FETAL PROGRAMMING IN CATTLE: THE EFFECTS OF VARYING MATERNAL PROTEIN INTAKE IN ADOLESCENT BEEF HEIFERS ON FETAL AND POSTNATAL GROWTH AND DEVELOPMENT OF THE CALF.

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (continued)

Page

LIST OF FIGURES

Chapter 1

Figure   **Page**

Chapter 2

Chapter 3

LIST OF FIGURES (continued)

Chapter 3

Figure   **Page**

Chapter 3

Chapter 4

LIST OF FIGURES (continued)

Chapter 5

Figure **Page**

LIST OF FIGURES (continued)

Chapter 5

Chapter 6

Chapter 7

LIST OF TABLES

Chapter 2

Table   **Page**

Chapter 4

LIST OF TABLES (continued)

Chapter 5

Table   **Page**

LIST OF TABLES (continued)

Chapter 5

Table   **Page**

Chapter 6

LIST OF TABLES (continued)

Appendix 1

Table   **Page**

ABSTRACT

Numerous studies, including those in agriculturally important species, have shown that maternal nutritional status during gestation influences the fetal development, postnatal growth pathways and metabolism and reproductive development of the offspring. Wide variation in pasture quality and quantity available for the cow herd throughout the breeding season and during gestation is common in extensive grass-fed beef production systems. In the northern Australian rangelands, protein, rather than energy, is the major limiting nutrient for cattle in dry-season pastures with protein supplementation of breeding heifers an animal husbandry requirement.

Few studies in cattle have specifically evaluated the effects of maternal protein restriction and/or supplementation on the fetal and postnatal development of the offspring. This thesis investigates the effect of low and high protein intake in nulliparous beef heifers during the peri-conception and first trimester of gestation upon feto-placental growth and the subsequent postnatal growth and reproductive development of the male offspring. Furthermore, the impact of maternal nutrition on appetite, feedlot performance and carcass traits was evaluated in the male offspring.

The outcomes of the first three chapters of this thesis highlight the importance of the peri-conception period and first trimester upon fetal and placental development. We have demonstrated that protein restriction (125 % versus 70 % of requirement) during the peri-conception period and first trimester decreased early fetal growth in the bovine in a sex-specific manner in association with maternal endocrine perturbations, and may contribute to early embryonic loss. Furthermore, we show that protein restriction during the peri-conception period and first trimester alters placenta parameters and produces asynchronous organ development in the bovine by 98 days post-conception. After feeding to meet nutritional requirements (but not realimentation) during the second and third trimester, the observed differences in fetal and gross placenta parameters measured at 98 days post-

conception were not present at birth. Intriguingly, the sex-related differences apparent at 98 days post-conception and normally observed in birth weight in the neonate were disrupted. This suggests sex- specific catch-up growth to term dependent upon early gestational dietary treatment. We propose that placental adaptations and an accelerated growth response in the restricted female fetus reduced the observed differences in fetal weight apparent at 98 days post-conception after the nutritional treatment ceased, thereby disrupting the normal disparity in birth weight between heifer and bull calves. However, as indicated by the differing fetal organ measures at the end of the first trimester, similarity in birth weight does not preclude that developmental programming of the structure and/or function of the fetal organs and tissues has already occurred following nutritional stress in early gestation. On the contrary, prior studies have shown that birth weight is not a satisfactory indicator of intrauterine growth restriction and that suboptimal maternal nutrition may have lasting effects on the post-natal growth and development and health of the offspring.

The second component of this study, which forms the final two chapters of the thesis, examined the post-natal growth and reproductive development of the non-castrated male offspring through to slaughter at 598 days of age. We demonstrated in the developing bull, that low dietary protein during the peri-conception period lowered sperm quality parameters during pubertal development with a concomitant delay in age of puberty. These effects were subsequent to lower FSH concentrations in this low peri-conception group. The circulating hormone data suggest that the peri-conception diet may have altered the development of the hypothalamic–pituitary-gonadal axis and the corresponding receptivity to circulating hormones. These male offspring underwent a 70-day residual feed intake feedlot test commencing at 528 days of age. Offspring from heifers that had a change in diet at the end of the peri-conception period from high protein to low protein, and *visa versa*, had 9% daily higher feed intake on test than offspring of mothers that remained on a constant low protein diet throughout the peri- and post-conception period. Offspring liveweight, feed efficiency and carcass weight were not different among bulls, however dressing percentage, estimated

retail beef yield and eye muscle (*longissimus dorsi*) area were all lower in offspring of heifers fed a low protein diet during the first trimester.

Significantly, these findings on the sex-specific effects of peri-conceptional dietary perturbations in cattle represent the first such data in a mammalian species with ovary structure, embryonic development and gestational length similar to the human. In combination, this work represents the first study to highlight the importance of maternal dietary protein intake during the peri-conception period and first trimester upon fetal and postnatal development in beef cattle. In a pasture-based production system where variation in feed quality through breeding and gestation occurs, quantification of these effects upon long term productivity of the progeny and elucidation of the causal mechanisms remains an important area of study.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or any other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text. In addition, I certify that no part of this work will, in future, be used in a submission in my name for any other degree or diploma in any university without prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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ABBREVIATIONS

ad libutum	to any desired extent
ADG	average daily gain
AMH	anti-müllerian hormone
ANOVA	analysis of variance
BNC	binucleate trophoblast giant cells
bPAG	bovine pregnancy-associated glycoprotein
BPD	bi-parietal diameter
bPL	bovine placental lactogen
BW	body weight
cm	cm
CP	crude protein
CNL	crown-nose length
CRL	crown-rump length
CV	coefficient of variation
d	days
DM	dry matter
DMI	dry matter intake
dpc	days post-conception
EBV	estimated breeding value
ELISA	enzyme linked immunosorbent assay
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotrophin-releasing hormone
hr	hour

gd	days of gestation
g	grams
IGF1	insulin-like growth factor 1
IGF2	insulin-like growth factor 2
kg	kilograms
L	litre
LD	*longissimus dorsi*
LH	luteinizing hormone
LSD	least significant difference
NEFA	non-esterified fatty acid
m	metres
MJ	megajoule
mL	millilitre
min	minute
mo	months
mRNA	messenger ribonucleic acid
n	sample size
NEFA	non-esterified fatty acid
P	probability
PERI	peri-conception period (-60 to 23 dpc)
POST	post-conception period (24 to 98 dpc)
PRL	prolactin
RIA	radioimmunoassay
RFI	residual feed intake
SC	scrotal circumference
SEM	standard error of the mean
ST	*semi-tendinosis*

vs.	versus
wks	weeks
wt	weight
χ^2	chi-squared distribution

Chapter 1

General Introduction

CHAPTER 1: General Introduction

Introductory background

Australian beef producers in both Northern and Southern regions are challenged by the large variation in pasture quantity and quality throughout the seasons (Dixon et al., 2011). Protein is often the limiting nutrient in cattle managed under extensive grazing systems. Bortolussi et al. (2005) reported that 76% of northern Australian properties require protein supplementation of their replacement heifers and this is a common management practice. Inadequate daily energy intake is also related to a reduction in performance in cattle managed under extensive grazing systems. Australian pastures generally have adequate energy levels, but are often low in protein during both the pre-breeding period and gestation of beef cows (Norman, 1963; Dixon et al., 2011). Furthermore, dry matter intake generally falls when the crude protein (CP) level of forage falls below 7%; provision of protein supplements can improve forage intake and therefore total energy intake.

Many studies have reported in different species, including domestic animals of agricultural importance and humans, the influence of maternal nutrition on progeny performance, health and reproduction (McMillen et al., 2001; McMillen et al., 2005; Wu et al., 2006; McMillen et al., 2008). Few studies however have evaluated the effects of protein specifically in the maternal diet (Funston et al., 2010). A recent study demonstrated for the first time that varying levels of maternal protein intake during early pregnancy in cattle affects post-natal production and reproduction traits in the offspring (Sullivan et al., 2009a; Sullivan et al., 2010b; Micke et al., 2011b, a).

These effects may be exacerbated in "adolescent" heifers (mated at 14 months of age) which require nutrients for continued growth and development along with their rapidly developing fetus (Wallace et al., 2004; Wallace et al., 2006a). This is of industry relevance as

heifers are increasingly mated to calve at two years of age; a practice that is an opportunity to increase herd profitability (Nunez-Dominguez et al., 1991).

Fetal, or developmental, programming is the concept that a maternal insult during a critical window of fetal development has long term effects on the offspring (Wu et al., 2006). This concept of the intrauterine environment influencing fetal developmental pathways and inducing permanent changes in the physiology of animals post-natally has its origins in the association between low birth weight and hypertension in adulthood in humans (Barker et al., 1993). In livestock, studies have reported sub-optimal maternal nutrition during gestation can result in reduced fetal and postnatal growth, altered meat quality, metabolic disorders, increased neonatal mortality, intestinal and respiratory dysfunction and altered reproductive development (Wu et al., 2006; Reynolds et al., 2010b; Bell and Greenwood, 2016). Knowledge of how early maternal nutrition influences fetal development and subsequent postnatal performance potentially represents an opportunity for livestock producers to breed animals better suited to their production environment utilising targeted nutrition at specific stages throughout the peri-conception period and gestation.

This chapter reviews the published literature on aspects of oocyte, embryo and feto-placental development, and how these are affected by maternal nutrient intake during the peri-conception period and gestation. The postnatal consequences of altered maternal nutrient intake, with particular emphasis on aspects of offspring birthweight, skeletal muscle and adipose tissue development, appetite and reproductive development will also be reviewed. The focus will be on the bovine, with information on research in other species discussed where findings in the bovine are limited or unknown. The studies reviewed were conducted in adults unless otherwise stated. This literature review aims to provide a background for understanding the research described in this thesis.

Peri-conception period

Introduction

The peri-conception period in cattle has been defined as the period from folliculogenesis to early embryo development before implantation (Velazquez, 2015). The embryonic period encompasses the period from fertilization at 1 day post-conception (dpc) (i.e. single cell embryo or zygote) to the completion of organogenesis at 42 dpc (DesCôteaux et al., 2010; Hopper, 2014); implantation in the bovine occurring between 18 to 22 dpc (Wathes and Wooding, 1980; Spencer and Hansen, 2015b).

The period prior to implantation is recognised as an important developmental stage for mammalian embryos (Hansen, 2015; Velazquez, 2015) and maternal nutritional signals during this period may influence epigenetic remodelling of fetal genes (Gardner and Lane, 2005; King, 2016). Epigenetic modifications to DNA (and to histones) do not involve changes in the DNA sequence but may affect gene expression and are potentially heritable, thus contributing to phenotypic variation in the offspring in response to environmental stimuli (Chavatte-Palmer et al., 2016).

Maternal diet and oocyte and embryo development

The bovine has been extensively used as an experimental model for embryonic research to study the effects nutrition and reproduction during the peri-conception and embryonic period (Hansen, 2015; Velazquez, 2015). Many studies in domestic livestock have shown that the maternal diet during the peri-conception period can affect follicular development, embryo development and survival (Fleming et al., 2011). The pre-implantation embryo is extremely sensitive to its environment; the fluid in the oviduct contains nutritional, metabolic and inflammatory markers that in turn reflect the mothers external environment (King, 2016). How maternal diet influences oocyte and embryo development is not fully understood but is thought to involve differences in hormones and intermediary metabolites in the body, induced by nutrition (Ashworth et al., 2009). Armstrong et al. (2001) investigated how dietary energy

and protein influence follicular dynamics and the developmental competence of oocytes in beef x dairy heifers and found that the nutritional regulation of follicular growth is mediated, at least partly, by the actions of circulating metabolic hormones on the ovarian IGF system. However, although a high energy diet increased circulating maternal insulin and IGF1 along with increasing growth rate of the dominant follicle, high dietary protein was found to decrease oocyte quality correlated with increased plasma urea concentrations. A later study linked oocyte quality and blastocyst yield in heifers to maternal body condition, with a high level of feeding being beneficial in heifers of low to moderate condition, but not in heifers of moderate to high body condition (Adamiak et al., 2005). Thus suggesting the impact of maternal nutrition on oocyte and embryo development is not only subject to maternal body condition (Fleming et al., 2012) but also the response differs between protein and energy.

Diets high in protein increase levels of circulating blood urea nitrogen (BUN) (Elrod and Butler, 1993; Borowicz et al., 2007) which is consequently reflected in the levels of ammonia and urea in the oviduct (Kenny et al., 2002) and follicular fluid (Santos *et al.* 2009). Urea is a by-product of dietary protein metabolism by the liver. Excess dietary protein increases ammonia production in the rumen, which is metabolised to urea by the liver (Huntington and Archibeque, 2000). Ammonia negatively influences embryo and subsequent fetal development and has been demonstrated to effect gene expression and the imprinting status of specific genes (Gardner and Lane, 2005). Elevated urea concentrations in peripheral circulation have been associated with impaired fertility in dairy cows receiving diets high in crude protein (17 to 19%) (Canfield et al., 1990; Butler et al., 1996; Larson et al., 1996). A series of experiments investigating the effects of urea on oocytes fertilised *in vitro* suggest that the detrimental effects may be mediated in part through the direct effect of urea on the maturing oocyte in the follicle (Rhoads et al., 2006; Santos et al., 2009) and furthermore, demonstrated urea may hinder meiosis in cultured oocytes with subsequent effects on fertilisation. Conversely, in beef heifers, elevated plasma urea at the time of conception and in early gestation has been reported in a number of studies to have no effect on embryo quality,

survival and/or pregnancy rates (Kenny et al., 2002; Gath et al., 2012; Amundson et al., 2016). Kenny et al. (2002) concluded that the previously reported adverse effects of urea upon early oocyte development in lactating dairy cattle may result from the interaction of negative energy balance and excess dietary protein intake rather than a direct outcome of elevated blood urea concentrations, whilst Gath et al. (2012) hypothesized that the negative effects of urea may be the collective outcome of events occurring earlier during oocyte development in the ovary rather than during fertilization or post-fertilization.

The Placenta

Introduction

The placenta develops during pregnancy and is the interface between the mother and the fetus in eutherian mammals (Mossman, 1987) having both maternal (endometrium) and fetal (chorioallantois) components to its structure (Peter, 2013). This organ enables the physiological exchange of nutrients, gases, hormones and waste products and modulates maternal physiological changes to support fetal growth (Gootwine, 2004).

Placental development

The bovine placenta is non-invasive and is classified according to histology as synepitheliochorial (Wooding, 1992) and gross morphology as cotyledonary and non-deciduate (Wooding and Flint, 1994). The placenta begins to form at 18 to 19 dpc. The bovine fetal placenta attaches along the uterine wall at multiple, pre-existing sites of non-glandular uterine epithelium known as caruncles. Caruncles are arranged in two dorsal and two ventral rows along the length of the uterine horns of non-pregnant animals and appear in the developing uterus of the female fetal calf from 4 months of gestation (Atkinson et al., 1984). Placental membranes attach at these sites via fetal chorionic villi known as cotyledons that become embedded into the crypts in the caruncle (Wooding and Burton, 2008) to form a placentome (the caruncle-cotyledon unit); forming the primary area of physiological exchange

6

between mother and fetus. The number of caruncles (approximately 100) is determined before birth (Schlafer et al., 2000). However, cotyledon number is dynamic and can vary widely amongst individual cows (Laven and Peters, 2001). It is unclear how the total number of placentomes in the ruminant placenta is determined (Sullivan et al., 2009c). By 30 dpc, the placentomes are small (20 to 40 μm wide) and either flat or slightly raised. The placentomes become raised by 33 dpc and the villi and crypts are visible. The villi are considerably longer with secondary branching by 42 dpc, resulting in a more complex association between the maternal and fetal tissue (Leiser et al., 1997) thus increasing the surface area available for exchange between the mother and fetus. Early studies reported that there is no change cotyledon number after 90 dpc in the bovine but the weight and length of the placentome continues to increase (Prior and Laster, 1979). A more recent study found however that cotyledon number at term was increased by dietary protein intake in the second trimester suggesting that the number of cotyledons can be altered beyond 90 dpc (Sullivan et al., 2009c).

