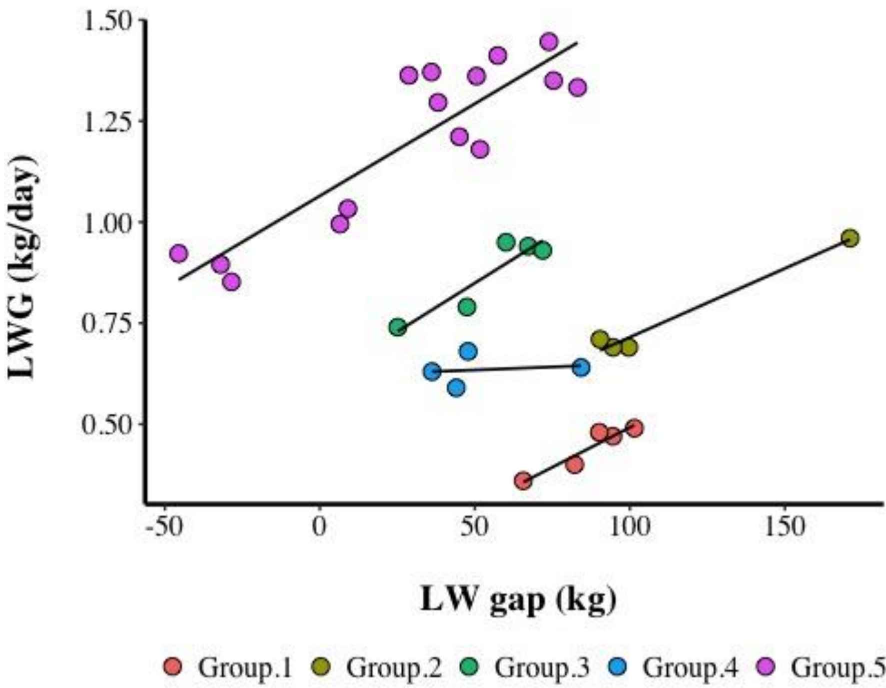


Figure 7-3 Relationship between the liveweight gap (difference between predicted liveweight by Model 5 and observed liveweight at the end of nutritionally restricted phases of Experiment 1 and 5) and liveweight gain observed during the subsequent compensatory growth period when the level of nutrition was improved. The regression equations generated by the different Groups[1] are described in Table 7-3.

[1]Description of groups is provided in materials and methods section 7.2.2.

Table 7-3 Regression equations within each animal group[1] of liveweight gain (LWG, kg/day; Y) during compensatory growth period on the difference between the predicted liveweight by Model.5 and the observed liveweight, the liveweight gap (kg; X) at the end of the restricted period.

	Equation	R^2	RSE	*P*-value
Group.1	Y=0.0755 + 0.0042X	0.95	0.07	<.0001
Group.2	Y=0.2841 + 0.0042X	0.95	0.07	<.0001
Group.3	Y= 0.6417 + 0.0042X	0.95	0.07	<.0001
Group.4	Y= 0.4121 + 0.0042X	0.95	0.07	<.0001
Group.5	Y= 1.0755 + 0.0042X	0.95	0.07	<.0001

[1]Description of groups is provided in materials and methods section 7.2.2.

7.4 Discussion

During nutritional restriction, growing cattle prioritize bone elongation over muscle or fat deposition. This pattern has been observed in Chapter 3 and 4 as well as Lawrence and Pearce (1964a), Berg and Butterfield (1968), Young and Sykes (1985) and McLennan (2014). The extent of the period of nutritional restriction creates a deviation from the liveweight-for-hip height relationship in continuously growing animals leading to cattle that are lighter than would be expected for their height. Figure 7-3 demonstrates that, during the compensatory growth period, previously restricted cattle increase LWG when returning to their expected LW-for-HH relationship. Despite the fact that cattle prioritize bone elongation during nutritional restriction, there is still a significant reduction in bone elongation rate (mm/100 days) and so HH increases slowly under nutritional restriction. In Chapters 4 and 5 it was demonstrated that a decrease in bone elongation rate is associated with smaller terminal hypertrophic chondrocytes and slower progression of endochondral ossification. A decrease in the rate of bone elongation leads to a delay in growth plate senescence (Lui et al., 2011). As a result, nutritionally restricted animals have a more physiologically immature growth plate, which allows it to exhibit faster bone elongation than unrestricted cohorts at the same chronological age once offered access to a higher level of nutrition - this process is known as catch-up growth. As discussed in previous chapters, some authors have adopted different terminologies to describe compensatory and catch-up growth and this can lead to problems of interpretation. Here the approach is to define compensatory growth as applying to weight and catch-up growth to bone length (by proxy HH) respectively to distinguish the two processes.

The initial goal of this study was to develop a liveweight-for-height relationship using data available for *Bos indicus* cattle from several studies. The use of body measurements such as hip height, hip width, heart girth and body length have been previously used to predict liveweight of cattle (Heinrichs et al., 1992; Enevoldsen and Kristensen, 1997). The models created in this study showed that sex and use of HGP implants have a significant effect on the LW-for-HH relationship especially as animals approach maturity. It is not the main objective here to discuss the reasons for such differences but it is important to point out that different nutritional levels utilized in these studies may have contributed to some of these differences. For instance, Exp.2 was the only experiment that utilized heifers and also the one with lower growth rates which probably contributed to an overestimation of the difference between steers and heifers (39.6 kg) observed in Model 5. Nevertheless, the models developed showed high correlation and good general fit to the observed data.

The second goal was to test the hypothesis that cattle that showed greater divergence from the LW-for-HH relationship during nutritional restriction have a greater potential to exhibit compensatory growth in weight during re-alimentation. In order to test this hypothesis the LW difference between the LW predicted by Model 5 according to the HH measured and the observed LW at the same time point at the end of the nutritional restriction – this parameter was named LW gap. A regression analysis then used the LW gap and the observed liveweight gain during the compensatory growth period of Exp. 1 and 5. The results showed a strong (R^2=0.95) correlation between LW gap and liveweight gain during compensatory growth (Figure 7-3 and Table 7-3). In addition, despite most groups (except Group 4) demonstrating the same pattern of an increased LWG associated with an increased LW gap, the LWG observed for a given LW gap differed greatly among groups. This difference is likely to be related to the nutritional level offered to cattle during the compensatory growth period in each of these scenarios and also different stages of maturity of the cattle. As an example, Group 5 in Figure 7-3 has the highest liveweight gain despite not displaying the greatest LW gap among the studied groups. Data from Group 5 was obtained from the experiment described in Chapter 4 where steers were pen fed lucerne chaff *ad libitum* during the compensatory growth period. Oppositely, Group 2 showed the greatest LW gap based on data from Exp. 4 in which steers grazed native wet season pastures. Interestingly, Group 4 did not show increases in LWG with increasing LW gap. However, this group had relatively fewer data points (n=4) and a longer period of time was used to calculate LWG compared to the other groups (197, 139, 151, 245 and 100 days for Group 1, 2, 3, 4 and 5 respectively).

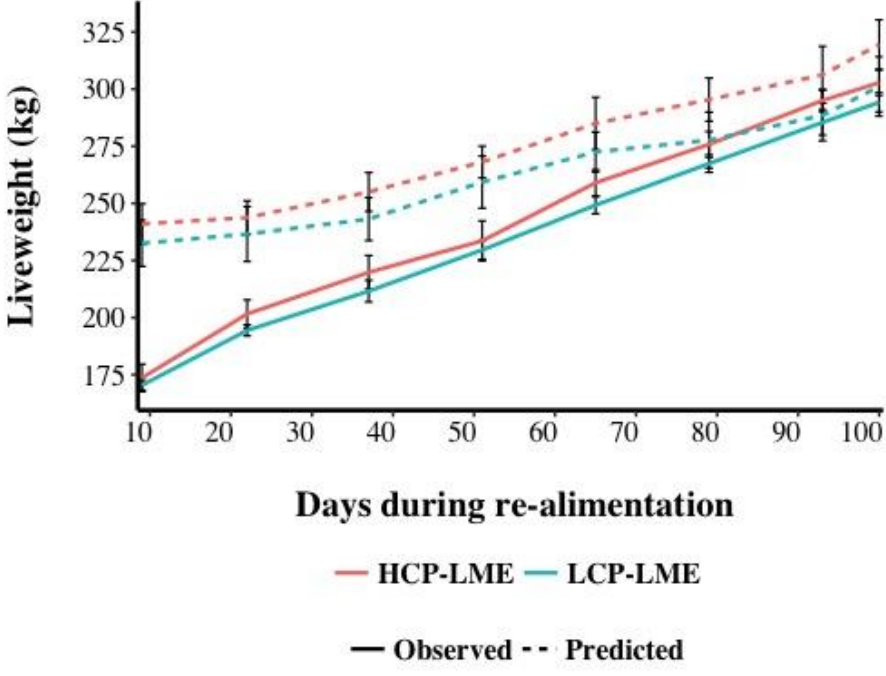


Figure 7-4 Observed (solid line) and predicted (dashed line) liveweight of nutritionally restricted steers in Exp.1 during the re-alimentation phase. Predicted liveweight was obtained by applying the

measured hip height values for each time point of individual steers to Model 5. Information regarding the nutritional treatments is provided in Chapter 4. Each data point represents the mean for each nutritional treatment (n=5) and errors bars represent standard error of the mean.

In Chapter 4 it was demonstrated that LWG during the compensatory growth period was highly correlated with the level of ME intake. When metabolizable energy intake was plotted against LW (Figure 4-3) previously restricted *Bos indicus* steers had a higher ME intake than the control group during the compensatory growth period. At the end of the re-alimentation phase both groups had a similar ME intake when compared at the same LW. For *Bos indicus* steers this occurred when observed LW approached their LW-for-HH (~270 kg LW, Figure 7-4). This observation is in accordance with the results reported by Ashworth (1969) when treating infants for protein-calorie malnutrition and provide a valuable insight to develop tools to predict the potential compensatory growth for a given animal. However the mechanisms whereby the muscle and bone length relationship affects intake are unknown. Ashworth and Millward (1986) suggested that appetite stimulation during the compensatory growth could be linked by an "aminostatic" mechanism whereby the increased protein deposition by tissues would lead to an accelerated removal of plasma amino acids that could induce an intake response.

The results presented in this chapter suggests that the potential for compensatory growth may be defined by the difference between the measured LW and the expected LW based on the animals LW-for-HH at the end of a period of nutritional restriction. In Figure 7-1 it can be observed that there is much less variation in early stages of development in relation to liveweight for a given hip height when compared to more mature animals. This is in agreement with the observation that compensatory growth is more evident in more mature cattle (Berge, 1991) and suggests a pattern where less mature animals are able to maintain a closer liveweight-for-hip height relationship than mature ones under nutritional restriction. This study and others have shown that factors such as sex, use of hormonal growth promotants and genotype are known to affect predictions of LW when based on body dimension measurements (Enevoldsen and Kristensen, 1997). Therefore, in order to have a more accurate prediction of LW-for-HH it is important that the model adopted be developed using representative data of the herd to be analysed. A relationship exists between LW and HH for continuously growing animals which is described using a polynomial function of the general form described by Equation 7-2:

Equation 7-2: $Y = 1896.2 - 37.9X + 0.199X^2 + 39.6$,

where Y is liveweight (kg) and X is hip height (cm) of a non-hormonal implanted *Bos indicus* steer. This implies that there is a strong relationship between muscle weight and bone dimensions according to the concept that muscle growth is controlled passively by a stretch mechanism associated with bone growth. The relationship can alter during nutritional restriction such that there is a decrease in the deviation from the generalised curve of continuously growing animals. Practically this is seen as large frame animals with low weight or body condition score. Other factors can cause deviation from this generalised curve and the current data set illustrated that sex and HGP implants had significant effects especially as animals approached maturity. Compensatory growth in weight is the process whereby a previously restricted animal increases growth rate and energy intake until reaching its LW-for-HH. The LWG during the compensatory growth period is affected by the difference between the actual LW and the expected LW-for-HH as well as the nutritive value of the feed offered during the compensatory growth period. The greater the deviation of the LW-for-HH, the greater the potential increase in LWG during the compensatory growth period, but the extent of that increase in LWG will depend on the level of nutrition, as influenced by feed quality, during this period.

7.5 Conclusion

It was concluded that a generalised equation of Model 5 would describe the relationship between LW and HH of continuously growing animals. Deviations from this relationship would enable animals to exhibit compensatory growth in LW when the level of nutrition was increased and the extent of the increase in LWG during this period depended primarily on the extent of the difference between actual and predicted LW based on the model that was developed for this genotype (*Bos indicus* crossbred cattle). In addition, the results presented here support the hypothesis that increased food intake during compensatory growth decreases once animals reach their expected liveweight for height during compensatory growth. However the physiological mechanisms that explain such phenomenon are unknown.