The uterine artery is the main supply source of nutrients and oxygen to the placenta and developing conceptus. Hence, the efficiency of the placenta in transporting nutrient is directly related to uteroplacental blood flow (Reynolds and Redmer, 1995). In early pregnancy, placental vascularisation is initiated and established, supporting the early survival of the embryo and subsequent fetal development. During the last trimester, uterine blood flow increases to support the rapid growth of the fetus. Hence, any alteration of uteroplacental blood flow may adversely impact fetal development as demonstrated by surgical restriction of fetal and placental growth in sheep through techniques such as uterine carunclectomy, placental embolisation and single umbilical artery ligation (Morrison, 2008).

In the bovine, growth of the placenta increases progressively throughout pregnancy (Vonnahme et al., 2007) although the absolute rate of increase in weight is much less than that for fetal weight in the second half of gestation. This contrasts to placental growth in sheep which follows an exponential pattern until 70 to 80 dpc before slowing significantly until

parturition (Reynolds et al., 2005). Vascularisation of the placentome in the bovine during mid to late gestation also differs to sheep (Reynolds et al., 2005; Borowicz et al., 2007). In sheep, whilst the placental weight is similar during mid to late gestation the density of the capillary area in the caruncle and cotyledon increases substantially (~ 200 and 400% respectively) (Borowicz et al., 2007). In the bovine caruncle, capillary number increases from 125 to 250 dpc whilst capillary area and size are decreased. In the cotyledon, capillary number, area and size all increase (Vonnahme et al., 2007; Funston et al., 2010). As differences exist between the ewe and the cow in both the placental growth trajectory and the patterns in placental angiogenesis, this must be considered when extrapolating experimental outcomes for ovine placental development to the bovine (Vonnahme, 2012).

Maternal diet and placental development

The placenta plays a major role in the regulation of fetal growth through the supply of nutrients and oxygen. Fetal weight near term and birth weight positively correlates with the weight of the placenta in many species, including cattle (Echternkamp, 1993; Zhang et al., 1999). The relationship between fetal weight and placental weight is suggested to represent a proxy measure of the efficiency of the placenta to support fetal growth (Fowden et al., 2009) and can be altered through changes in fetal weight, placental weight or both (Fowden et al., 2009). Furthermore, the capacity of the placenta for nutrient transfer to support fetal growth is reflected not just by placenta weight or size, but also depends on other factors including placental vascularity, uteroplacental blood flow and the abundance of nutrient transporters (McMillen and Robinson, 2005; Fowden et al., 2006; Wu et al., 2006; Reynolds et al., 2010a; Vaughan et al., 2011; Vonnahme and Lemley, 2011).

It is well established that maternal nutrient intake affects placental development and thus may impact upon the ability of the placenta to support fetal growth. Considerable evidence exists to suggest that the placenta adapts both morphologically and functionally to adverse maternal conditions to optimise substrate supply and thus survival of the fetus

(Burton and Fowden, 2012). Experimental studies show that the specific effects on placental parameters depend on the type of nutritional challenge (i.e. global restriction vs. protein) along with the severity, duration and timing of the challenge (Sferruzzi-Perri and Camm, 2016). The effectiveness of these adaptations ultimately determines the flow of substrates to the fetus; if delivery is still inadequate this may contribute to structural and functional changes in physiological systems at all levels in the fetus.

The sheep is widely used as an experimental model for placental insufficiency (Morrison, 2008). In sheep, extensive studies have shown both nutritional restriction and overfeeding during pregnancy may result in altered placental growth and/or function (Fowden et al., 2006) and consequently contribute to fetal growth restriction (Morrison, 2008). Maternal dietary treatments have been shown to reduce umbilical and/or uterine blood flow (Lemley et al., 2012) and increase arterial indices of resistance (Lekatz et al., 2012) but capillary density remains similar (Eifert et al., 2015).

In the bovine, studies are more limited but nutrient restriction in both early and mid-gestation has been reported to impact upon placental development (Perry et al., 1999; Vonnahme et al., 2007; Zhu et al., 2007; Sullivan et al., 2009c). Protein restriction in the first trimester followed by increased dietary protein in the second trimester enhanced placental development and fetal growth (Perry et al., 1999), whilst Sullivan et al. (2009c) reported that the number of cotyledons and placental weight at term were susceptible to maternal protein intake in the first and second trimester; the response however depending on heifer genotype.

Given that the development of an adequate placental-fetal vascular interface is essential for normal fetal growth (Reynolds et al., 2005), surprisingly few studies have investigated the effects of dietary perturbations on uterine and umbilical blood flow and vascular development of the bovine placenta. Vonnahme et al. (2007) demonstrated that global nutrient restriction of cows from 30 to 125 dpc did not affect measures of capillary vascularity in cotyledon or caruncular tissue at 125 dpc. Following realimentation, capillary vascularity was found to be altered at 245 dpc; an indication the placenta compensated after

nutritional restriction ceased. Total placentome weight in this study was decreased at 125 dpc following nutrient restriction and remained suppressed at 245 dpc after realimentation (Vonnahme et al., 2007; Zhu et al., 2007). However, whilst the weight of both the cotyledonary and caruncular portions of the placentome were adversely impacted at 125 dpc only cotyledonary tissue weight was decreased at 245 dpc. The two main pathways controlling angiogenesis through cell proliferation and growth were also found to be upregulated in the cotyledon but not the caruncle (Zhu et al., 2007).

The development of new technologies such as colour Doppler sonography present new opportunities to explore uteroplacental blood flow during bovine pregnancy utilising a relatively non-invasive technique (Herzog and Bollwein, 2007). To date, only limited studies on small numbers of animals have evaluated the technique's potential to monitor umbilical or uterine blood flow in the bovine (Herzog et al., 2011). A recent study in cattle examined the effect of maternal realimentation after nutrient restriction during early to mid-gestation (30 to 85 dpc) on fetal blood flow utilising the Doppler technique. Nutrient restriction did not affect umbilical blood flow during the period of restriction but following realimentation blood flow to the ipsilateral horn was increased (Camacho et al., 2014). The adaptations in the bovine following nutritional perturbations discussed in the preceding section may therefore improve substrate supply to the developing fetus through increased maternal blood flow and nutrient transport. Increased understanding of placental adaptations to suboptimal nutrition during gestation in the bovine is still required.

Placental and metabolic hormones

During a normal pregnancy, significant amounts of hormones are produced in the maternal, placental and fetal compartments. Concentrations in maternal and fetal circulations change in response to environmental factors and are able to directly and/or indirectly modulate the maternal and fetal metabolism, the growth of the placenta and nutrient transport in the placenta (Fowden et al., 2009). The placenta itself also acts as an endocrine organ regulating

fetal growth (Chavatte-Palmer et al., 2014) and maternal metabolism (Tarrade et al., 2015). The placenta may sense perturbations such as altered nutrient or hormone concentrations (i.e. IGF1 and leptin) in the maternal compartment via nutrient signalling agents and modify the nutrient supply to the fetus via the activity of nutrient transporters (i.e. amino acid transporters) in the trophoblast (Jansson and Powell, 2013). In the bovine, the placentally produced bovine placental lactogen (bPL) and bovine pregnancy-associated glycoprotein (bPAG) stimulate repartitioning of substrates to the fetus and are considered indicators of placental growth and function (Bertolini et al., 2006). The predominantly ovarian produced steroid hormone progesterone also plays a role in placental growth and function, and along with the metabolic hormones, IGF and leptin, is known to be nutritionally sensitive (Anthony et al., 1995; Sullivan et al., 2009b).

Bovine placental lactogen and bovine pregnancy-associated glycoprotein

Binucleate trophoblast giant cells (BNC) are a characteristic of the placenta in ruminants (Wooding, 1992). In cattle, BNC's form 15 to 20% of the total cell population in the fetal trophoblast, playing a key role in placental growth and remodelling through their direct involvement in modifying the uterine epithelium (Wooding, 1992). They migrate through the fetal-maternal interface to fuse with the maternal epithelium and secrete their Golgi formed cytoplasmic granules, containing the glycoproteins bPL and bPAG, into the maternal circulation (Wooding, 1982). These proteins stimulate repartitioning of maternal nutrients to the fetus (Bertolini et al., 2006) and concentrations of these proteins in maternal circulation are considered to reflect the growth and remodelling activity of the trophoblast (Breukelman et al., 2005).

Bovine placental lactogen (bPL): The glycoprotein hormone bPL is a member of the growth hormone (GH) / prolactin (PRL) gene family (Nakaya et al., 2009) and has both somatogenic (GH like) and lactogenic (PRL like) biological activity (Byatt et al., 1992; Takahashi, 2006).

Bovine placental lactogen is considered to play a role in fetal growth, stimulating repartitioning of maternal nutrients to the fetus and modulating fetal substrate use (Byatt et al., 1992; Anthony et al., 1995; Bertolini et al., 2006; Takahashi, 2006). It is thought that bPL may act through components of the IGF system as the main stimulus of IGF during fetal development; correlations between bPL and IGF have been observed in cattle (Anthony et al., 1995; Hossner et al., 1997; Weber et al., 2007).

Bovine placental lactogen is only produced during pregnancy and is secreted into both fetal and maternal circulation (Gootwine, 2004). It is not detected in maternal peripheral circulation until ~ 90 to 110 dpc following the attachment of the trophoblast to the endometrium (Bolander et al., 1976; Guilbault et al., 1988). In the early to mid-stages of gestation, bPL is preferentially secreted into the fetal circulation, peaking at mid gestation (5 to 25 ng/mL). This corresponds to the phase of maximum fetal growth when perhaps it exerts a growth promoting effect (Byatt et al., 1992; Wallace, 1993; Patel et al., 1996). Fetal bPL concentrations then plateau or gradually decline (Bolander et al., 1976; Byatt et al., 1992).In comparison, maternal bPL concentrations in the bovine are lower but increase during gestation, peaking between 200 to 232 dpc (Bolander et al., 1976; Wallace, 1993; Hossner et al., 1997) before plateauing and then declining just prior to term (Wallace, 1993). This coincides with the rapid decline in BNC number known to occur prior to parturition (Wooding, 1992). In comparison to the cow, maternal concentrations of PL are 100 to 1000-fold higher in sheep and goats (Byatt et al., 1992).

Placental lactogens in sheep and cattle share common features in their synthesis along with their lactogenic and somatogenic properties (Byatt et al., 1992). However, species-specific differences exist in structure, abundance and glycosylation (and therefore molecular weight) (Byatt et al., 1992) and it has been suggested that subsequently PL may have different functions between the species.

Factors regulating the secretion and function of bPL in cattle are not precisely understood (Takahashi, 2006) but placental mass (weight) and nutrition may play a role

(Byatt et al., 1992). Relationships with bPL have been shown to exist with fetal weight (Bertolini et al., 2006), calf birthweight (Guilbault et al., 1988; Patel et al., 1996; Hossner et al., 1997), placental weight (Bertolini et al., 2006), milk production (Erb et al., 1980; Patel et al., 1996) and breed of the calf sire/dam (Guilbault et al., 1988; Hossner et al., 1997). It is thought bPL may also act as a luteotropin in the cow (Anthony et al., 1995) and has been associated with increased corpus luteum size and consequently increased plasma progesterone concentrations (Lucy et al., 1994).

In cattle, nutritional restriction during the second trimester, at levels sufficient to alter body condition score to thin, resulted in elevated bPL concentrations later in pregnancy compared to cows of moderate condition (Rasby et al., 1990). Similarly, heifers fed a low protein diet in early gestation had increased plasma bPL concentrations in the second trimester (Sullivan et al., 2009b). Concentrations of bPL in the second trimester tended to be correlated with calf birthweight but not placental weight.

Bovine pregnancy-associated glycoproteins: Pregnancy-associated glycoproteins (PAGs) are a large family of aspartic proteinase molecules (Xie et al., 1991) that are secreted by the BNC of the placenta similar to bPL (Wooding, 1992). They are restricted to the placenta of even-toed ungulates with a number of different PAG genes identified in the bovine (bPAG's) (Wallace et al., 2015). Pregnancy-associated glycoproteins are abundantly expressed in the placenta and are released during the fusion of the BNC with uterine epithelial cells in the early implantation period (Wathes and Wooding, 1980). The precise role of bPAG's is unknown (Wallace et al., 2015) however it is speculated that they may initially play a role in the attachment of trophoblasts to the endometrium (Wooding et al., 2005). During pregnancy, bPAG is thought to have an ongoing role in feto- placental growth and development (Wooding et al., 2005; Hashizume et al., 2007; Wallace et al., 2015), with changes in PAG glycosylation pattern at different stages of gestation (Klisch et al., 2006) postulated to potentially indicate specific functions including immunomodulatory activity (Dunbar et al.;

Wooding et al., 2005) although this remains unclear (Klisch et al., 2006). Experimental evidence is also suggestive of a luteotrophic role in both sheep (Weems et al., 2003) and cattle (Del Vecchio et al., 1996; Wallace et al., 2015).

Bovine pregnancy-associated glycoproteins can be detected in the maternal circulation beginning at approx. 24 dpc (Green et al., 2005; Wallace et al., 2015). Secretion of bPAG follows a biphasic pattern; the first peak corresponds to the initial formation of cotyledons (a rapid phase of placental growth) with the second major peak in the third trimester when the transport of substrates in the placenta increases to meet the demands of rapid fetal growth (Green et al., 2005). Maternal bPAG levels peak immediately before parturition (Zoli et al., 1992; Green et al., 2005) coinciding with de-granulation of BNCs in the last 10 days of pregnancy associated with placental maturity (Schlafer et al., 2000). Maternal plasma bPAG concentrations are correlated to the stage of gestation (Zoli et al., 1992; Breukelman et al., 2005) and have been used to monitor embryonic or fetal viability, placental function and for early diagnosis of pregnancy (25 to 30 dpc) in cattle (Sasser, 1986; Zoli et al., 1992; Green et al., 2005; Perry et al., 2005; Pohler et al., 2013; Wallace et al., 2015; Pohler et al., 2016)

Relationships have been demonstrated between maternal bPAG concentrations during gestation with a range of factors including breed, weight and parity status of the dam, calf birthweight, fetal sex and fetal number (Zoli et al., 1992; Mialon et al., 1993; Patel et al., 1997; Breukelman et al., 2005; Echternkamp et al., 2006; Mercadante et al., 2013). In sheep, correlations exist between ovine PAG and placental weight, and are influenced by maternal nutrition (Wallace et al., 1997). In cattle, low dietary protein intake in the first trimester in beef heifers increased circulating bPAG, which the authors ascribed to an increase in placental function (Sullivan et al., 2009b). Once this dietary perturbation ceased, however, the relationship between nutritional treatment and placental function was no longer observed. In contrast to previous studies, no relationships existed between bPAG concentration and birth weight, fetal sex or genotype.

Insulin –like growth factors (IGFs): Insulin –like growth factors (IGFs) are single chain polypeptides produced by the placenta and maternal and fetal tissues (Gicquel and Le Bouc, 2006). The IGFs influence fetal growth acting in an autocrine, endocrine and paracrine manner and have been shown to affect aspects of both placental morphology and function with subsequent effects on placental efficiency (Fowden, 2003; Sferruzzi-Perri et al., 2006) The IGFs (IGF1 and IGF2) are regulated by the IGF-binding proteins (IGFBP's) and they interact to modulate fetal growth (Sferruzzi-Perri et al., 2006). IGF1 regulates fetal growth in response to nutrient availability whilst IGF2 stimulates placental growth and differentiation (Fowden, 2003; Sferruzzi-Perri et al., 2011). The maternal IGF system has been reported to be modulated by many factors including dietary protein (Perry et al., 2002) and endocrine signals (Osgerby et al., 2002) including those produced by the placenta associated with fetal development (Guilbault et al., 1988; Bertolini et al., 2006). In turn, the maternal IGF system has been shown to play a role in modulating placental growth and development, influencing the nutrient partitioning between conceptus and maternal tissues (Sferruzzi-Perri et al., 2006; Sullivan et al., 2009d; Sferruzzi-Perri et al., 2011; Sferruzzi-Perri et al., 2017). There are at least six different IGFBP's (Sferruzzi-Perri et al., 2011; Bach, 2015) that modulate the IGF's action at the target cell level. The IGFBP's are thought to act in the plasma as transport proteins; modulating IGF's access to their receptors in addition to regulation of the metabolic clearance of IGF in the circulation (Silva et al., 2009; Sferruzzi-Perri et al., 2011; Bach, 2015).

In early pregnancy in the bovine, there is a significant rise in maternal peripheral concentrations of IGF1 and IGF2.The increase in IGF1 concentration has been reported to vary dependent on maternal nutrition. Concentrations of IGF2 are less dependent on maternal diet, increasing sharply in the second trimester before falling at the end of gestation (Perry et al., 2002; Sullivan et al., 2009d). Differences exist in the IGF1 and IGF2 concentration profile between species. In contrast to the bovine, concentrations remain high in guinea pigs and humans during late pregnancy (Sferruzzi-Perri et al., 2006; Sferruzzi-Perri et al., 2007). IGF

receptor concentrations and the abundance of IGFBP's are also reported to be influenced by the stage of gestation (Wathes et al., 1998; Sullivan et al., 2009d).

Nutrient intake has been found in a number of studies to be directly related to plasma IGF concentrations (Yelich et al., 1996) in mature (Richards et al., 1991) and primiparous beef cows (Ciccioli et al., 2003) and in beef heifers (Yelich et al., 1996). Postpartum protein supplementation was found not to alter plasma concentrations of IGF1 either during or after the feeding period (Lents et al., 2008) but concentrations tended to be greater in fatter cows .

The pattern of IGF changes in pregnancy can also be modulated by nutrition. In cattle, restriction of maternal nutrition reduces peripheral levels of IGF (Houseknecht et al., 1988; Perry et al., 2002; Lents et al., 2005; Sullivan et al., 2009d). This response in both the pregnant and the post-partum bovine however appears to vary dependent on the balance of protein and energy in the diet (Houseknecht et al., 1988; Spicer et al., 1991; Lents et al., 2008; Sullivan et al., 2009d).

Fetal growth in cattle has been shown to be negatively influenced by maternal IGF concentrations (Hossner et al., 1997; Sullivan et al., 2009d). Sullivan and associates reported that fetal-crown rump length was negatively associated with maternal IGF1 whilst cows pregnant to bulls with genetically high expected birth weights had reduced levels of circulating IGF1 (Hossner et al., 1997). Furthermore, fetal calf weight was found to be negatively correlated with maternal IGF1 concentrations. There are conflicting reports in the literature on the relationships between bPL and IGF1 in the bovine; Hossner et al. (1997) reported a negative relationship whilst Weber et al. (2007) demonstrated a positive relationship.

Progesterone: The steroid hormone progesterone plays a role in placental growth, differentiation and function (Hoffmann and Schuler, 2002). Furthermore, progesterone has a pivotal role in the establishment and maintenance of pregnancy (Spencer et al., 2007; Lonergan, 2011; Lonergan and Forde, 2015) through modulation of the luteolytic signal,

regulation of conceptus growth and development, and the production of interferon-tau (Chagas e Silva and Lopes da Costa, 2005). It is well established that circulating progesterone concentrations depend on the quality of the corpus luteum and are influenced by dietary intake, energy balance and liver metabolism (O'Callaghan and Boland, 1999). The corpus luteum is the main source of circulating progesterone throughout gestation in the bovine (Chew et al., 1979; Hoffmann and Schuler, 2002) with placental contribution only minor (Ferrell, 1991) and restricted to late in gestation (Chew et al., 1979). Elevated progesterone early post-estrus induces changes in the uterine environment that promote conceptus elongation (Lonergan and Forde, 2015) whilst progesterone directly affects the early growth and development of the bovine conceptus (Garrett et al., 1988). Inadequate levels have been associated with increased levels of early embryonic loss in dairy cattle (Lonergan, 2011; Lonergan et al., 2016) whilst progesterone concentrations are reported to be positively associated with bovine placental weight (Guilbault et al., 1988) but not calf birth weight (Guilbault et al., 1988; Hoffmann and Schuler, 2002) or calf sex (Echternkamp, 1993).

Circulating progesterone levels are also regulated by the rate of metabolism of progesterone primarily in the liver due to changes in liver blood flow and dry matter intake (Wiltbank et al., 2006; Wiltbank et al., 2014) as demonstrated by negative associations in sheep (Parr et al., 1993) and lactating dairy cattle (Wiltbank et al., 2006). In beef heifers, the effects of adequate vs. restricted intake on circulating progesterone levels appear more variable (Spitzer et al., 1978; Villa-Godoy et al., 1990; Kenny et al., 2002). Heifers in negative energy balance have been reported to have lower progesterone levels (Villa-Godoy et al., 1990), however progesterone may also be released during mobilisation of adipose tissue in periods of low intake (Boland et al., 2001). Progesterone concentrations increased in heifers fed a diet high in protein during early and mid-gestation (Sullivan et al., 2009b) and concentrations were correlated with placental function in late gestation (Sullivan et al., 2009b).

17

Collectively this demonstrates that endocrine regulation of the placenta, therefore, depends on the changes of multiple hormones in the maternal, placental and fetal compartments with many of these changes occurring interdependently. The body of evidence discussed in the proceeding section indicates that further studies are required to fully understand how the bovine placenta adapts and compensates for adverse maternal nutrition.

Fetal development

Fetal growth is influenced by maternal and fetal genotype, maternal factors, uterine environment and maternal and fetal hormones (Wu et al., 2004). The growth of the bovine fetus follows an exponential pattern (Prior and Laster, 1979) with fetal weight gain reaching a peak between 230 dpc (dairy) (Eley et al., 1978) and 232 dpc (beef) (Prior and Laster, 1979) before declining to less than 100g/day (male and female fetuses) by parturition (Eley et al., 1978). Growth of individual tissues in the fetus, regardless of type, primarily occurs through an initial period of cellular hyperplasia (increase in the number of cells) followed by cellular hypertrophy (increase in the size of cells). Hyperplasia is reported to continue throughout gestation in the bovine fetus (Prior and Laster, 1979) declining rapidly near gestation, whilst hypertrophy (as indicated by protein/DNA and RNA/DNA ratios) continues to increase.

Fetal organ formation (organogenesis) occurs simultaneously with placental development in early gestation (Funston et al., 2010). In the bovine, organogenesis is complete by 42 dpc (Hopper, 2014) with a heartbeat visible from 21 to 22 dpc followed by a sequential development of other organs (Hubbert et al., 1972; Funston et al., 2010). Seventy-five percent of the growth of a ruminant fetus occurs during the last 2 mo of gestation (Robinson et al., 1977). The minimal nutrient requirements for fetal development in early gestation, led historically to considerable research effort focusing on the effects of nutrition in the bovine in the last trimester. However, during early gestation adequate maternal nutrition is critical for establishing normal development of all organs and tissues in the fetus (Reynolds and Redmer, 1995). The growth trajectory of individual tissues in the developing fetus differs,

with maximum periods of growth occurring at different stages of gestation (Godfredson et al., 1991). The variation in the growth trajectories of each of these tissues suggests that each tissue is vulnerable to insults, such as maternal undernutrition, at critical windows of development (Redmer et al., 2004). Moreover, a hierarchy is thought to exist under adverse conditions with partitioning of nutrients favouring the development of certain organs or systems in the fetus important for immediate survival i.e. brain, heart and liver (McMillen et al., 2001). By comparison, muscle development is thought to be particularly vulnerable due to its lower priority in nutrient partitioning (Zhu et al., 2006).

Maternal diet and fetal development

The major substrates for fetal growth are oxygen, glucose, amino acids and lactate; a reduction in substrate supply can cause alterations or reductions in fetal growth (Bell and Ehrhardt, 2002). Information on the specific nutrient requirements of the bovine fetus and the influence of maternal diet on organogenesis is limited compared to considerable research undertaken in the ovine fetus (Hopper, 2014). As in other species, experimental studies have shown that normal bovine fetal growth, including organogenesis, can be disrupted by maternal nutrition (Long et al., 2009; Meyer et al., 2010; Micke et al., 2010b; Mossa et al., 2013). The reported effects vary depending on the severity and duration of the nutritional perturbation, and the age of the dam. Development of fetuses that experience intrauterine growth restriction (IUGR) can be characterised by symmetrical or asymmetrical growth patterns (Platz and Newman, 2008). Whilst symmetrical growth (where all organs and tissues are reduced in size) has been linked to genetic conditions, asymmetrical growth is thought to be caused by a sub-optimal maternal or placental environment (Platz and Newman, 2008). Asymmetrical fetal growth restriction, whereby body weight is reduced more than crown-rump length or girth, can be characterised by the preservation of vital organs (such as the brain) despite an overall decrease in fetal body size (Greenwood et al., 1999; McMillen et al., 2001). These relationships have been extensively studied in sheep, and as in humans (Platz

and Newman, 2008), the relative growth of key organs show differential patterns as fetal body weight decreases (McMillen et al., 2001). In cattle, protein restriction of mature heifers from 0 dpc resulted in altered fetal growth (measured by ultrasound) from as early as 39 dpc (Micke et al., 2010b). In a study with a fetal endpoint, global nutrient restriction between 30 and 125 dpc (Long et al., 2009) was found to markedly influence early fetal development, reducing fetal weight and abdominal circumference along with absolute heart, liver, lung and brain weight at 125 dpc in female IUGR fetuses from 3.5 yr old dams. When corrected for fetal weight, fetal brain weight was reported to be increased. However these effects were not observed in nutrient restricted fetuses from mature dams (Long et al., 2009). Following realimentation, organ weights at 245 dpc in a further subset of fetuses were all similar. In the kidneys, glomerular number and glomeruli per gram of tissue were found to be reduced in the 245 dpc fetus despite similar size and weight. As reported above cotyledonary weight and caruncle weight were reduced in the nutrient restricted IUGR cohort at 125 dpc (Vonnahme et al., 2007; Zhu et al., 2007); potentially this may have reduced placental nutrient transport capacity as previously observed in adolescent sheep (Wallace et al., 2002a; Wallace et al., 2002b). After realimentation from 125 to 245 dpc, placental adaptations occurred thereby increasing blood flow and nutrient transport with concomitant compensatory growth of the fetus (Long et al., 2009). Furthermore, the younger dams were hypothesised to be more susceptible to nutrient restriction as cattle continue to grow, albeit slowly, until reaching mature size at about 4 years of age (Arango et al., 2002).

Female offspring of heifers that experienced relatively mild nutrient restriction (60 vs. 120% of maintenance; 11 d prior to conception until 110 dpc) had enlarged aortas at 94 dpc and at slaughter at 95 wks of age concomitant with increased arterial blood pressure despite similar fetal size, placental weights, birthweight and postnatal growth rates (Mossa et al., 2013). Fetal gastro-intestinal tissue development has also been shown to be responsive to global maternal nutritional restriction (30 to 125 dpc followed by realimentation) including altered proliferation and vascularisation (Meyer et al., 2010). These changes were

hypothesised to potentially contribute to differences in postnatal growth, survival and efficiency in calves which have experienced growth restriction *in utero*. The repartitioning of nutrients under growth restrictive environments to support fetal development has been demonstrated in many species to have long term effects on post-natal growth pathways and metabolism including development of hypertension, insulin resistance and increased adiposity (McMillen et al., 2001; McMillen and Robinson, 2005; Hanson et al., 2011).

Calf birthweight

Pre-natal development has been described by many studies as a contributing factor in neonatal survival. Many of the causes associated with increased mortality and morbidity in farm animals are, in turn, consequences of the pre-natal environment (Sinclair et al., 2016) including premature birth (Bloomfield et al., 2003), low birthweight (Morris et al., 1986), dystocia (Hickson et al., 2006; Zaborski et al., 2009), altered neonatal behaviour (Kleemann et al., 2015) and poor adaptation to post-natal life i.e. altered immunity or thermoregulation (Carstens et al., 1987; Holland and Odde, 1992).

Dystocia is one of the main causes of calf mortality at parturition in cattle [see reviews (Hickson et al., 2006; Arnott et al., 2012)] and has also been linked to an increased risk of postnatal mortality and morbidity (Rice, 1994). The incidence of dystocia in nulliparous beef heifers is higher than in mature cows (Philipsson, 1976; Morris et al., 1986) with fetal-maternal disproportion the major contributor to dystocia in heifers (Philipsson, 1976; Rice, 1994). Calf birthweight (Arthur et al., 2000) and heifer size (Zaborski et al., 2009) are considered the primary factors causing fetal-maternal disproportion.

The immaturity of the adolescent heifer has been found to influence calf birthweight, suggesting the growing heifer and rapidly developing fetus compete for nutrients during pregnancy. The birthweights of first parity progeny are generally lower compared to the offspring born to mature cows (Bellows and Short, 1978). However, the parity of the dam has been reported to have no effect on fetal growth rate when heifers were mated at a more mature

bodyweight at 21 mo of age compared to 15 mo of age (Tudor, 1972). This suggests that the maturity of the heifer rather than parity may influence fetal growth (Wu et al., 2006).

Maternal diet and calf birthweight

Altered calf birthweight following maternal dietary perturbations has been described by a number of studies (Tudor, 1972; Bellows and Short, 1978; Rasby et al., 1990; Cafe et al., 2006; Funston et al., 2010). As previously discussed, calf birthweight is directly affected by placental size and functional capacity; both factors that are influenced by maternal nutrient intake during gestation (Rasby et al., 1990; Reynolds and Redmer, 1995). However, reports on the effects of maternal nutrient restriction and/or excess nutrition during gestation upon calf birthweight are conflicting which may reflect differences in age and parity of the dams studied, nutritional regimens and/or experimental design (Holland and Odde, 1992; Hickson et al., 2006; Micke et al., 2010b).

Early studies on the effects of maternal diet on bovine fetal development and calf birthweight focused predominately on interventions during late gestation (Tudor, 1972; Bellows and Short, 1978; Anthony et al., 1986; Rasby et al., 1990; Holland and Odde, 1992); this period corresponding to the most rapid increase in fetal weight (Prior and Laster, 1979). Dietary perturbations imposed only in early gestation appear to consistently result in similar birthweights at term (Long et al., 2010c; Mossa et al., 2013) compared to dietary interventions during the second and/or third trimester where the birthweight responses are variable (Corah et al., 1975; Cafe et al., 2004; Stalker et al., 2006; Micke et al., 2010b; Shoup et al., 2015). This variability in birthweight responses to dietary treatments in later gestation is thought to be linked to the treatment severity as well as the body reserves of the dam and thus her capacity to adapt and buffer the neonate from the effects of the dietary treatment on nutrient supply (Robinson et al., 2013).

The influences of specific dietary nutrients (i.e. energy source and protein) have also been explored in relation to calf birthweight (Larson et al., 2009; Micke et al., 2010b; Radunz

et al., 2010; Summers et al., 2015b). Protein supplementation in mid to late gestation has been reported to have no effect on birthweight (Anthony et al., 1986; Stalker et al., 2006; Martin et al., 2007; Summers et al., 2015b) whilst birthweight increased moderately following protein supplementation in the second trimester in heifers (Micke et al., 2010b), and after supplementation in late gestation to cows grazing winter pasture (Larson et al., 2009).

Importantly, studies have shown that even when birthweight is similar, maternal nutrition may have already adversely impacted fetal development at an earlier stage of gestation with potentially long-term consequences for the progeny as reported in many species (McMillen et al., 2001; McMillen and Robinson, 2005) including sheep (Ford et al., 2007; Jaquiery et al., 2011) and cattle (Martin et al., 2007; Larson et al., 2009; Lamb et al., 2010; Mossa et al., 2013).

Post-natal growth

Post-natal growth pathways and body composition are important drivers of economic outcomes in the production of meat animals. Decreased growth, reduced muscling or increased adiposity are all considered detrimental outcomes in livestock production systems but have been shown in domestic animals of agricultural importance to be influenced by nutrition during gestation (Wu et al., 2006; Greenwood and Cafe, 2007; Funston et al., 2010; Funston and Summers, 2013; Bell and Greenwood, 2016).