Chapter 8. General discussion and conclusions

8.1 Introduction

Nutrition is the most significant factor affecting cattle production and variations in nutritional plane are a common challenge in tropical grazing systems. Cattle exhibit a normal sigmoidal growth curve over the lifetime of the animal to maturity (Brody, 1945) with fluctuations in nutrient supply resulting in deviations from this pattern. In some cases cattle exhibit accelerated LWG after a period of nutrient restriction and this is often called compensatory growth. Whether this also occurs for skeletal growth is not known. As reviewed in Chapter 2, little is known about the effect of nutrition on skeletal development in cattle during periods of limited nutrient supply and subsequent skeletal elongation when the level of nutrition is increased.

The initial goal of this thesis was to determine how the type (Chapter 4) and severity (Chapter 5) of nutritional restriction affect bone development, in particular skeletal elongation as measured by hip height, and how this might be influenced by stage of maturity. Secondly, it aimed to understand if the restrictions imposed on bone development and elongation affect compensatory growth in LW and catch-up growth in bone elongation.

8.2 Compensatory and catch-up growth terminology

As reviewed in Chapter 2, many attempts have been made in order to generate a terminology that describes the pattern of growth recovery after nutritional restriction. The difficulty in such a task arises from the lack of understanding of the biological mechanisms that drive growth acceleration and deceleration following a period of nutritional restriction. Moreover, the distinct adaptive physiological changes of tissues within the body (e.g. bones, muscles and organs) add another level of complexity to the process of describing this phenomenon. Historically, the term compensatory growth has been used mostly in the agricultural context to describe a faster LWG of a previously restricted animal compared to an age matched unrestricted counterpart (Bohman, 1955; Wilson and Osbourn, 1960; Sainz et al., 1995; Hornick et al., 2000). The term catch-up growth has been mostly applied to bone growth to describe faster bone growth in a previously restricted animal compared to an age-matched unrestricted counterpart (Gafni et al., 2001; Emons et al., 2005; Lui et al., 2011). However, some authors have also utilized the term catch-up growth to describe faster growth rates in both LW and height (Prader et al., 1963; Ashworth and Millward, 1986) while others have also

suggested that both terms (i.e. catch-up and compensatory growth) are synonymous (Yambayamba and Price, 1991b; Boadi and Price, 1996). Jobling (2010) on the other hand, suggested that differentiation between catch-up, compensatory and recovery growth to be based on the analysis of growth trajectories over time. In this concept, the terms could be used interchangeable between tissues with no specific nomenclature for measures of weight or size. The author defined compensatory growth as the phenomenon when an animal exhibits a faster growth rate than a size or weight matched unrestricted counterpart, whereas catch-up growth would be the convergence of different growth trajectories. In this thesis I propose the separation of these terms when describing liveweight gain and skeleton growth and discount the need for a comparison at an equivalent size (i.e. weight, height) basis. The necessity for this distinction is justified by the following reasons:

1 - The biological mechanisms that sets the limits of LWG and HHG (i.e. as a proxy for skeletal growth) following nutritional restriction are independent from each other. The underlying concept is that muscle growth in weight is dependent on the stretch mechanism of bone elongation (Goldspink, 1977; Hooper, 1978; Holly et al., 1980; Young and Sykes, 1985; Goldspink et al., 1995) and so variations from the "normal" growth curve of muscle weight (by proxy LW) to bone length (by proxy HH) result in faster gains in weight than an animal undergoing an uninterrupted growth in weight and bone elongation. Liveweight gain is highly correlated with ME intake during this period but also likely to be affected by the requirement for maintenance energy. The pattern of LWG during re-alimentation follows closely the pattern of DM intake over time and is best described by a cubic function with an initial acceleration reaching a peak approximately 40 to 60 days after the start of re-alimentation. The decrease in DM intake, hence LWG, is related to the moment when animals approach their "normal" liveweight-for-hip height relationship. Therefore, it seems that the length of compensatory growth is dependent on how fast an animal reaches its "normal" liveweight for hip height relationship. The rate of skeletal growth, on the other hand, is governed by the endochondral ossification process at the growth plate. More specifically, skeletal growth is the final result of the action of multiple growth plates within the body, which may differ greatly from one to another for any given point in time during skeletal development (Wilsman et al., 2008). Nevertheless, it seems that under normal circumstances (i.e. animal not suffering from any disease or other factors that may restrict growth) the growth rate of a given growth plate will be first limited by its maturity and secondly by nutrition. The quantification of growth plate maturity is not as straightforward as nutritional intake but it can be understood as the degree of senescence of a given growth plate. Growth plate senescence is a term utilized to describe the postnatal development program that occurs at the growth plate. This is composed of progressive loss of function (e.g. decline in the rate of chondrocyte proliferation) as well as structural involution (e.g. decline in volume of terminal hypertrophic

chondrocytes) which sets a limit to the rate of bone elongation (Nilsson and Baron, 2004). The most accepted hypothesis to explain the mechanism whereby the growth plate "stores" the information regarding its previous growth story (i.e. growth plate senescence) is at the progenitor chondrocytes at the resting zone. Lui et al. (2011) suggested that a cell-cycle counting system could be the mechanism regulating chondrocyte proliferation at the resting zone and therefore growth plate senescence. An example of such a mechanism is the telomere shortening (Olovnikov, 1996). It follows that in eukaryotes cells, telomeres shorten with each cycle of cell division leading to incomplete replication of linear chromosomes by conventional DNA polymerases. In most cell types (except germ and stem cells), the shortening of telomeres progresses until a specific critical length, after which, the cells lose viability (Olovnikov, 1996). Therefore, skeletal growth rate after a period of nutritional restriction is increased by the increased level of nutrient intake as demonstrated in Chapter 3 but this response is dependent upon the degree of growth plate maturation (or senescence). On the other hand, compensatory growth is also a function of increased nutrient intake however the limit for the increased intake seems to be set by the relationship between the animal's liveweight to its skeletal size.