Maternal diet and post-natal growth

Evidence in sheep indicates that maternal undernutrition in mid to late pregnancy can alter the early postnatal growth trajectory of the progeny. By contrast unless dietary treatments imposed in early pregnancy are severe or prolonged, the effects upon birthweight and the post-natal growth trajectory in sheep are limited (Kenyon and Blair, 2014; Bell and Greenwood, 2016). Singleton and twin progeny of ewes fed to achieve weight loss of 10 to 15% of bodyweight between 60 d before conception and 30 dpc had similar birthweights and

weaning weights (Jaquiery et al., 2011). In the adult offspring at 3 to 4-years, there were no long- term effects on post-natal growth but males exposed to undernutrition were fatter (Jaquiery et al., 2012)

In cattle, long-term severe maternal undernutrition from 80 dpc to term and/or through lactation resulted in fetal growth retardation, reduced birthweight and lower bodyweight until slaughter at 30 mo (Cafe et al., 2006; Robinson et al., 2013). In comparison, shorter periods of dietary interventions in mid to late gestation have resulted in variable outcomes on post-natal bovine growth (Robinson et al., 2013). Maternal protein restriction during the second trimester in beef heifers reduced birthweight and very early post-natal growth (Micke et al., 2010a; Micke et al., 2010b). Steers from this study were subsequently heavier from 191 d to slaughter at 657 d but heifer siblings were lighter from 552 d until slaughter. However, in multiparous beef cows, nutrient restriction in early to mid-gestation (70% NRC; 45 to 185 dpc) with, and without, protein supplementation, did not alter birthweight or bodyweight at slaughter.

Body composition - muscle and adipose tissue development

Skeletal muscle

Skeletal muscle has a lower priority in nutrient partitioning which increases its vulnerability to altered maternal nutrition (Zhu et al., 2006). The fetal period is critical for muscle development as it is believed there is no net increase in the number of muscle fibres after birth (Zhu et al., 2004) with post-natal muscle growth occurring through hypertrophy (increase in fibre size) rather than increased fibre number (Buttery et al., 2000). Myocytes, adipocytes, and fibroblasts originate from a common progenitor, the mesenchymal stem cells, during early embryogenesis (Maltin et al., 2001; Biressi et al., 2007; Du et al., 2013). Mesenchymal stem cells are capable of committing to different cell lineages. During muscle development they commit to the myogenic cell lineage and become myoblasts through the expression of

regulatory factors including transcription factors paired box 3 (*Pax3*) and *Pax7* (Biressi et al., 2007; Messina and Cossu, 2009). In the absence of *Pax3* and *Pax7*, muscle development is arrested and only the embryonic myotome muscle forms (Relaix et al., 2005). Subsequently the proliferating myoblasts differentiate to form myotubes during primary myogensis. In the bovine, a limited number of primary muscle fibres form during embryonic development (Russell and Oteruelo, 1981; Du et al., 2010). Secondary myogenesis overlaps with primary myogenesis beginning around 90 dpc in the bovine; precursor cells surrounding primary myofibres continue to proliferate before fusing to form secondary myofibres (Robelin et al., 1991). The majority of muscle fibres form during secondary myogenesis, only limited muscle fibres form after approximately 7 mo of gestation and growth of the muscle occurs due to an increase in muscle size and length (Du et al., 2010).

Maternal diet and skeletal muscle development

Any alteration to the proliferation of the myogenic precursor cells impacts upon the formation of muscle fibre numbers with long-term effects on muscle growth (Zhu et al., 2006). Nutrient restriction of ewes from 29 to 78 dpc (50% of NRC requirement) reduced the ratio of secondary to primary fibres in fetal lambs at 78 dpc (gestation length in sheep being approx. 145 d). This occurred in association with the downregulation of the mechanistic target of rapamycin (mTOR) in the nutrient restricted fetal muscle; mTOR plays a known role in sensing of nutrient availability for muscle growth (Bodine et al., 2001). Maternal nutrient restriction of multiparous cows (60% of NRC requirement; 30 dpc to 85 or 140 dpc with realimentation) reduced myogenic progenitor cell density and fetal IGF1 expression. Realimentation supported some compensatory growth of the fetal muscle dependent on the duration of restriction (Gonzalez et al., 2013). Micke et al. (2011b) reported phenotypic changes (namely an increase in the ultrasound measure of the cross-sectional area of the ST and LD muscle in male progeny between 65 and 462 d) resulting from the variation in maternal protein intake during the first trimester in primiparous heifers, were associated with

greater expression of IGF1 and IGF2 mRNA in ST muscle at 680 d. These effects were sex-specific and were proposed to represent a compensatory response to the low nutrient environment. Expression of IGF1R mRNA in ST of all progeny was also greater following exposure to maternal diets low in protein during the second trimester.

The effects of the impact of varying maternal intake in pregnancy on skeletal muscle development in the bovine are similar to those reported in studies of maternal nutrient intake undertaken in sheep (Costello et al., 2008; Quigley et al., 2008). Intriguingly, evidence from prior studies in the sheep suggests that the effects of lowering maternal nutrient intake on fetal skeletal muscle development begin during the peri-conception period; maternal nutrient restriction to 60% of metabolic requirements from 89 d before conception to 133 dpc decreased the weights of individual fetal skeletal muscles by 19 to 23.5% at 133 dpc (Quigley et al., 2005a) whilst restriction to 50% of requirement from 6 d before conception to 7 dpc increased fibre diameter of secondary fibres at 90 dpc without affecting fibre type (Sen et al., 2016). Furthermore, maternal undernutrition during the peri-conception period resulted in changes to expression of mRNA and/or protein abundance of factors that regulate myogenesis and protein synthesis in muscle of the sheep fetus during the late gestation (Lie et al., 2015). Skeletal muscle in vertebrates forms from the mesoderm layer of the embryo (Maltin et al., 2001) with the majority of muscle fibres forming between 60 dpc and 210 dpc in the bovine (Du et al., 2010); the effect of peri-conception diet on muscle fibre number and size in cattle is unknown.

Adipose tissue

Fetal adipocyte development begins in early to mid-gestation but the majority of fetal adipose tissue is not deposited until late pregnancy (Symonds et al., 2007). The initiation of adipose tissue formation occurs slightly later than primary myogenesis. The commitment of progenitor cells to the adipose cell lineage overlaps with secondary myogenesis (Muhlhausler et al., 2007b; Bonnet et al., 2010). The major stage of adipocyte formation occurs late in

gestation to early weaning stages (~ 250 d). Adipocytes are detected firstly in visceral depots, then subcutaneous followed by the formation of intramuscular fat (Du et al., 2013). The number of adipocytes is fixed at adolescence; the number and size of the adipocytes determines fat mass (Spalding et al., 2008).

Maternal diet and adiposity

There are a limited number of studies that have investigated the effects of maternal undernutrition on post-natal adiposity in the bovine. In the same study described above, maternal nutrient intake during gestation had permanent sex and depot-specific effects on the expression of adipogenic and adipoctytokine genes and offspring adiposity assessed at 680 d (Micke et al., 2011a). Exposure in the first trimester to a high protein maternal diet resulted in a decreased IGF1R mRNA expression in subcutaneous fat in all progeny but was associated with an increase in the depth of rump fat in males. Conversely, leptin mRNA expression in omental fat and IGF2 mRNA in perirenal fat of all progeny was decreased following exposure to high protein diets during the second trimester. Exposure in the second trimester to a high protein maternal diet also increased IGF1R mRNA in perirenal and omental fat in all progeny, leptin mRNA in perirenal fat in males and IGF2 and IGF2R mRNA in the omental fat of all progeny. Similarly, in the study reported by Long et al. (2012), steer progeny of cows nutrient restricted between 45 to 185 dpc also showed depot-specific effects but the effects were not sex-specific. Adipocyte size was increased by nutrient restriction (with or without protein) and yield grade was reduced.

The causal mechanisms of these effects are unclear; the increased adipocyte size and reduced muscle mass may contribute to increased adiposity and an altered metabolism in later life (Zhu et al., 2006; Du et al., 2010; Long et al., 2012) as observed in other species. However, it is not clear to what extent these effects represent the effects of maternal nutrient intake on the early myogenic and adipogenic cell lineages in fetal life and to what extent they

represent effects on the neuropeptide system in the developing hypothalamus which regulates energy intake in postnatal life (McMillen et al., 2007).

Appetite

Early gestation is an important period of development for the programming of appetite and voluntary food intake. Food intake allows the post-natal animal to meet its requirements for basal metabolism, growth, reproduction and fat deposition. Glucose, insulin, leptin and ghrelin act through a range of mechanisms in the hypothalamus to regulate appetite and thereby energy balance (Cone et al., 2001; Williams et al., 2001). Appetite, and thus voluntary food intake, is controlled by the arcuate nucleus (ARC), part of the hypothalamus. The ARC integrates diverse peripheral signals through neurons that terminate beyond the brain-blood barrier near the carotid sinus, in contact with the circulation (Cone et al., 2001). The ARC has two important populations of neurons that produce appetite-stimulating (orexigenic) neuropeptides and appetite-suppressing (anorexigenic) neuropeptides. The orexigenic neuropeptides, mainly neuropeptide Y (NPY) and agouti-related peptide (AGRP) stimulate feeding behaviour. The anorexigenic neuropeptides, mainly pro-opiomelanocortin (POMC) gene products and cocaine- and amphetamine-regulated transcript (CART), inhibit feeding when activated (Grove and Smith, 2003; Muhlhausler et al., 2006). The two major neuronal pathways are regulated by feedback from signals of the animals' nutritional status through peripheral levels of circulating metabolites and metabolic hormones, including leptin and ghrelin (Cone et al., 2001). The brainstem also functions in the control of feeding behaviour via the caudal nucleus of the solitary tract (NTS) which integrates gastrointestinal, circulatory and central cues (Berthoud, 2004; Minor et al., 2009).

Maternal diet and appetite regulation

The appetite-regulating neural and feedback pathways develop in early fetal life. The hypothalamus is distinguishable by the end of the first trimester and gene expression of

neuropeptides has been reported in fetal sheep from as early as mid-gestation (81 dpc) (Muhlhausler et al., 2004; Muhlhausler et al., 2005; Adam et al., 2008). Subsequently the development of the neuronal pathways may be susceptible to nutritional challenges *in utero* with the persistence of such changes contributing to the programming of an altered appetite in adulthood; this hypothesis forming the foundation for investigations into the fetal origins of human obesity (Ong and Muhlhausler, 2014).

Multiple experimental models investigating developmental programming have been shown to result in altered postnatal appetite in the offspring. Many of the studies examining the programming of adult appetite phenotype through altered maternal nutrition focus on outcomes in laboratory species. In altricial species, the hypothalamus is relatively immature at birth (Muhlhausler et al., 2004). Hence, extrapolation of experimental outcomes in rodents to livestock species must account for the distinct developmental differences (Sinclair et al., 2016).

In sheep, expression of appetite-regulating neuropeptides has been shown to be altered following infusion of glucose into fetal lambs between 130 and 140 dpc (Muhlhausler et al., 2005), increased levels of maternal nutrition from 115 dpc (Muhlhausler et al., 2006) and early maternal undernutrition from 4 dpc (and subsequent realimentation) (Adam et al., 2015). In the study by Adam et al. (2015), changes were normalised by realimentation and furthermore, males in this study tended to have greater orexigenic gene expression compared to the females. The persistence of such changes in hypothalamic gene expression in the postnatal sheep has been variable (Muhlhausler et al., 2006). Furthermore, ovine studies on voluntary food intake in progeny that have experienced prenatal nutritional restriction or excess have also shown variable results dependant on the age when appetite was assessed, the maternal dietary regimen and the post-natal management of the animal (Muhlhausler et al., 2006; De Blasio et al., 2007).

In cattle, only a small number of studies have reported on progeny feed intake and feed efficiency following maternal dietary interventions. Feed efficiency in cattle can be

explored through feed conversion ratios (feed consumed: weight gained), the efficiency of gain or residual feed intake (RFI; or net feed intake (NFI) as it is referred to in Australia) (Arthur et al., 2004). Residual feed intake is the difference between actual dry matter intake (DMI) and expected DMI based on bodyweight and growth rate (Arthur et al., 2004). Variation in RFI is thought to stem from a range of mechanisms including feeding behaviour, body weight and composition, feed intake, rate of average daily gain (ADG), metabolism, physical activity and heat increment (Herd and Arthur, 2009). Heifers from cows that grazed dormant winter pasture supplemented with CP in late gestation and fed hay in a drylot during early lactation had a higher DMI and RFI than non-supplemented but were not different to those from cows (supplemented and non-supplemented) that grazed improved pasture in early lactation (Martin et al., 2007). This indicates they ate more and their growth was less efficient even though their ADG was higher. The steer progeny from the same study were not different for ADG, DMI or feed efficiency (Stalker et al., 2006). The interaction of the pre- and post-partum treatments in this study may also increase the complexity of uncoupling the effects of the maternal diet and subsequent lactation (Bell and Greenwood, 2016).

In many species, there is a characteristic surge of peripheral leptin levels in the neonate. In rodents, it has been demonstrated that leptin exposure in the neonatal period alters the development of neuronal connections between the ARC and hypothalamic nuclei, which mediate changes in feeding behaviour (Bouret et al., 2004; Bouret, 2010) i.e. in male rats born to protein restricted mothers, low levels of leptin were associated with a hyperphagic state (Qasem et al., 2012). In lambs born to overfed ewes (150% of NRC requirements for 60 d prior to conception to term) the characteristic leptin surge was not present (Long et al., 2011). Subsequently, the offspring displayed increased appetite, weight gain and adiposity under ad libitum feeding conditions at 19 mo (Long et al., 2010a).

In cattle, the neonatal leptin profile has recently been characterised (Long and Schafer, 2013). Plasma leptin concentrations increased from birth until 2 d and then decreased until 16 d. Sex-related differences were evident with bull calves exhibiting elevated plasma leptin

concentrations compared with heifers, unlike sheep where leptin concentrations in the neonate are similar between sexes (Long et al., 2011). Offspring of multiparous cows that experienced maternal nutrient restriction (without protein supplement) in late gestation (last 100 d) had lower birthweights and reduced leptin concentrations at birth compared to offspring from control and nutrient restricted + protein supplemented cows (LeMaster et al., 2017). The long-term implications are unknown, but as in other species, these differences in the neonate may affect the appetite centres of the hypothalamus subsequently influencing appetite and weight gain (Long and Schafer, 2013).

Reproductive development

The ability to reproduce is dependent on the successful orchestration of development during sensitive periods of cellular proliferation, differentiation and maturation in the developing fetus and in early post-natal life. The hypothalamic-pituitary-gonadal (HPG) axis is the primary regulator of reproductive function in both males and females. This reproductive axis, and its hormonal control systems, develop and progress through a series of maturational and functional changes during early fetal life (Deligeorgis et al., 1996). This occurs in association with sexual differentiation and fetal testis development. Potentially therefore, the development of the gonads and/or gametes and the hypothalamic-pituitary-gonadal axis in the fetus may also be affected by maternal nutrition.

Pre-natal development of the reproductive tract

To ensure reproductive function and capacity in the adult is maximised, the proper developmental continuum from sex determination in the embryo through to a reproductively competent male in adulthood is critical (Klonisch et al., 2004). As noted, it has been suggested that there are critical windows of development in the fetus whereby the periods of vulnerability to perturbations during gestation may differ for each specific organ, tissue or system (Rhind et al., 2001; Redmer et al., 2004; Rhind, 2004; Nathanielsz, 2006; Symonds et al., 2007).

There are at least five distinct morphological stages of testis development in the fetus; genital ridge development, indifferent (bipotential) gonad formation, sex determination, seminiferous cord formation and the development of a functional testis (Skinner and Griswold, 2004). The precursors of the gonads, the genital ridges, first appear in the bovine embryo between approximately 27 to 32 dpc (Wrobel and Süß, 1998; Ross et al., 2009). The primordial germ cells, which are the precursors of spermatozoa and oocytes, migrate from the

hindgut, proliferate and populate the undifferentiated gonad from 27 dpc where they continue to accumulate until 40 dpc (Hopper, 2014).

During the very early stages of gonad development in the mammalian embryo, there are no distinguishable morphological differences between males and females. Whilst the sex of the embryo is genetically determined by the presence or absence of the 'Y' chromosome i.e. embryos with two X chromosomes develop into females (XX) and those with an X and a Y develop into males (XY), the urogenital system develops in both sexes from the intermediate mesoderm (Capel, 2000). Two pairs of genital ducts, the Wolffian (mesonephric) and the Müllerian (paramesonephric), form from the intermediate mesoderm prior to sexual differentiation of the reproductive tract. In males, anti-Müllerian hormone (AMH) is secreted by the Sertoli cells of the developing fetal testis. The Müllerian ducts subsequently regress due to the inhibitory effects of AMH allowing the Wolffian ducts to differentiate to form structures of the male reproductive tract including the seminal vesicles, epididymis and vas deferens. In the female, the developing ovary does not produce AMH allowing the Müllerian ducts to persist and differentiate into the female reproductive tract (oviducts, uterus, cervix and the upper part of the vagina).

The trigger for sexual differentiation is the start of the expression of the gene *SRY* (sex determining region on the Y) in the genital ridge (Koopman et al., 1991; Gutiérrez-Adán et al., 1997) at 37 dpc and peaking at 39 dpc in the bovine (Ross et al., 2009). This transcription factor starts a cascade of gene expression, including SOX9 (*Sry*-related HMG box containing gene 9), and signalling events leading to testis differentiation. Differentiation includes the establishment of the Sertoli cell lineage from bipotential precursor supporting cells in the indifferent gonad and the start of testis morphogenesis [formation of the testis cords (distinguishable in the bovine embryo by 42 dpc (Rajakoski and Hafez, 1964; Ross et al., 2009), establishment of testis-specific vasculature and the appearance of fetal Leydig cells and peritubular myoid cells (Koopman et al., 1991; Yao et al., 2015)]. The interstitial cells of the differentiating testis, the Leydig cells, then begin to secrete testosterone which contributes

to the embryo developing as a male (Habert et al., 2001; Klonisch et al., 2004; O'Shaughnessy and Fowler, 2011). The germ cells become encased within the somatic testis cords (the future seminiferous tubules) and transition from primordial germ cells to prespermatogonia from day 50 to 80 dpc. From 80 to 200 dpc germ cell multiplication decreases before the prespermatogonia enter a phase of relative mitotic quiescence until 4 wks of age in the post-natal bull calf (Wrobel and Süß, 1998; Wrobel, 2000; Ross et al., 2009; Hopper, 2014).

The hypothalamic-pituitary-gonadal axis also begins to develop during early fetal life (Klonisch et al., 2004; O'Shaughnessy and Fowler, 2011) and is functional in the bovine fetus by 120 dpc (Klonisch et al., 2004). Developmentally this occurs comparatively much earlier than in altricial species (Figure 2) and also earlier than in sheep (Klonisch et al., 2004).
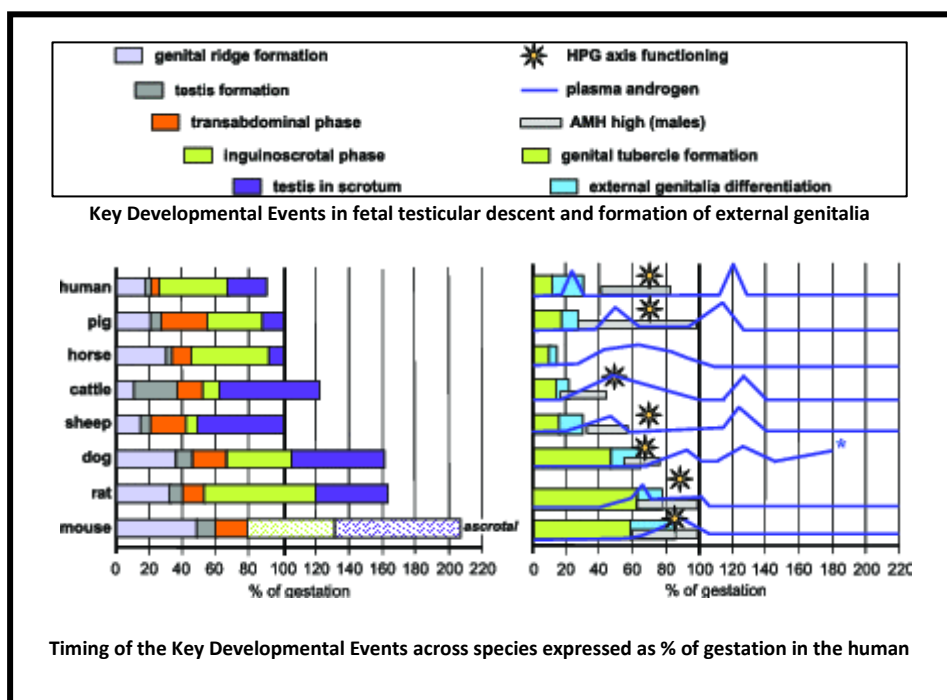
Figure 2. A comparative review of the timing of key developmental events across species. Genital ridge formation and testis descent across species are expressed as percentages of gestation in the human (left-hand panel). Plasma concentrations of anti-Mullerian hormone (AMH; only high levels indicated) and androgen levels in males (right-hand panel). These are given as a developmental function of fetal/perinatal testicular Sertoli and Leydig cells [adapted from (Klonisch et al., 2004)].

Post-natal reproductive development of bulls

Post-natally, the testicular development of Sertoli cells, Leydig cells and seminiferous tubules, culminating in the initiation of spermatogenesis at puberty, is regulated mainly by the interaction between gonadotrophins (LH and FSH) and testosterone in association with the maturation of the hypothalamus-pituitary-gonadal axis (Rawlings et al., 2008). The hypothalamus secretes GnRH in a pulsatile manner, which in turn induces pulsatile release of the gonadotrophs LH and FSH from the anterior pituitary. The gonadotrophs enter the circulation and reach the testes exerting influence over the Leydig and Sertoli cells as discussed in detail below.

Endocrine changes during post-natal reproductive development of the bull

Hormones play an important role in the establishment and maintenance of male reproductive function in bulls (Lunstra et al., 1978; Perry et al., 1991; Moura and Erickson, 1997; Brito et al., 2007c, d). Post-natal reproductive development in the bull based on the endocrinological changes during sexual maturation and can be divided into three distinct periods: infantile, pre-pubertal and pubertal.

During the early post-natal, or infantile period, (0 - 8 wks of age) the reproductive organs are quiescent (Amann, 1983) with few changes in testicular cell proliferation and differentiation; this period is associated with low levels of gonadotrophins and testosterone (Rawlings et al., 2008).

The pre-pubertal period (8 – 20 wks of age) is characterised by significant changes in hypothalamic, pituitary and gonadal function leading to puberty (Amann, 1983). There is a transient increase in gonadotrophin secretion (early gonadotrophin rise) and a concurrent increase in testosterone secretion (Barth et al., 2008). Peripheral Luteinising hormone (LH) concentrations increase transiently from 4 to 5 wks of age, to a post-natal peak at approximately 12 – 16 wks of age, followed by a decline in LH pulse frequency and amplitude (Miyamoto et al., 1989) to approximately 25 wks of age, with LH concentration

thereafter remaining at a low (but variable) baseline level through puberty (Rawlings et al., 1978; Rawlings and Evans, 1995; Bagu et al., 2006). The early post-natal rise in LH secretion is attributed to an increase in LH pulse frequency (Evans et al., 1995) triggered by an increase in the frequency of pulses of gonadotrophin-releasing hormone (GnRH) secretion (Rodriguez and Wise, 1989). Peripheral concentrations of FSH in the bull during early the early gonadotrophin rise generally increase before subsequently declining to baseline by approximately 25 wks of age (Amann and Walker, 1983; Evans et al., 1995). The pattern of FSH concentrations during this period is less marked and more variable (Amann and Walker, 1983; Rawlings et al., 2008) than the corresponding changes in LH. Furthermore, secretion of FSH is not pulsatile (Evans et al., 1996; Bagu et al., 2006). There is reported to be a concomitant rise in high testicular FSH receptor concentration and affinity associated with low FSH concentration in the pubertal bull suggesting a high Sertoli cell sensitivity to FSH, which ensures the progression and sustenance of spermatogenesis when FSH concentrations are low (Bagu et al., 2006).

The early gonadotrophin rise is considered critical for reproductive development in the bull (Barth et al., 2008; Rawlings et al., 2008) as it occurs prior to the phase of rapid testicular growth; the pre-pubertal rise in LH stimulating the Leydig cells to secrete androstenedione and testosterone (Evans et al., 1995). Testosterone levels increase slowly from birth to approximately 20 wks of age during the pre-pubertal period. Peripheral concentration then increases markedly, starting between 20 and 35 wks of age reaching adult concentrations by approximately 40 wks (Rawlings et al., 1978; Amann, 1983; Miyamoto et al., 1989; Evans et al., 1996). This marked rise in testosterone corresponds to the period of rapid testicular growth with the decline in gonadotrophins by 25 wks of age in the pre-pubertal bull attributed to increased sensitivity of the hypothalamus to negative androgen feedback and subsequent downregulation of GnRH release (Amann, 1983; Rawlings et al., 2008).

The period of rapid reproductive development, lower gonadotrophin secretion and marked rise in testosterone secretion described above characterise the pubertal period which is

considered to extend from around 20 to 24 wks of age until puberty (Amann, 1983; Amann and Walker, 1983; Evans et al., 1996; Rawlings et al., 2008).
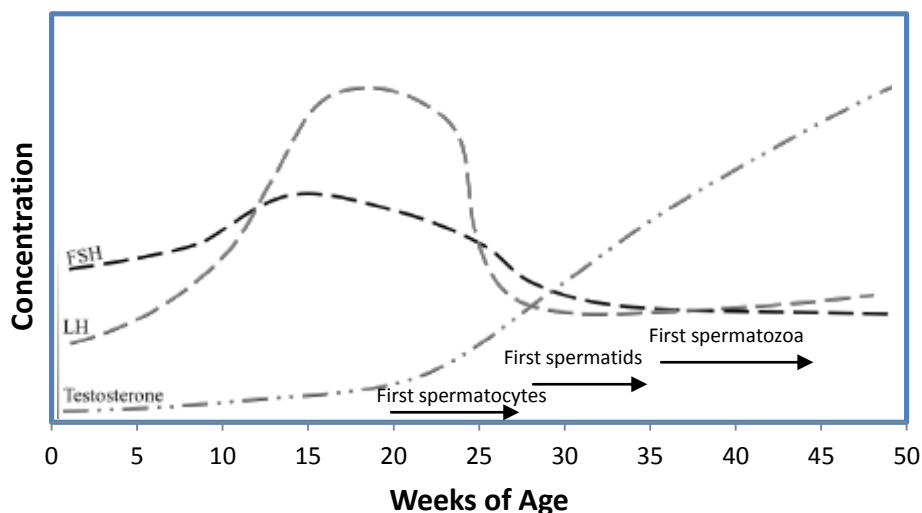
Figure 2. Endocrine changes during the reproductive development of the bull in relation to production of the first spermatozoa [adapted from Rawlings et al. (2008)].

Testicular changes during post-natal reproductive development in the bull

Post-natal testicular growth follows a sigmoid pattern, growing relatively slowly till around 25 wks of age (Coulter and Foote, 1979). This is followed by a marked increase in testicular growth until puberty, at 37 to 50 wks of age, slowing as the bull reaches maturity characterised by adult levels of sperm production (Amann, 1983; Rawlings et al., 2008). As gonadotrophin levels are relatively low during the pre-pubertal period, it appears testicular development at this stage is regulated by a mechanism independent of gonadotrophin (Brito et al., 2007d)

The testicular parenchyma has two compartments; tubular and interstitial (Senger, 2005). The tubular compartment comprises Sertoli cells, seminiferous epithelium, developing germ cells, peritubular cells and the basement membrane The interstitial compartment comprises Leydig cells, mesenchymal-like cells, peritubular cells, fibroblasts, capillaries,

37

lymphatic vessels and connective tissue (Senger, 2005) During the early post-natal phase of slow testis growth (prior to 25 wks of age) the testis is comprised of prespermatogonia, spermatogonia, undifferentiated Sertoli cells and Leydig cells (which appear independent of gonadotrophins). During the period of rapid testicular growth, after 25 wks of age, the seminiferous tubules distinctly increase in diameter and length along with the dramatic proliferation and differentiation of germ cells. The adult population of Leydig cells is developed by 30 wks of age, Sertoli cells by 30 to 40 wks and mature spermatozoa appear between 32 and 40 wks (Rawlings et al., 2008).

Sertoli cells: The proliferation of Sertoli cells occurs in two distinct phases in the bull. One occurs during fetal life and the other during the peripubertal period giving rise to two discrete Sertoli cells types which differ in the age at which they appear in the testis, their morphology and their function. The fetal population of Sertoli cells is in place at birth. At 4 to 8 wks of age, this population undergoes rapid cell division which subsequently declines and proliferation ceases by 12 to 24 wks (Wrobel, 2000). These undifferentiated Sertoli cells then undergo terminal differentiation and maturation to form the adult Sertoli cell. This is characterised by a loss in proliferative activity and morphological and functional changes (Sharpe et al., 2003). Hence, Sertoli cell number in the post-pubertal bull is determined prior to puberty as only the undifferentiated fetal population can proliferate. Ultimately this determines testis size, daily sperm production and the efficiency of spermatogenesis as these are all influenced by Sertoli cell number (Sharpe et al., 2003).

The development and proliferation of Sertoli cells is predominately regulated by FSH (Orth, 1984; Skinner and Griswold, 2004) via cAMP-dependent kinase (PKA) and mitogen–activated protein kinase (MAPK) pathways (Crepieux et al., 2001). Other factors known to influence Sertoli cell number include IGF1, thyroid hormone and growth factors (Skinner and Griswold, 2004; Griffeth et al., 2014). Emerging evidence suggests the insulin/IGF1

signalling pathway may also be integral for mediating the action of FSH in Sertoli cell proliferation (Pitetti et al., 2013).

Leydig cells: Two distinct populations of Leydig cells appear during testis development; fetal and adult (Habert et al., 2001). At birth, degenerating fetal Leydig cells and newly formed adult Leydig cells are both present. Within 8 wks of age, only post-natal adult Leydig cells are present as mesenchymal-like precursor cells transform into Leydig progenitor cells in the first stage of Leydig cell differentiation. The transformation of mesenchymal-like cells into Leydig cells proceeds to account for 17% of testicular volume by 16 wks. Progenitor cells differentiate into newly formed adult Leydig cells that grow to become immature adult Leydig cells. The adult Leydig cells produce testosterone and subsequently become mature Leydig cells; this population is in place by 30 wks of age. This process of growth and maturation is influenced by many factors including LH, IGF1, steroidogenic factor (SF-1) and platelet-derived growth factor (PGDF) (Griffeth et al., 2014), and precedes the onset of spermatogenesis (Moura and Erickson, 1997). The Leydig cells respond by a gradual increase in secretion of circulating testosterone from 6 to 35 wks of age and then a marked increase associated with the production of mature spermatozoa (Amann, 1983; Evans et al., 1996; Chandolia et al., 1997).