2 – Due to the different biological mechanisms regulating liveweight and hip height gain after a period of nutritional restriction, the growth trajectories of these measurements are inherently different and shouldn't be compared under the same perspective. For instance, the results of Chapter 4 provide evidence that liveweight gain during compensatory growth is greater than the rate of gain of an unrestricted cohort at the same age. In addition, the liveweight gain may also be transitorily higher than a non-restricted animal compared at the same liveweight (e.g. *Bos taurus* steers fed the treatment LCP-LME). However, the skeletal growth rate, measured as the hip height gain, seems to be unable to be faster than that of a size matched unrestricted cohort. Nevertheless, during catch-up growth the skeletal elongation rate is faster than that of an age matched unrestricted cohort. Evidences of catch-up growth in cattle were observed during the re-alimentation of restricted steers (i.e. HCP-LME and LCP-LME) in Chapter 4 as well as during the second wet season of non-supplemented heifers (i.e. T1) of Chapter 5. Probably the best way to exemplify this difference is plotting the rate of skeletal growth of unrestricted and restricted animals during re-alimentation against their age as well as their height. Unfortunately, it is not possible to execute such comparisons with the current data of this thesis because the exact date of birth of animals utilized in both experiments is unknown. In addition, the experimental length (Chapter 4) or the variation in nutritional during the studies (Chapter 5) are also restrictive factors for such comparison. However, the classical work of Moulton et al. (1921) which published the result of several investigations conducted between 1907 and 1912 at the Agriculture College of the University of Missouri can be used to exemplify the concepts previously

mentioned. This publication includes an array of long time measurements (i.e. more than 4 years) on liveweight and body measurements of cattle growing continuously fed the same diet or subjected to periods of nutritional restriction and re-alimentation. This publication provides the original data of these investigations and they were utilized to generate Figure 8-1.

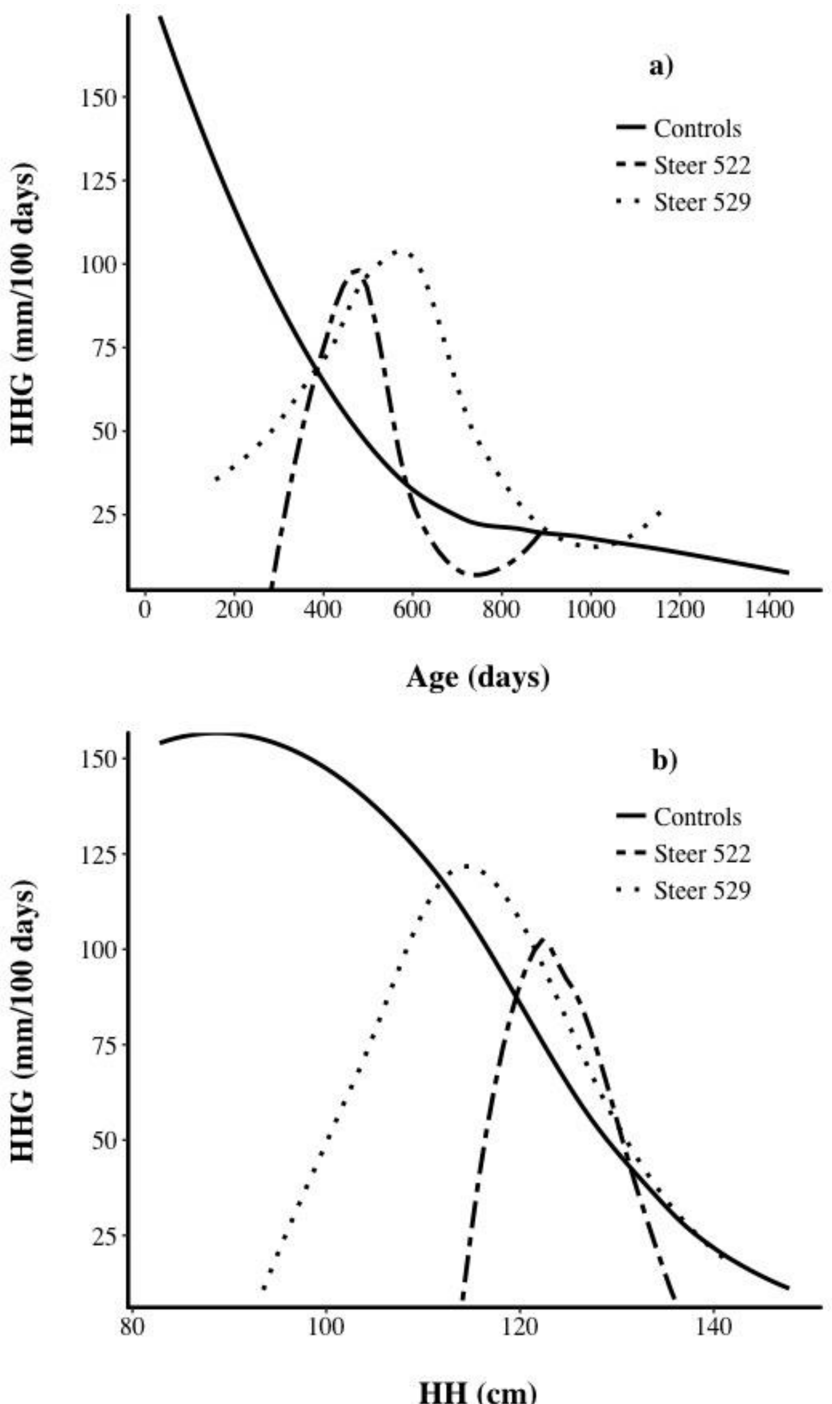


Figure 8-1 The relationship between hip height gain (mm/100 days) and age (a) as well as hip height (b; HH) of steers fed *ad libitum* high quality diets throughout (controls, solid line, n=7) compared to steers 522 and 529. Steer 522 was fed to achieve liveweight maintenance from about 250 to 400 days old, from 400 to 670 days of age this steer had *ad libitum* access to the same diet as controls steers and again subjected to liveweight maintenance from 670 to 880 days old. On the other hand, steer 529 was fed to achieve liveweight maintenance from 180 until 390 days old and then offered *ad*

libitum access to diet offered to control steers. Data utilized in this figure was adapted from Moulton et al. (1921).

Figure 8-1.a demonstrates the pattern of skeletal elongation rate of cattle when offered the same unlimited amount of high quality diet throughout (Controls, solid line) as well as during nutritional restriction and re-alimentation (dashed and dotted line). It is clearly observed that rate of skeletal growth increases after the removal of the nutritional restriction even at older ages when unrestricted cattle normally present a much lower rate of skeletal elongation. However, when skeletal growth is plotted against hip height (Figure 8-1.b) it shows that rate of skeletal elongation progressively increases after the removal of the nutritional restriction but the peak of velocity reached is similar to the rate of skeletal elongation of an unrestricted cattle at the same hip height. In addition, Figure 1 provides evidences to support the hypothesis that skeletal elongation rate is primarily a factor of the degree of growth plate senescence (i.e. growth plate maturity) and not age. These collations exemplify the concepts discussed previously about the reasons why compensatory and catch-up growth process should be named as two distinct processes and are in agreement with the results presented in Chapter 4 and 5.

This thesis has demonstrated examples of nutritional manipulation affecting growth in terms of skeletal development and liveweight in cattle. It has also demonstrated some of the mechanisms, which are involved in the physiological and morphological responses during and after nutritional restriction. It has shown that liveweight can be lost or maintained under nutritional restriction but that skeletal elongation will still occur even under nutritional restriction albeit at a lower rate than when nutrition was not limiting. Following nutritional restriction when animals are allowed access to a period of high nutrition both weight and skeletal elongation show faster growth but while the liveweight gain is faster than that of controls, skeletal elongation reaches a maximum dependent on nutrition but this value, measured as rate of change in hip height (mm/100 days) does not appear to be faster than controls at the same hip height. Thus there appears a maximum rate of elongation set by growth plate dynamics of maturity and nutrition. Overall, the results of the experiments described in this thesis, allied with previous work reported in the literature, indicate two distinct processes occurring following a period of nutritional restriction. The understanding of these processes is crucial for enhancing efficiency of animal production systems in environments where animals are subjected to periods of nutritional variation such as tropical grazing systems. Therefore, it is proposed that compensatory growth should be defined as the process whereby the body compensates for deviations in the relationship between soft tissue (e.g. muscle and organs) and skeletal size. Liveweight gain during compensatory growth is higher when compared to an unrestricted age matched cohort and may

be transitorily higher than cattle at the same liveweight. This is usually accompanied by an increase in food intake expressed as percentage of liveweight when compared to unrestricted animals as well as higher feed efficiency. The length of the compensatory period will depend upon of the LW gap (i.e. actual LW – LW predicted by LW-for-HH) and quality and quantity of nutrition available during re-alimentation as demonstrated in Chapter 7. The Figure 8-2 represents a theoretical concept of this model.

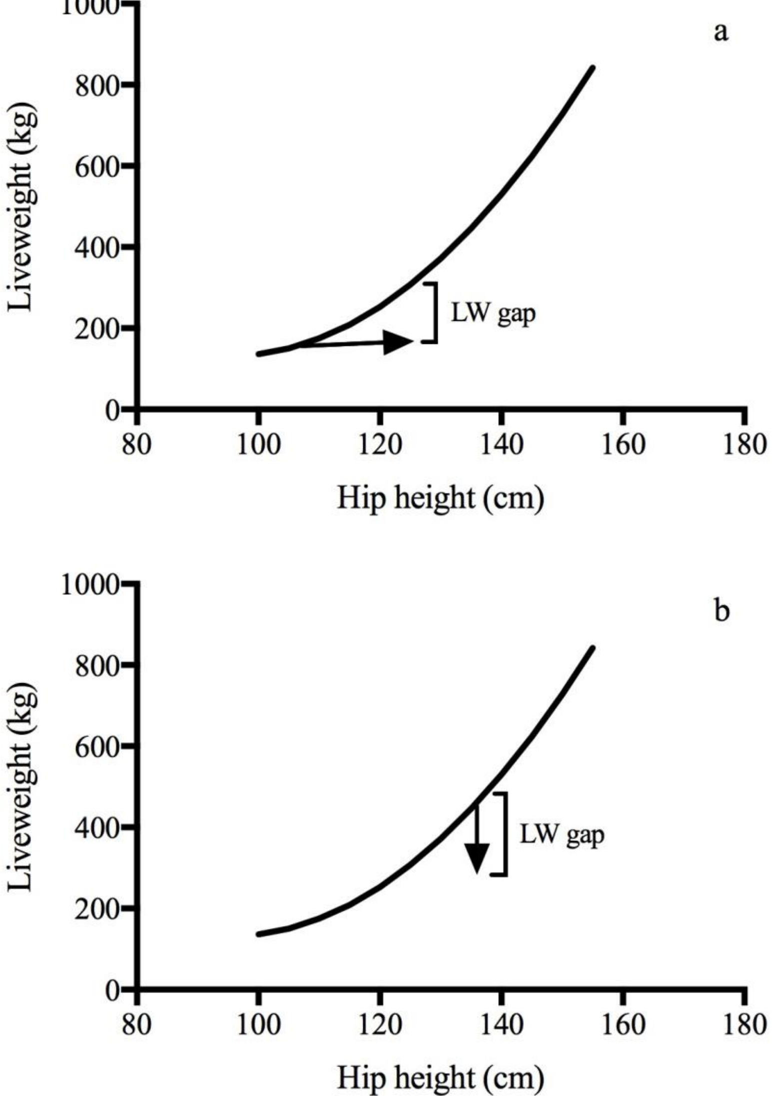


Figure 8-2 Liveweight for hip height representation of two hypothetical representations that may lead to compensatory growth. The first Figure (a) represents a weaner on liveweight maintenance but positive gain in hip height during nutritional restriction similar to the pattern observed in restricted steers in Chapter 3 and 4. The second Figure (b) illustrates a situation where a heavier cattle loses liveweight without increasing in hip height even though stasis in hip height has not been observed in any of the current studies. In both simulations, there is a deviation from the "normal" liveweight for hip height relationship and the difference between the actual liveweight and the predicted liveweight for its hip height is named LW gap.

Catch-up growth may be defined as the process whereby previously nutritional restricted cattle demonstrate increased skeletal growth rate when compared to an age matched unrestricted cohort. However, the rate of skeletal growth is not greater than unrestricted cattle with similar body dimensions. Food intake during catch-up growth may also be greater when compared to unrestricted cattle however it seems that it won't be greater than an unrestricted animal compared at the same liveweight.

8.3 Effect of nutrition on bone development of cattle

Very few studies have focused on exploring the effect of nutrition on the bone development of cattle and to investigate its effect in the context of animal production. The great majority of previous work has looked at the effect of nutrition on size, proportion in relation to other tissues and shape of bones while some others have investigated the relationship between muscle development and bones (Young and Sykes, 1987; Young, 1988). The present work showed that the process of endochondral ossification and bone remodelling are severely affected by nutrition.