Germ cells: Post-natally germ cell proliferation resumes by 4 to 5 wks of age (Evans et al., 1996; Bagu et al., 2006) and between 4 and 15 wks postpartum, the diameter of the seminiferous tubules grow from 40 to 80 micron (Wrobel, 2000). The predominant germ cell at 12 wks of age is a gonocyte which are subsequently replaced by prespermatogonia and a-spermatogonia by 20 wks (Curtis and Amann, 1981). Primary spermatocytes are present at approx. 20 wks of age, secondary spermatocytes at 20 to 30 wks, round spermatids between 25 and 30 wks, long spermatids at 25 to 35 wks and mature spermatozoa at 32 to 40 wks of age (Curtis and Amann, 1981; Evans et al., 1996; Bagu et al., 2006). This timeline was

established in *Bos taurus* beef bulls (Evans et al., 1996; Bagu et al., 2006) and Holsteins (Curtis and Amann, 1981) but occurs at a later age in *Bos indicus* breed bulls (Aponte et al., 2005). The complex process of male germ cell proliferation and differentiation from diploid spermatogonia to haploid spermatozoa is called spermatogenesis; the final stage of differentiation from a round spermatid into spermatozoa is termed spermiation (Wrobel, 2000). Spermatogenesis and the continuous production of spermatozoa in the adult male occurs through the self-renewal of the niche of spermatogonia stem cells in the epithelium of the seminiferous tubules (Yoshida, 2010). In males, reproductive performance and fertility is dependent on the success of spermatogenesis; this in turn requires complete and normal sexual differentiation and development.

Puberty

Age of puberty in beef production is a driver of efficiency; the use of yearling bulls (and heifers) is an opportunity to rapidly shorten the generation interval, improving genetic gain (Barth and Ominski, 2000) and increasing overall lifetime productivity (Yilmaz et al., 2006). At puberty bulls have the ability to produce spermatozoa capable of fertilizing an oocyte and exhibit the sexual behaviour required to satisfactorily breed a female (including ejaculation) (Lunstra et al., 1978). Experimentally puberty in bulls is commonly defined as the age at which a bull first produces an ejaculate containing $\geq$ 50 million sperm with $\geq$ 10% motility (Wolf et al., 1965). Sperm characteristics (i.e. concentration and quality) continue to improve linearly for some time after this criterion has been met (Rawlings et al., 2008). These improvements in semen quality have been shown to be highly correlated ($r = 0.44$ to 0.75) with the post-pubertal increase in scrotal circumference (Lunstra and Echternkamp, 1982). Bulls attain puberty, on average, when their scrotal circumference reaches 28 to 30 cm and this age is often used as a proxy measure for puberty (Lunstra et al., 1978; Lunstra et al., 1992). Variation in timing of puberty and the age at which a mature spermiogram is achieved has implications for the adequate reproductive performance of yearling bulls. Variability in

the reproductive performance of yearling bulls may be the result of immature sexual behaviour and serving ability (Chenowith et al., 1988) but poor semen quality associated with incomplete testicle development is considered to be the major factor (Lunstra et al., 1978). Earlier age of puberty in bulls is thus a desirable production characteristic and advancement in age of puberty by even a short time period is advantageous in production systems utilising yearling bulls to maximise genetic gain.

Age of puberty in bulls has been shown to be influenced by a range of different factors including post-natal nutrition, genetics and management. Links between nutrition and reproductive development, age of puberty and reproductive performance in both the pre-pubertal and post-pubertal bull are well established (Barth et al., 2008). Metabolic hormones (e.g. leptin, insulin and IGF1) and nutrients are thought to be detected by GnRH neurons via a "metabolic sensor" signalling information on the animals nutritional and energy status to the hypothalamus-pituitary-gonad axis thereby affecting sexual development (Blache et al., 2003; Zieba et al., 2005; Brito et al., 2007a; Barth et al., 2008). Whilst peripheral concentrations of leptin and insulin are moderately correlated with SC and paired testis volume indicative of a role in promoting testis development; based on experimental evidence their role (if any) in regulating GnRH secretion in the pre-pubertal bull is now considered permissive at most (Barth et al., 2008) Collectively, studies have found that calfhood nutrition during the pre-pubertal period (0 to 6mths of age) is critical for maximising reproductive potential in bulls; improved nutrition hastens puberty, increases LH secretion and increases yearling SC (Brito et al., 2007b; Brito et al., 2007d, c; Barth et al., 2008). Interestingly, improved nutrition post-weaning cannot overcome poor nutrition during this period (Brito et al., 2007b).

Genotype has also been shown to strongly influence reproductive development in bulls. *Bos indicus* genotypes are slower to reach puberty than *Bos taurus* breeds due to a longer pre-pubertal period (Chase et al., 1997; Aponte et al., 2005). This delay in sexual development between *Bos taurus* and *Bos indicus* breeds has been associated with a later onset in spermatogenesis although the spermatogenic process, once started, occurs at similar

rates (Aponte et al., 2005). Yearling *Bos indicus* bulls have smaller sized testicles than *Bos taurus* (Lunstra and Cundiff, 2003) and at puberty, have been found to be older, heavier, have a larger scrotal circumference and have increased paired testicle volume compared to *Bos taurus* bulls (Chase et al., 1997). Genetic differences in testosterone occur and concentrations are higher in *Bos taurus* than *Bos indicus* breeds (Chase et al., 1997).

Endocrine manipulation of puberty in bulls has been explored by a number of studies. Exogenous GnRH given to Hereford bulls every 2 h for 14 days from 4 to 6 wks of age enhanced testicular development and hastened sexual maturity (Chandolia et al., 1997). Similar effects were observed when 4 to 8 wk old calves were given GnRH, twice daily, for 14 days increasing testicular growth rates and advancing sexual maturity by 4 wks compared to the controls (Madgwick et al., 2008). Furthermore, exogenous FSH administered to bull calves from 4 to 8 wks increased testicular growth, hastened the onset of puberty, increased Sertoli cell number and enhanced spermatogenesis (Bagu et al., 2004). Collectively these studies confirm the early gonadotrophin rise has an integral role in sexual maturity in bulls (Hopper, 2014).

Semen quality

Following spermatogenesis, spermatozoa are released into the lumen of the seminiferous tubules then passage into the epididymis undergoing further maturational changes (Curtis and Amann, 1981; Kaya et al., 2014). The morphology of the sperm is critical for normal cell function and the production of normal spermatozoa is known to be negatively influenced by external factors including temperature, nutrition, altered hormonal milieu, DNA modifications and genetics (Barth and Oko, 1989; Barth and Bowman, 1994; Barth et al., 2008; Kaya et al., 2014).

Morphological deviations from normal structure can be identified by microscopic evaluation (Barth and Oko, 1989), often undertaken as part of a bull breeding soundness evaluation (BBSE) (Fordyce et al., 2006). Abnormal morphology is associated with sub-

fertility (or sterility) in bulls depending on the defect and the frequency (or percentage) of the abnormalities in the ejaculate (Barth and Oko, 1989; Perry et al., 1990; Holroyd et al., 2002; Kaya et al., 2014). Semen quality in peripubertal bulls is generally poor with proximal cytoplasmic droplets and abnormal sperm head sperm defects commonly prevalent in ejaculates from young bulls (Lunstra and Echternkamp, 1982; Aravindakshan et al., 2000b; Entwistle and Fordyce, 2003; Brito et al., 2012a). In the pubertal bull, there is normally gradual improvement in both sperm motility and the number of morphologically normal sperm in the ejaculate (Barth and Oko, 1989; Brito et al., 2012a). The time taken between reaching puberty [$\geq$ 50 million sperm with $\geq$ 10% motility; (Wolf et al., 1965)] to maturity [i.e. producing an ejaculate of adequate semen quality; $\geq$ 30% motility and $\geq$ 70% morphologically normal sperm (Brito et al., 2004; Brito et al., 2012a)] is reported to be approximately 50 d in *Bos taurus* bulls (Brito et al., 2012a) and approximately 110 d in *Bos indicus* bulls (Brito et al., 2004).

Indicators of reproductive potential

Testicular measures

Age-adjusted scrotal circumference (SC) is considered to be a useful method of assessing reproductive function in bulls because of positive relationships with sperm traits (Brinks et al., 1978; Silva et al., 2011) and fertility (Mackinnon et al., 1991). Measurement of SC is highly repeatable and is recognised as the best method of assessing testicular development (Barth, 2000). Testis growth and, as a consequence, SC are related to growth rate in males (Bourdon and Brinks, 1986; Evans et al., 1995; Aravindakshan et al., 2000a; Brito et al., 2012b) and to attainment of puberty in males (Perry et al., 1991). Increased scrotal circumference is also positively genetically correlated with age of puberty in half-sibling female cattle (Perry et al., 1990; Johnston et al., 2009).

Scrotal circumference can be used as an indicator of puberty (Barth et al., 2008) due to the reported correlation with age of puberty (Lunstra et al., 1978; Lunstra and Echternkamp, 1982); on average *Bos taurus* bulls have been shown to reach puberty when their scrotal circumference is 28 cm (Lunstra et al., 1978) although considerable variation does exist in SC at puberty (Hopper, 2014). Testis size is highly heritable (Lunstra et al., 1988) and bulls with larger testicles are expected to have a higher total sperm output as the efficiency of spermatogenesis (number of sperm produced per gram of testicular parenchyma tissue) is relatively constant especially in young bulls ($10\text{-}14 \times 10^6$ sperm/g parenchyma) (Brito, 2014). Total daily sperm production, therefore, is determined by paired testis weight which is positively correlated to SC (Curtis and Amann, 1981; Brito, 2014).

As multiplication of Sertoli cells in the testis ceases between 20 to 25 wks of age in bulls, adult testis size may already be determined in calfhood (Barth et al., 2008). Testis diameters measured at 5 mo of age in bull calves are correlated with measures taken at 12 mo (Moura and Erickson, 1997). Furthermore, generally bulls that have small or large testes at 12 mo of age are comparatively sized when later assessed at 2-years of age (Curtis and Amann, 1981).

Hormones

Based on the evidence in the literature on the physiological and endocrinological processes that occur during post-natal reproductive development, circulating concentrations of hormones (i.e. gonadotrophins (LH and FSH), testosterone, AMH and inhibin, and IGF1 in the developing bull may potentially serve as biomarkers of reproductive development and indictors of age at puberty (Phillips, 2005; Burns et al., 2011).

Luteinising Hormone (LH). Luteinising hormone, secreted by the pituitary, is linked to testosterone secretion and influences the onset of puberty (Evans et al., 1995; Bagu et al., 2006). The reported relationships between LH concentrations and age at puberty are

conflicting. Greater pituitary/hypothalamic activity early in life, may account for higher pre-pubertal basal LH concentrations in faster maturing compared to slower maturing bulls (Evans et al., 1995; Aravindakshan et al., 2000a). The LH response to GnRH stimulation, however, was observed to be lower in early maturing bulls (Bagu et al., 2006). Negative correlations between LH and testosterone and paired testis volume have been reported in bulls between 10 and 70 wks of age (Brito et al., 2007c) and LH concentrations during the pre-pubertal stage are inversely related to age of puberty (Amann, 1983; Evans et al., 1995). Measurement of basal LH concentrations requires repeated samplings due to the pulsatile nature of LH (Aravindakshan et al., 2000a). By contrast, GnRH- stimulated LH requires only a single sample taken 0.5 to 1 hr following the injection of a GnRH analogue (Moura and Erickson, 1997; Bagu et al., 2006; Burns et al., 2013). The majority of studies utilising this technique however have only monitored bulls until yearling age (Burns et al., 2011).

FSH. The spermatogenic capacity of the testis is influenced by FSH through its effects on Sertoli cells (McLachlan et al., 1995). Basal FSH concentration in pre-pubertal bulls is reported to be correlated with later yearling measures of testis diameter, Sertoli cell number and number of round spermatids per Sertoli cell, whilst concentrations of GnRH-stimulated FSH in pre-pubertal bulls are positively correlated with testis diameter and cell ratios in the seminiferous tubules (Moura and Erickson, 1997). Treatment of bull calves with FSH at 4 to 8 wks of age has been reported to increase scrotal circumference, hasten the onset of puberty and enhance spermatogenesis (Bagu et al., 2004). Pre-pubertal basal FSH (Evans et al., 1995) and GnRH-stimulated FSH levels (Aravindakshan et al., 2000b) however, do not differ between early and late maturing bulls (based on age of puberty). In the post-pubertal bull, basal FSH levels increase; a rise thought to be associated with the age-related improvement in semen quality and quantity (Matsuzaki et al., 2000). Interestingly, in post-pubertal primates and humans, FSH levels have also been reported in a number of studies to be associated with sperm quality parameters including altered motility and morphology (Plant and Marshall, 2001).

Testosterone. In pre- and post-pubertal bull calves, peripheral testosterone concentrations are positively associated with testis weight and the diameter of the seminiferous tubules (Evans et al., 1996), and correlations exist with SC and age of puberty (Moura and Erickson, 1997; Aravindakshan et al., 2000a). Testicular steroidogenesis (testosterone concentrations) in the pubertal bull reflects Leydig cell number and/or function (Barth et al., 2008) and the age related rise in testosterone in pubertal bulls is hastened by high nutrition, possibly mediated by LH and IGF1 (Barth et al., 2008).

AMH. AMH is one of the first genes to be switched on in Sertoli cells after differentiation in fetal life and expression continues whilst Sertoli cells remain immature (Sharpe et al., 2003). Expression of AMH in Sertoli cells and its secretion into the bloodstream has been shown to be down regulated at puberty in mammals (Rey and Josso, 1996; Rey et al., 2003). Circulating AMH levels are known to decline sharply in the pubertal bull (Rota et al., 2002). Persistent high peripheral concentrations of AMH may indicate failure of maturation of Sertoli cells or, as has previously been suggested, reflect deficiencies in androgen action (Sharpe et al., 2003).

Inhibin and Activin. Pre-pubertal serum inhibin level in bulls has been proposed as a potential early biomarker of testis development and function (Sharpe et al., 2003; Burns et al., 2011; Fortes et al., 2013). Inhibin is produced exclusively by Sertoli cells in the testes (Kaneko et al., 2001; Sharpe et al., 2003; Phillips, 2005). It may act as a regulator of Sertoli cell differentiation and control FSH secretion during early pre-pubertal development (Kaneko et al., 2001) and is linked to the regulation of spermatogenesis (Phillips, 2005). Relationships have been reported between levels in the pre-pubertal bull with sperm production parameters and SC in the mature bull (Sharpe et al., 2003). However, in the post-pubertal stage, inhibin levels are low and relatively constant suggesting that it has no direct role in testicular function in the mature bull (Matsuzaki et al., 2000). In contrast to the actions of inhibin, activin stimulates FSH secretion (Mather et al., 1992), proliferation of sperm (Mather et al., 1990)

and Sertoli cells (Boitani et al., 1995) and theoretically may also have value as a an early biomarker of reproductive development.

IGF1. As previously discussed previously, the metabolic hormone IGF1 has important autocrine and paracrine roles regulating mitosis, apoptosis and differentiation at the cellular level (Griffeth et al., 2014). In pre-pubertal *Bos taurus* bulls, IGF1 concentrations were reported to be positively correlated with adult SC and sperm motility (Yilmaz et al., 2004). Concentrations of IGF1 in the bull account for a high proportion of variation in testicular size suggesting IGF1 may play a specific role as a testicular mitogen (Brito et al., 2007a). Furthermore, nutritional studies have also demonstrated relationships between circulating IGF1 concentrations and male reproductive development. Low nutrition in calfhood reduced peripheral IGF1 concentrations and delayed the rise of testosterone (Brito et al., 2007c). Temporal associations were also found between IGF1 and LH concentrations; it is unknown however if IGF1 can promote GnRH secretion in bulls (Barth et al., 2008).

Maternal nutrition and reproductive development and function.

Evidence to support the link between suboptimal maternal nutrition and altered fetal and/or post-natal reproductive development has been reported in a range of experimental studies in laboratory and domestic animals including rats (Zambrano et al., 2005; Toledo et al., 2011; Rodríguez-González et al., 2012), rabbits (Dupont et al., 2014), sheep (Da Silva et al., 2001; Bielli et al., 2002; Kotsampasi et al., 2009) and cattle (Wilkins et al., 2006; Martin et al., 2007; Sullivan et al., 2009a; Mossa et al., 2013). The reported effects vary dependent on the timing and composition of the dietary intervention, the sex of the conceptus and the species (Dupont et al., 2012; Chavatte-Palmer et al., 2014; Mossa et al., 2015). Such effects are however only of practical importance in domestic animal production if they translate into altered reproductive function and efficiency in later post-natal life.