8.3.1. *Endochondral ossification*

Endochondral ossification is the process whereby longitudinal growth occurs. This appears to be the first study that investigated the effect of nutrition on the growth plate of cattle. Since skeletal growth in length is estimated to account for 77% of the changes in muscle volume (Young and Sykes, 1987) the factors leading to changes in bone elongation must be better understood in order to develop techniques to improve efficiency in animal production.

Nutrition had a significant effect on the morphological growth plate parameters of trabecular bone as measured by using a biopsy technique of the tuber coxae, however the impact of this factor on cortical bone is unknown. The effect on the PZ was more variable within and between experiments than the HZ. It is assumed that bone elongation occurs through a process of cell proliferation at the PZ followed by cell elongation through hypertrophy at the HZ and the changes can be followed through histological examination of growth plate morphology (Wang et al., 1999; Gat-Yablonski et al., 2008). In Chapter 4, only *Bos taurus* steers showed reductions in terms of PZ thickness when fed energy restricted diets and this effect was independent of the CP content in the diet. Despite the different response between genotypes on height of the PZ, restricted diets resulted in very similar rates of skeletal growth independent of cattle genotype. In Chapter 5 the lowest level of supplementation resulted in reductions in the height of the PZ only in early-weaned heifers. The variation in response

of height of PZ in response to nutritional restriction was also evident in the correlation analysis in Chapter 6. In contrast, the height of terminal chondrocytes at the HZ showed a consistent positive relationship across both experiments with rate of skeleton rate measured as HHG. This observation is in agreement with the suggestion that volume of the terminal hypertrophic cells is the main driver of skeleton elongation rate (Breur et al., 1991). However, the proportional reduction observed in height of terminal hypertrophic cells in both experiments is lower than the relative reduction in HHG. For instance in Chapter 4 *Bos indicus* steers showed reductions of 12 and 35% in diameter of terminal hypertrophic chondrocytes when offered HCP-LME and LCP-LME diets respectively and compared to control group (i.e. HCP-HME). The reduction in HHG however was relatively greater namely 54 and 67% respectively for the same groups. Similarly, in Chapter 5 non-supplemented heifers (i.e. T1) had the height of terminal hypertrophic chondrocytes reduced by 10 and 14% in early and normally weaned heifers respectively compared to supplemented heifers (i.e. T5). On the other hand, the HHG was reduced by 28 and 20 % in the same groups respectively. This difference may indicate that during nutritional restriction the contribution of cell proliferation to skeletal growth rate has a greater impact than during unrestricted growth. This observation is in agreement with Wilsman et al. (1996) who proposed that the relative contribution of cell division, matrix synthesis and chondrocyte hypertrophy to growth rates changes when comparing fast and slow growing growth plates. In addition, a slower rate of proliferation at the growth plate of restricted cattle would also be in accordance with the hypothesis of growth plate senescence to explain catch-up growth (Baron et al., 1994; Lui et al., 2011). However, the rate of cell proliferation was not assessed in the experiments of this thesis and the height of PZ does not appear to be related with rate of proliferation.

Nevertheless, based on the results of this thesis it can be concluded that nutrition has a marked effect on the bone endochondral ossification process. Crude protein intake explained 82% of the variation in hip height gain in cattle independent of the genotype. Moreover, high crude protein diet during energy restriction increased the rate of skeletal elongation, although not a large quantitative increase, and this was associated with larger terminal hypertrophic chondrocytes. Despite a significant correlation with changes in bone elongation rate at specific moments in time, the concentration of circulating hormones such as IGF-1, T3 and insulin are not able to explain different rates of skeletal elongation in the long term. The physiological changes at the growth plate, during the process of growth plate senescence, seem to affect the response of the growth plate from external stimulus such as nutritional level or endocrine concentration. The pace of growth plate senescence may be reduced during restricted nutrition in cattle, as it is in rodents and rabbits, and it may be the mechanism that allows catch-up growth in cattle.

8.3.2. *Trabecular bone turnover*

Bone formation and resorption are the processes responsible for bone turnover or remodelling and it is constantly renewing the mineralized structure of bones. The assessment of the bone turnover is not only important because of the relevance of physical structure of bones but also due to its function as a regulator of the mineral storage balance in the body. Phosphorus is a very important mineral in cattle nutrition and it has been estimated that approximately 80% of the total body content of P is stored in the mineralized matrix of bones (Freer et al., 2007). Bone remodelling affects cortical and trabecular bone, however, trabecular bone accounts for approximately 80% of the total skeletal surface area. The greater surface area leads to a greater surface area exposed to the action of osteoblasts and osteoclasts. This difference translates into a significant difference in the proportion of these two different bone types that undergo remodelling every year. Its estimated that between 2 to 3% of cortical bone is remodelled in a year opposed to 25% of the trabecular bone mass (Swaminathan, 2001). Therefore, understanding the physiological processes that regulate bone remodelling balance is relevant to animal science.

It has been shown in Chapter 4 that limited energy intake leads to lower bone volume, trabecular thickness, bone surface as well as greater trabecular separation. In addition, animals fed a high protein diet during energy restriction showed a greater bone volume than cattle fed a low protein diet. Independent of the protein content in the diet, cattle had a similar concentration of BAP in plasma during energy restriction, however animals fed high protein diets had significantly lower concentration of PYD in plasma which may indicate lower bone resorption activity. This observation is in agreement with results obtained with other animal species (Sukumar et al., 2011). No differences in terms of trabecular bone structure were found in Chapter 5 and as discussed in Chapter 7 this could be related to the overall lower nutritional plane offered to supplemented and un-supplemented heifers in this experiment. Bone formation in growing cattle seems to be the process most affected by level of nutrition. However, this assumption could only be confirmed by a dynamic analysis of bone formation in such conditions. Nevertheless, the changes in trabecular structure as well as the plasma concentrations of bone metabolism biomarkers indicate that during nutritional restriction trabecular bone loss at the tuber coxae is a result of a greater reduction in bone formation than increased bone resorption.

Bone metabolism biomarkers are preeminent tools to assess bone turnover in cattle. However, there is no information available in the literature about the relationship of the concentration of such molecules in cattle with related changes in bone structure that may indicate changes in bone formation or resorption. In Chapter 7, a significant positive correlation between the concentration of BAP in

plasma and bone volume and trabecular thickness was demonstrated. In addition, a significant negative correlation with the concentration of PYD in plasma was found for the same trabecular bone parameters. Osteocalcin, total deoxypyridinoline crosslinks and CTX-I concentration in the plasma showed no significant relationship with structural changes in trabecular bone morphology assessed by histomorphometry. However, in Chapter 5 the overall level of nutrition was low and this may have influenced the result of CTX-I thus this biomarker should be tested again across a greater range of nutritional levels. Nevertheless in growing cattle, the concentration of BAP and PYD in plasma can be utilized as biomarkers for bone formation and resorption respectively.