Experimental studies on the effects of prenatal nutrition upon reproductive development in cattle are very limited as recently reviewed (Chavatte-Palmer et al., 2014;

47

Mossa et al., 2015; Sinclair et al., 2016). Furthermore, the majority of studies to date have focused primarily on outcomes in the female offspring rather than male. Maternal nutritional restriction (11 days prior to 110 dpc) in beef heifers was associated with lower antral follicle count, lower AMH and greater FSH concentrations (collectively taken to represent markers of reduced ovarian reserve) in the female progeny before and after puberty (Mossa et al., 2013). These effects occurred independently of birthweight, post-natal growth rates (to 95 wks of age) and age of puberty, which were similar the offspring from both restricted and control mothers (0.6M vs. 1.2M).

Several studies have reported that protein restriction and/or supplementation during gestation in beef cattle affects the reproductive development and performance of the heifer progeny. A three-yr study on the effects of protein supplementation during late gestation under rangeland grazing systems, showed offspring from multiparous *Bos taurus* composite bred cows grazing low quality forage that received protein supplement during late gestation had higher pregnancy rates, and shorter time to calving, but did not differ in age of puberty from the progeny of cows that were unsupplemented (Martin et al., 2007). In contrast, protein restriction during the first trimester of gestation followed by protein supplementation in the second trimester in mature *Bos indicus x Bos taurus* composite bred heifers was associated with negative effects in the heifer progeny on follicle size and density as adults (Sullivan et al., 2009a).

As previously noted, the effect of pre-natal nutritional perturbations may vary between male and female offspring; in the male cohort of the Sullivan et al. (2009a) study, protein restriction during early gestation was reported to have collectively positive effects on the pre-pubertal development of the bull offspring. This was evidenced in measures collected immediately prior to, and at castration at 5 mo of age by associations with increased testicular volume (when restriction in the first trimester was followed by protein supplementation in the second trimester) and increased plasma concentrations of the gonadotrophin FSH (pre GnRH stimulation) (Sullivan et al., 2010a). Although suggested to potentially indicate an improved

lifetime reproductive capacity, the long-term implications of these observations in the pre-pubertal bull on later reproductive development and function are unknown. A recent study investigated the effects of maternal overnutrition in multiparous cows (ad libitum vs moderate intake; 60 dpc to 241 dpc) on gonadal development and pituitary-gonadal gene expression in fetuses at 139 dpc, 199 dpc and 241 dpc (Weller et al., 2016). Overnutrition affected ovarian follicular growth and follicle number in the female fetuses; primordial and total follicle numbers were lower with the reverse observed for preantral and antral follicles. This outcome is similar to that observed in sheep by (Da Silva et al., 2003). In males, testicular development was disturbed by overnutrition as assessed by measures of the fetal seminiferous cords with lower volumetric proportion and diameter of the seminiferous cords. The underlying mechanisms were reported to involve differential expression of multiple genes: P450 aromatase, *StAR, BMPR2, TGFBR1, GDF9, FSHR, Bax, CASP3, HSD17B3, IGF1, IGF2, and IGF1R.* A significant limitation of this study is the lack of statistical power. Additional studies utilising adequate experimental numbers are required to confirm these results and evaluate the functional meaning of the differential gene expression.

Comparative studies in rams have found a range of effects dependent on the timing of the maternal nutritional perturbation and the developmental stage at which the effects were assessed i.e. in the fetus at mid-gestation or in the post-natal animal. Nutritional restriction of ewes during gestation does not affect basal pituitary gonadotrophin secretion in the fetal lamb (Rae et al., 2002b; Andrade et al., 2013) but has been reported to result in a reduced LH response to GnRH in fetal (Rae et al., 2002b) and pre-pubertal lambs (Deligeorgis et al., 1996), and an increased FSH response to GnRH in ram lambs at 10 mo, but not at 2 and 5.5 mo (Kotsampasi et al., 2009). In the ram lamb, maternal undernutrition from mid-pregnancy to term has been associated with a reduction in the number of both seminiferous cords and Sertoli cells per testis in association with a lower birthweight (Bielli et al., 2002). In contrast, maternal undernutrition from 31 to 100 dpc did not alter birthweight or age of puberty but decreased Sertoli cell number and the size of the seminiferous tubules in ram lambs at 10 mo

(Kotsampasi et al., 2009). In the overfed adolescent ewe model, placentally-mediated fetal growth restriction had no effect on fetal testis development (assessed at 103 dpc) (Da Silva et al., 2003). In the pubertal offspring however, testis growth, seasonal increase in testosterone and peak testosterone concentration were delayed until the rams had attained similar weights to the control lambs at puberty (Da Silva et al., 2001). This was attributed to the combined effects of altered development of the hypothalamic-pituitary-gonadal axis and carryover effects of the placentally-mediated nutrient restriction on post-natal growth.

A recent study found fetal testis development, as assessed by fetal testis Sertoli cell numbers and expression of gene products that regulate apoptosis, was not affected in the nutrient restricted (mating to 110 dpc) male lamb fetus at 110 dpc (Andrade et al., 2013). This window of maternal undernutrition in the sheep fetus includes the period of sexual differentiation, the onset of pituitary function and gonadotrophin secretion and a major portion of the period of fetal Sertoli cell proliferation. The lack of effect in the fetal testis contrasts to the altered development of the fetal ovary observed in previous studies using the same experimental model (Rae et al., 2001; Lea et al., 2006) providing evidence that differences may exist in the sensitivity of the developing ovary and testis to maternal nutrition effects, dependent on the timing of the dietary perturbation. In the absence of effects on fetal testis morphology, the authors postulated that the reported effects of early fetal undernutrition on post-natal reproductive development in ram lambs (Da Silva et al., 2001; Rae et al., 2002a; Kotsampasi et al., 2009) may therefore, in-part, result from changes in the expression of genes which regulate the onset of post-natal hypothalamic - pituitary activity at puberty (Andrade et al., 2013).

Few studies on the effects of maternal nutrition on male post-natal reproductive development have assessed measures of reproductive function beyond puberty in sheep. Undernutrition (High: 100% vs. Low: 50% of ME requirements) *in utero* (0 to 95 dpc) had no effect on adult reproductive capacity in 20 mo adult rams as assessed by measures of sperm concentration and motility at a single time point (Rae et al., 2002a). Furthermore, there was

50

no effect of maternal undernutrition on birthweight, liveweight, testicular size, gonadotrophin response to GnRH, basal LH, basal testosterone and LH stimulated testosterone levels but there was associated with a higher basal FSH profile. The biological significance of this difference was considered uncertain (Rae et al., 2002a).

In male rats, maternal protein restriction during fetal development and/or lactation (0 − 21 post-natal days) delays testicular descent, reduce LH and testosterone levels plus reduce fertility rate and sperm count in adulthood (Zambrano et al., 2005). In comparison, protein restriction *in utero* only (0 - 21 gestational days) reduced Sertoli cell number, sperm count and motility and increased the incidence of sperm abnormalities in adult male rats (Toledo et al., 2011). No comparable studies exist on the effect of maternal nutrition during pregnancy on sperm parameters in progeny in ruminants.

Collectively, this body of evidence suggests that a suboptimal maternal nutritional environment (i.e. undernutrition or overnutrition) may negatively impact offspring reproductive development. Few studies in domestic animals of agricultural importance have comprehensively considered these effects in both the fetus and adult offspring. This is due, in part, to the length of time required to allow offspring to reach puberty as compared to laboratory species (Mossa et al., 2015). In bulls, the effects of maternal nutrition on the fetal testis and long-term reproductive development and function remain to be fully evaluated.

Sex differences

Sex differences in responses to suboptimal *in utero* environments have been reported in a range of species including humans (Clifton, 2005; Hodyl et al., 2010), rodents (Kwong et al., 2000; McMullen and Langley-Evans, 2005), sheep (Gilbert et al., 2007) and recently, cattle (Micke et al., 2010a; Hernandez-Medrano et al., 2015; Micke et al., 2015).

Studies have found that differences exist in growth rate, metabolism and gene expression level between female and male embryos (Gutierrez-Adan et al., 2006). Evidence suggests that embryos may be affected differently by environmental conditions dependent on

their sex (Kwong et al., 2000; Bermejo-Alvarez et al., 2011) and furthermore, that the male bovine preimplantation embryos may be more responsive than females under stress conditions (Gutierrez-Adan et al., 2006). Supplementation of glucose in culture of bovine conceptuses resulted in a preferential loss of females with a greater proportion of males surviving *in vitro* to the blastocyst stage (Gutiérrez-Adán et al., 2001); in the bovine preimplantation embryo therefore, survival may already be influenced in a sex-dependent manner even before the development of gonads (Aiken and Ozanne, 2013). The underlying mechanisms regulating the differences between the sexes in their response to the early-life developmental environment are not fully understood. Whilst proposed that sex differences in developmental programming studies result from the differing ability of the male and female conceptus to respond to an environmental insult, an additional consideration is perhaps the mothers response differs dependent on the sex of the fetus they are carrying (Aiken and Ozanne, 2013; Rosenfeld, 2015). It has therefore been recommended that offspring sex be considered in the design, interpretation and review of future developmental programming studies (Clifton, 2010; Aiken and Ozanne, 2013; Rosenfeld, 2015).

Summary, aims and hypotheses

There is considerable evidence demonstrating that altering the level and composition of maternal intake during pregnancy may have long-term consequences for the progeny. In species of significant agricultural importance, such as the bovine, these effects may impact production traits and the reproductive capacity of the progeny thereby generating significant economic loss for the agricultural and meat industries. There have been relatively few studies that specifically evaluate the effects of maternal protein restriction and/or supplementation on the fetal and post-natal development of the offspring in the bovine. Dietary protein is of particular importance as it is the major limiting nutrient observed in rangeland cattle production. Thus this study is of particular interest to industry as it examines the long-term effects of maternal dietary protein intake.

The overall aim of the current study was to investigate the impact of maternal dietary protein intake during the peri-conception period and first trimester of pregnancy in the adolescent heifer, upon measured outcomes in both the dam and fetus during gestation and in the subsequent post-natal calf.

Placental and fetal growth

Protein deficiency is common in extensively-managed beef herds in Australia (Norman, 1963), with the provision of protein supplement to replacement beef heifers now an established management practice (Bortolussi et al., 2005). The effect of protein restriction and/or supplementation upon metabolic outcomes, fertility and early embryo development has been well tested in the literature, particularly in dairy cattle (Laven et al., 2007). There are currently limited studies that have examined the effects of these nutritional treatments in beef heifers (Gath et al., 2012). Furthermore, metabolic outcomes in response to dietary protein treatments during early and mid-gestation in mature beef heifers are also suggested to mediate changes in placental development and maternal-fetal nutrient capacity (Sullivan et al., 2009b; Sullivan et al., 2009c) affecting fetal development (Micke et al., 2010b). There are currently no studies that have monitored the effects of dietary protein treatments commencing prior to conception and extending to early gestation on feto-placental development in adolescent beef heifers where the demands for growth of the heifers may compete for nutrients with their developing fetus. In **Chapters 2 to 4** therefore, the aim of the experiment was to determine the effects of maternal dietary protein intake during the peri-conception period and first trimester of pregnancy upon the growth and physiology of the developing yearling heifer, placental function and fetal development. The hypotheses tested included:

- Protein restriction during the peri-conception period and early gestation would alter the metabolic and endocrine profiles of the dams and increase early embryonic loss.

53

- Protein restriction would reduce placental and fetal development in a gender-dependant manner with subsequent deleterious effects upon placental parameters and fetal morphometry at 98 dpc and at term.

Reproductive development of the male progeny

It is well-established that maternal nutrition can affect fetal development with long-term consequences (McMillen and Robinson, 2005). Varying levels of maternal nutrition during critical windows of development can affect gonad development, gamete quality and hormonal status through effects upon different cellular components of the developing testis and hypothalamus (Sullivan et al., 2009a; Sullivan et al., 2010b; Dupont et al., 2012; Mossa et al., 2013). The development of the testis and circulating gonadotrophin levels in the pre-pubertal bull have been shown to be sensitive to maternal dietary protein intake (Sullivan et al., 2010b). However, the long-term implications for reproductive development and performance in pubertal and adult cattle progeny are not known. In **Chapter 5** therefore, the aim of this experiment was to examine the effects of maternal dietary protein intake during the peri-conceptional period and first trimester in adolescent heifers on the reproductive development of male progeny though puberty. The hypothesis tested was that maternal dietary protein restriction would delay puberty with deleterious effects upon testicular development and sperm production. Furthermore, this would be associated with alterations to the hormonal milieu in the developing bull.

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Post-natal growth and carcass traits of the male progeny

In altricial species, such as the rodent, there is evidence that altering the level and composition of maternal intake during pregnancy results in changes in body mass and body composition in post-natal life. There have been few studies, however, on the impact of altered maternal nutrition on the programming of appetite and body composition in the bovine. In **Chapter 6** therefore, the aim of this experiment was to evaluate the effects of maternal

dietary protein intake during the peri-conception period and the first trimester in adolescent heifers on post-natal growth pathways, appetite, feedlot performance and carcass traits in the male progeny. The hypothesis tested was that maternal dietary protein restriction would decrease post-natal growth, feedlot performance and carcass traits whilst increasing feed intake.

The overall purpose of the experiments in this thesis is to provide greater insight into the effects of varying levels of maternal protein intake on fetal and post-natal development in the progeny of adolescent beef heifers. Gaining a better understanding of both the production outcomes and the causal mechanisms behind the programming effects will ultimately allow the design of targeted dietary interventions to maximise productivity.

Chapter 2

Fetal programming in 2-year-old calving heifers
: peri-conception and first trimester protein restriction alters fetal growth
in a gender-specific manner.

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Overall percentage 70 (%)	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 30 / 10 / 16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

     i.   the candidate's stated contribution to the publication is accurate (as detailed above);

     ii.   permission is granted for the candidate in include the publication in the thesis; and

     iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Fetal programming in 2-year-old calving heifers: peri-conception and first trimester protein restriction alters fetal growth in a gender-specific manner

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Abstract. Protein restriction in early bovine gestation affects post-natal reproduction and production traits in progeny. This experiment evaluated the effects of dietary protein restriction during the peri-conception period and first trimester in yearling heifers on conceptus growth and development; this period of dietary intervention being earlier than any previous bovine fetal programming studies. Three-hundred and sixty primiparous 12-month-old Santa Gertrudis heifers were individually fed high [14% crude protein (CP)] or low (7% CP) diets for 60 days before conception. At 23 days post-conception (dpc), each high (HPERI) or low (LPERI) group was again split into high (HPOST) or low (LPOST) protein groups yielding four treatment groups in a 2×2 factorial design. From the end of the first trimester of gestation (98dpc), the pregnant heifers were individually fed a 12% CP diet until parturition. Forty-six fetuses were excised at 98dpc. Sixty-four heifers went on to calve. Conceptus development was assessed via transrectal ultrasound from 36dpc, fetal necropsy at 98dpc and live calf measures at term. At 36dpc, HPERI diet increased fetal crown–rump length (CRL) ($P < 0.05$) and at the 60dpc scan, biparietal diameter (BPD) tended to be increased by HPOST diet ($P < 0.1$) though the greater effect upon BPD was still the HPERI diet ($P < 0.05$). At 60dpc, BPD in the male fetus was affected by the peri-conception diet ($P < 0.05$), while in females, BPD was not different among nutritional groups. These ultrasound measures of fetal growth were validated by measures of the excised fetus at 98dpc. Fetal weight was heavier ($P < 0.01$) in those whose mothers were fed the HPOST diet than their LPOST counterparts. Males fetuses were heavier than female fetuses ($P < 0.001$). Fetal CRL was increased by HPERI diet ($P < 0.05$) and tended to be increased by HPOST diet ($P < 0.1$). Fetal BPD tended to be increased by HPERI diet ($P < 0.1$). In males, BPD tended to be increased in those fetuses whose mothers were fed HPERI ($P < 0.1$). For females, maternal nutrition during PERI or POST did not affect BPD at 98dpc ($P > 0.1$). At term, no dietary effect on birthweight was observed ($P > 0.1$) and males were not heavier than females ($P > 0.1$). These results suggest that maternal protein intake during the peri-conception (−60 to 23dpc) and first trimester (24–98dpc) may influence early conceptus growth and development in the bovine. The long-term effects on offspring metabolism and post-natal development of this dietary intervention are yet to be determined.