8.4 Permanent stunting after nutritional restriction

One of the main concerns of cattle producers and nutritionists is the impact of restricted nutrition at early stages of development on the mature size of a given animal. This topic is very relevant in northern Australia where weaning predominantly occurs at the beginning of the dry season when pasture quality and availability declines. In this environment, early weaning (i.e. 100 to 150 kg LW) is recommended in order to decrease nutritional requirements of dams and enhance the probability of re-conception but it presents a greater nutritional challenge for the immature calves. In the medical context, an individual is consideered permanently stunted when the closure of the epiphyseal growth plate occurs and the height is two standard deviations below the reference population for its age (UNICEF-WHO-WB, 2016). The epiphyseal closure is affected by the rate of growth plate senescence and it is anticipated in situations where the decline in number of chondrocytes at the resting zone is accelerated, such as during estrogen treatment (Nilsson et al., 2014).

A cell-cycle counting system has been suggested as a possible mechanism regulating chondrocyte proliferation at the resting zone and therefore growth plate senescence (Lui et al., 2011). Assuming that the hypothesis proposed by Lui et al. (2011) is true, it seems biologically impossible for an animal that suffered from nutritional restriction during growing stages of development to achieve its genetic potential mature size, due to the fact that bone elongation is prioritized in growing animals even under conditions of liveweight maintenance or loss. Therefore, continuous bone elongation during nutritional restriction would result in an inefficient use of the limited replications of chondrocyte because the level of nutritional intake would reduce the growth generated by each chondrocyte during hypertrophy. However, the length of nutritional restriction necessary to cause a significant impact on the mature frame size of cattle may not be commonly achieved under field conditions. In addition, after a period of nutritional restriction cattle, as other mammals, extend their period of skeletal growth for their age therefore a relative long time frame would be necessary in order to detect a possible

permanent stunting. In Chapter 5, a group (T1) of early and normally weaned heifers were fed only a mineral urea block lick over the first dry season. There was no evidence that the skeletal growth or mature frame size of these cattle was in some way impaired when compared to cattle that received supplementation over the first dry season (T5). All heifers independent of whether they received a supplement or not over the first dry season were still growing by the end of the experiment. Moreover, applying the definition of stunting proposed by UNICEF-WHO-WB (2016) (i.e. shorter than minus two standard deviations than the average height of reference population) to the present study and considering T5 of early and normally weaned heifers as the reference population, it can be concluded that non-supplemented heifers were not stunted by the end of the experiment.

8.5 Dry matter intake

There are only two ways that an animal is able to gain liveweight faster than another animal. The first is having a higher nutrient intake and the second is having a greater feed conversion, however these factors are often associated. A more efficient feed conversion can be caused by several factors such as: a higher intake, a higher digestibility, a higher efficiency of use of absorbed nutrients and/or a lower maintenance energy requirement (Yambayamba et al., 1996a) or a difference in tissue deposition pattern which affects the efficiency of use of energy for growth and fattening (Fox et al., 1972). In cattle, much of the research investigating compensatory growth has pointed to food intake as one of the main factors leading to the occurrence of this phenomenon (Sainz et al., 1995). The results of Chapter 4 are in agreement with these previous reports and show a linear relationship between the ME intake and liveweight gain which is also agreement with more recent work on continuously growing cattle (Salah et al., 2015). Interestingly, this was independent of the type of restriction (ME or crude protein) and genotype of steers. In addition, the ME intake of previously restricted steers (i.e. diet treatments HCP-LME and LCP-LME) was transitorily higher during compensatory growth when compared on the same liveweight basis to control steers (HCP-HME). To answer which of these factors (food intake or feed conversion) had the greater impact on liveweight gain during compensatory growth is a challenging task since both are correlated. However, the results from Chapter 4 showed that a great proportion of the variation in LWG is explained by ME intake rather than FCR (92 *vs* 78 %, respectively).

The results from Chapter 7 show that increases in LWG and MEI during compensatory growth are associated with deviations from the LW-for-HH relationship. Overall these results seem to indicate that food intake could be a response to potential for protein deposition (primarily muscle) rather than being the reason for this increased protein synthesis. Despite not having hard evidence from the thesis

experiments to support this hypothetic relationship there is some evidence in the literature for it. For instance, Therkildsen (2005) showed that concentration of RNA and DNA in the muscle as well as the fractional breakdown rate of protein from previously restricted steers had the same pattern during re-alimentation. Both protein synthesis and breakdown started the re-alimentation period lower than controls and peaked 5 weeks after the steers had access to the *ad libitum* diet which also mimicked the pattern of liveweight gain during the period. This observation is similar to the results observed in Chapter 4 in terms of liveweight gain and dry matter intake (Figure 8-3) as well as the general pattern of liveweight gain during compensatory growth described by Hornick et al. (2000). Similarly, intake expressed per unit of liveweight achieves its highest values in early stages of body development when skeletal growth is at its maximum rate and muscle synthesis is also accelerated in order to keep pace with bone elongation and maintain an allometric relationship. Following this rationale, we can assume that any factor reducing the speed of skeletal growth will consequently reduce metabolisable energy intake due to a decrease in rate of muscle growth (i.e. protein synthesis). Interestingly, several studies in different species have shown that both calcium and phosphorus deficiency promote a significant reduction in food intake in growing animals (Shapiro and Heaney, 2003; Quigley and Poppi, 2013b). A decrease in energy intake due to a slower bone growth rate would be expected since the energy balance of an animal must obey the first law of thermodynamics. Energy in excess cannot be easily dissipated from the body so the main regulation is given by decreases in energy intake. In contrast, excess of protein can be easily catabolized and excreted in urine (Mertens, 1994).

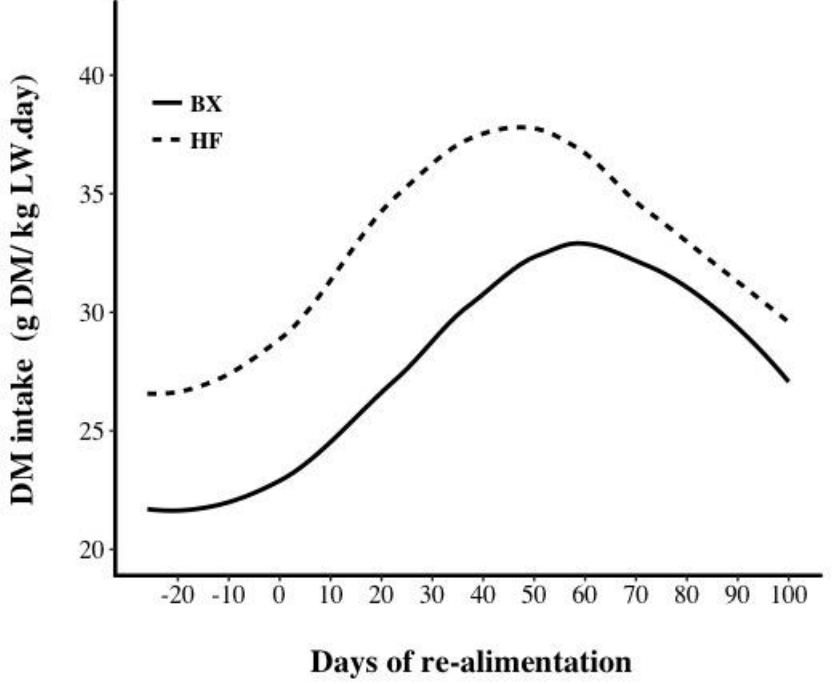


Figure 8-3 Pattern of dry matter (DM) intake of *Bos indicus* (BX, solid line) and *Bos taurus* (HF, dashed line) steers during re-alimentation after 93 days of nutritional restriction. Day zero on the graph represents the first day of re-alimentation period. Data was obtained from the experiment described in Chapter 4 and represents the average of five repetitions of each genotype. During

nutritional restriction (93 days) steers were fed low CP-low ME (Mitchell grass hay) *ad libitum* and during re-alimentation high CP-high ME *ad libitum* (Lucerne chaff).

Leptin and insulin are hormones related with nutritional status and are well known to affect food intake (Schwartz et al., 2000). However, in Chapter 4 and 5 there was no evidence that the concentration of these hormones was related to intake during compensatory growth. In fact leptin concentration was not reduced by nutritional restriction in any of the experiments. On the other hand, insulin concentration was lower in all nutritionally restricted treatments in both experiments. The transport of insulin into the central nervous system has been shown to be in proportion to the plasma concentration (Baura et al., 1993). Moreover, the deficiency of this hormone in the brain leads to hyperphagia (Sipols et al., 1995). Thus, the lower concentration of insulin in nutritionally restricted cattle by the end of the restricted phase could be acting as a stimulus to increase intake during compensatory growth. Insulin was not measured during the compensatory phase in Chapter 3 so no conclusions can be drawn from it in this regard. However, in Chapter 5 the concentration of insulin in previously restricted heifers was already equal to supplemented ones at the start of the first wet season (i.e. beginning of re-alimentation phase). At this moment the recover index (Equation 2-1) of un-supplemented heifers was only 12% compared to 44% by the end of the first wet season. This means that the greater proportion of liveweight gain in the compensatory growth period was obtained after the insulin concentration reached similar values between supplemented and un-supplemented heifers. Therefore, it seems that the change in insulin concentration is not able to explain the greater liveweight gain, hence dry matter intake, of heifers during compensatory growth in Chapter 5.

The proposed model of feed intake regulation based on protein synthesis - and indirectly skeleton growth - is still crude and speculative but nevertheless provides a hypothesis on regulation of food intake in the long term which may be tested in the future. Nevertheless, Radcliffe and Webster (1979) previously suggested that Zucker rats regulate food intake during growth so as to sustain a maximal rate of protein deposition provided that intake was not restricted by other nutrients. Research with lambs have indicated that they are able to select their diets in order to maximize growth and meet their crude protein requirements. It also demonstrated that sheep and lamb would adapt the protein intake accordantly to the physiological state such as pregnancy and maturity (Kyriazakis and Oldham, 1993; Cooper et al., 1994).

In conclusion, the increased voluntary intake during compensatory growth seems to be at least in part a factor of the enhanced potential for protein synthesis which in turn derives from the unbalanced

relationship between soft tissue weight for the skeletal size (i.e. liveweight to hip height ratio). However the physiological mechanism that regulates this response is unknown.

8.6 Perspective for future research

In order to study the effects of nutrition on the growth plate and trabecular bone in cattle a novel bone biopsy procedure in cattle was developed in this thesis. The procedure has been described in Chapter 3 and showed to be an effective way to obtain tuber coxae specimens *in vivo* from growing cattle. Moreover, the morphological changes observed at the growth plate and trabecular bone level due to nutritional manipulation in both experiments (i.e. Chapter 4 and 5) were consistent with changes in body measurements, and also in agreement with more detailed studies in different species (e.g. mice, rat and rabbit). Thus the tuber coxae biopsy seems to be a valuable methodology that may allow future studies in endochondral ossification as well as trabecular bone turnover in cattle.

The proposed description of compensatory and catch-up growth provided in this thesis may help to enhance the understanding of the processes occurring after nutritional restriction during re-alimentation in cattle. The relationship between liveweight and skeletal size and its effect on animal physiology needs to be further explored in future research. Specifically, the mechanism whereby a change in liveweight-to-hip height may affect voluntary intake seems to be a preeminent topic to be addressed in the future. In addition, the introduction of the concept of liveweight-to-hip height may bring practical benefits for the cattle industry. For instance, using the model developed in Chapter 7 is possible to obtain an estimation of the compensatory growth potential (i.e. liveweight gap) of a given animal based on its liveweight and hip height measures. This analysis provides a prediction of the liveweight that can be achieved at the end of the compensatory growth based on animal's hip height. However, it is important to notice that the liveweight-for-hip height relationship is affected by several factors such as sex and type of hormonal implant as demonstrated in Chapter 7. Therefore, in order to have a more accurate prediction it is important that the model adopted to generate the liveweight-for-hip height relationship be developed using representative data of the herd to be analysed.

Probably the most relevant aspect of this thesis was to demonstrate the importance of the impact of nutrition on endonchondral ossification in cattle. Not many studies have focused on understanding the impact of nutrition on skeletal development and its effect on cattle performance. Skeletal growth represents high nutritional demand to cattle and dictates the rate of body growth, including muscles and organs; therefore future research may aim to develop nutritional strategies to maximize

endochondral ossification (i.e. skeletal growth) in growing cattle without affecting growth efficiency by increments in fat deposition.

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