Additional keywords: beef, embryo, fetus, nutrition, oocyte.

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Introduction

Protein is the major limiting nutrient in range beef cattle in Australia. It has previously been established that protein restriction in early gestation may affect post-natal reproduction and production traits in progeny (Sullivan *et al.* 2009, 2010; Micke *et al.* 2010*a*, 2011; Mossa *et al.* 2013). Further, studies have also shown effects of maternal diet upon fetal growth to 123 days post-conception (dpc) after dietary intervention 30–123dpc (Long *et al.* 2009) although this effect was no longer apparent at birth (Long *et al.* 2012).

Critical windows exist during oocyte, embryo and fetal development where decreased maternal dietary protein may reset early cell lineages (Wu *et al.* 2006). Maternal nutrition affects both the number of oocytes that ovulate and their quality (Ashworth *et al.* 2009), which has then been associated with differences in embryo survival (Borowczyk *et al.* 2006). The gender of an embryo has also been shown to affect the susceptibility of the embryo to altered nutrition before mating (Vinsky *et al.* 2006). Gender-specific differences in embryo DNA methylation pattern (Dobbs *et al.* 2013), expression of

key developmental genes (Kwong *et al.* 2006), fetal and placental perfusion (Prior *et al.* 2013) post-natal carcass traits (Micke *et al.* 2011) have all been reported. Intriguingly, neonatal and fetal death in cattle is increased if the fetus is male, as with most mammalian species.

The aim of this study is to further evaluate the critical windows during development where dietary protein may alter fetal development. By examining the effects of protein diets similar to those experienced in range conditions in supplemented and non-supplemented breeders during the period from oocyte development in the ovary to the end of the first trimester we aim to establish the effects of such restriction.

This paper focuses on the effects of protein restriction in early life upon conceptus growth and development. We hypothesise that low maternal protein during the peri-conception period and the first trimester will reduce fetal growth in a gender dependent manner.

Materials and methods

This project was approved by the University of South Australia IMVS Animal Ethics Committee (Approval number 18/11) on 11 March 2011.

Experimental animals

Three-hundred and sixty primiparous 12-month-old Santa Gertrudis (*Bos taurus* × *Bos indicus*) heifers were selected based on weight and age from SK Kidman herds at Glengyle and Morney Plains, south-western Queensland. All heifers were vaccinated against viral and bacterial diseases on two occasions

4 weeks apart before transport. The heifers were transported to Sedan, South Australia to purpose-built, shaded feedlot pens, acclimatised to diet, and trained to individual stall feeding.

At 12 months of age, 60 days before artificial insemination (AI), the heifers were randomly assigned to two equal groups stratified by weight. Each heifer was individually fed a high [14% crude protein (CP)] or low (7% CP) protein diet consisting of pellet diet fed in stalls and straw (5% CP) *ad libitum* in pens. The digestible energy content of the diet was as similar as possible in the ruminant and supplemented with a vitamin and mineral commercial preparation (Table 1).

Heifers underwent an 8-day progesterone-based oestrous synchronisation program. On Day 0, heifers were AI sequentially with frozen semen from one low birthweight Santa Gertrudis bull, selected on the basis of estimated breeding value.

At 23dpc each high (HPERI) or low (LPERI) group was again split into high (HPOST) or low (LPOST) protein groups yielding four treatment groups [high high (HH), high low (HL), low high (LH), low low LL)] in a 2 × 2 factorial design. Detailed composition of the heifer rations is given in Table 1.

Pregnancy was positively diagnosed in 124 heifers (HH – 33; HL – 37; LH – 30; LL – 28) at 36dpc via transrectal ultrasound and non-pregnant heifers were removed from the trial. At 60dpc fetuses were sexed via ultrasonography enabling equal numbers of sex fetuses to be excised at slaughter from each of the six treatment groups. From the end of the first trimester of gestation (98dpc), the remaining pregnant heifers were individually fed at the NRC recommended ration for weight containing 12% CP until parturition.

Table 1.   Ingredients and nutrient content of heifer rations for peri-conception and during the first trimester of gestation

	Induction	Peri-conception		First trimester	
		High	Low	High	Low
Ration as fed					
Wheat (kg)	0.66	0.48	1.81	0.56	2.12
Canola meal (kg)	2.23	–	–	–	–
Soybean meal (kg)	–	1.83	0.48	2.14	0.56
Barley straw (kg)	7[B]	6.7	5.5	10.7	10.2
Molasses (g)	90	72	72	84	84
Biofos MDCP (g)	–	–	19	–	22
Salt (g)	15	12	12	14	14
Vitamin/trace mineral premix (g)	3	2	2	3	3
Dry matter	9.1[C]	8.3	7.2	12.3	11.8
Total energy (MJ ME)	–	71	63	102	98
% of energy requirements[A]	–	96	85	142	136
Total crude protein (kg)	–	1.18	0.62	1.49	0.88
% of protein requirements[A]	–	127	67	123	72
% CP (total diet)	–	14.2	8.6	12.1	7.4
Total calcium (g)	–	26	22	38	37
% of calcium requirements[A]	–	130	110	190	185
Total phosphorus (g)	–	17	17	21	21
% of phosphorus requirements[A]	–	130	130	160	160

[A]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (2007, CSIRO). Input values were based upon nutrient analysis of component ingredients in the total diet, liveweight and age of heifers at each diet change, mature cow weight of 550 kg and the desired growth target.
[B]Assumed value.
[C]Predicted value.

Fetal calf measurements

Fetuses were measured using transrectal ultrasound (Sonosite M-Turbo; Sonosite Inc., Bothell, Washington, DC, USA) at 36, 60 and 95dpc. Fetal sex was determined at 60dpc by rectal ultrasound. Measurements of the conceptus were taken at the time of ultrasonography (Micke *et al.* 2010*b*). All fetal body measurements were in centimetres. Crown–rump length (CRL) was measured from a lateral view of the fetus from the tip of nose to the base of the tail. Biparietal diameter (BPD) was measured from a dorso-ventral view of the cranium perpendicular to the sagittal crest at the widest span between the most lateral parts of the parietal bone.

Fetal excision 98dpc

A subset of heifers (HH – 12; HL – 15; LH – 10; LL – 9) were killed in a commercial abattoir at 98dpc and used for human consumption. Tissues for examination were immediately collected from the uterus and fetus after removal from the kill floor. The fetus was excised from the uterus, cord blood collected, weighed, measured and then dissected. Sex of fetus was recorded during dissection. Measures of BPD and CRL were obtained from fetuses using sliding calipers. The remaining heifers were taken to term.

Calf measurements

At calving, heifers (*n* – 64; 19 female, 45 male) were monitored individually and assistance provided where necessary. Calves were collected within 15 min of birth, before sucking, and sex, birthweight and CRL recorded.

Statistical analyses

Multifactorial ANOVA using STATA SE version 11 (Stata Corporation, College Station, TX, USA) was used to interpret the main effects of peri- and post-conception diet, sex and their interaction terms on ultrasound measures of embryo and fetal body dimensions from 36dpc, fetal measures at 98dpc and live calf measures at term. Individual calf was considered the experimental unit, with gestation length as a co-variate for birth measures only. Males and females were analysed together and independently. A probability of 5% ($P < 0.05$) was accepted as the level of significance and trends reported as $P < 0.1$. Data presented as mean ± standard errors of the mean.

Results

Fetal measures *in utero*

At 36dpc high protein (14% CP) preconception diets increased fetal crown–rump length (CRL) compared with low protein (7% CP) ($P < 0.05$) (Fig. 1) and by 60dpc BPD tended to be increased by diet post-conception ($P < 0.1$) though the greater effect upon BPD was still peri-conception diet ($P < 0.05$) (Fig. 1). In males, BPD was increased in those fetuses whose mothers were fed HPERI ($P < 0.05$). For females, maternal nutrition during PERI or POST did not affect BPD at 60dpc ($P > 0.1$).

Weight gain during peri- and post-conception

Initial weight of the heifers at acclimatisation was 350 kg. The average daily gain of heifers was 0.4 kg/day during the
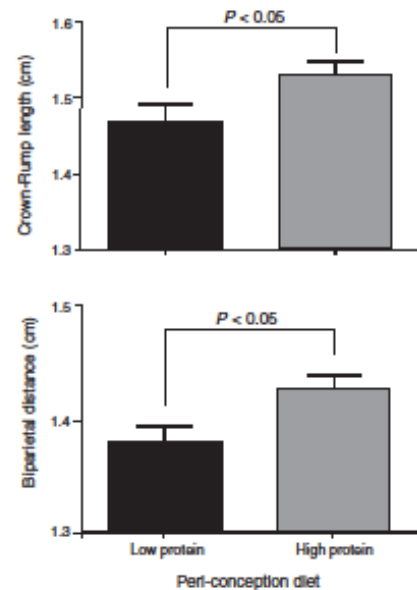
Fig. 1. The effects of peri-conception dietary protein; high (14% CP), low (7% CP) upon fetal growth at 36 and 60 gd (days gestation). Values are unadjusted means ± s.e.m.

high and 0.2 kg/day during the low protein treatment periods (Fig. 2).

Fetal size at 98dpc

In measures of the excised fetus at 98dpc, fetal weight was heavier ($P < 0.01$) in those whose mothers were fed HPOST diet (HH + LH – 326.3 ± 6.7 g) compared to their low counterparts (HL + LL – 301.2 ± 7.6 g). Male fetuses were heavier than female fetuses ($P < 0.001$). Fetal CRL at 98dpc was increased by HPERI diet compared to LPERI ($P - < 0.05$) and by this stage of development also tended to be increased by HPOST diet compared to LPOST ($P < 0.1$). Fetal BPD tended to be increased by HPERI diet ($P < 0.1$). In males, BPD tended to be increased in those fetuses whose mothers were fed the HPERI diet compared to LPERI ($P < 0.1$). For females, maternal nutrition during PERI or POST did not affect BPD at 98dpc ($P > 0.1$).

Calf size at birth

There was no effect of dietary treatment on birthweight, CRL or head measures (all $P > 0.1$). Gestation length did however affect birthweight ($P < 0.05$). Male calves were not larger or heavier at birth than female calves ($P > 0.1$).

Discussion

The present study demonstrates a significant difference in bovine conceptus size as early as 36 days post-conception in
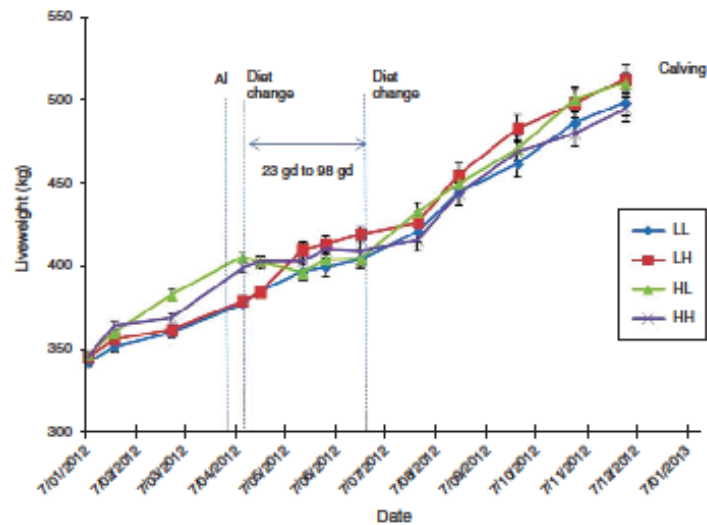
Fig. 2.　Liveweight over time of heifers in each dietary treatment group. HH = high protein peri-conception and first trimester, LL = low protein peri-conception and first trimester, HL = high protein peri-conception and low protein first trimester, LH = low protein peri-conception and high protein first trimester.

response to maternal dietary protein restriction during peri-conception and first trimester. Further, this effect was gender specific there being a greater effect of early dietary intervention on measures of male fetal growth at least until the end of the first trimester.

Nutritional intervention

The 360 heifers were individually fed for a total of 14 months (including acclimatisation) and inseminated via fixed time AI over 1.5 days. Thus, the nutritional regimens were instigated at exactly the same time points in gestation and were controlled with heifers being fed individually from 60 days before AI and throughout gestation until term. This is similar to previous fetal programming studies reported by (Sullivan *et al.* 2009; Micke *et al.* 2010*a*) but not others (Cafe *et al.* 2006; Greenwood and Cafe 2007; Hickson *et al.* 2008; Long *et al.* 2009, 2012). Furthermore, in these latter three studies, realimentation of the restricted heifers was applied so that all calved at similar weight. In this study the high protein diet group had average daily gain of 0.4 kg/day and low protein 0.2 kg/day over the treatment period. After this time they continued to be individually fed throughout gestation at positive weight gain [unlike studies with negative gain in the low treatment (Cafe *et al.* 2006)]; there being also no attempt, to attain similar liveweight at parturition. This difference in (1) nutritional window of dietary intervention and (2) severity and period of restriction applied, may explain the apparently conflicting results in fetal development reported from bovine fetal programming studies.

Sexual dimorphism

This period of dietary intervention is earlier than any previous studies investigating bovine fetal growth trajectory in utero. The sexual dimorphism observed, such that the effect peri-conception protein restriction effect was greater in the male, supports our previous findings in beef cattle (Micke *et al.* 2011, 2014). This suggests that the male embryo is more susceptible to early gestational intervention than the female, as seen in the *in vitro* embryo (Bermejo-Alvarez *et al.* 2011), and that this effect continues until at least 98dpc. The reason for this gender difference may be that the pattern of DNA methylation during embryo development in the two sexes is different over time making susceptibility to epigenetic change both gender and time dependent (Dobbs *et al.* 2013). We note however, that the expected gender effect on birthweight where the male is larger and heavier than the female was not observed in this study.

Birthweight

The finding that maternal peri-conception and first trimester nutrition did not affect birthweight at term may be an important dystocia management consideration when considering timing of supplementation to heifers. This is in agreement with previous reports following first trimester intervention (Micke *et al.* 2010*b*) and between 30 and 123dpc (Long *et al.* 2009). If restriction is applied during the second trimester (Micke *et al.* 2010*b*), however, or second and third trimester (Cafe *et al.* 2006) birthweight has reportedly been affected.

The lack of effect on gross morphology at birth, however, does not necessarily reflect the impact of early gestational intervention upon long-term growth, development and health in the offspring (Wu *et al.* 2006) as we have previously reported. In sheep, peri-conceptional undernutrition does not necessarily result in altered birthweight but has been associated with altered fetal growth trajectory and altered post-natal growth, metabolic and endocrine regulation (Jaquiery *et al.* 2011). The post-natal effects of similar periods of peri-conceptional undernutrition in the bovine are less well understood. Further studies of the post-natal development of the progeny are under way.

Acknowledgements

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63

Chapter 3

Maternal peri-conceptional and first trimester protein restriction in beef heifers:

1. Impacts upon maternal performance and early fetal development.

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Principal Author

Name of Principal Author (Candidate)	Katrina Copping		
Contribution to the Paper	Contributed to experimental design and collection of data, performed statistical analysis and interpreted data, wrote the manuscript.		
Overall percentage 70 (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	30/10/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Andrew Hoare		
Contribution to the Paper	Collected fetal ultrasonography data		
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Contribution to the Paper	Contributed to initial funding application and concept.		
ignature		Date	14/10/16

Contribution to the Paper	Co-supervisor. Contributed to initial funding application. Contributions to sample collection and manuscript evaluation.
Signature	Date 12/9/2016

Name of Co-Author	Viv Perry
Contribution to the Paper	Principal supervisor, advisory role, overall project design and co-ordination, contributions to experimental design, procedures, animal monitoring, sample collection, evaluation and editing of the manuscript.
Signature	Date 20/11/2016

Maternal peri-conceptional and first trimester protein restriction in beef heifers: 1. Impacts upon maternal performance and early fetal development[1]

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ABSTRACT: The effect of dietary protein restriction during the peri-conception period (PERI) and first trimester (POST) upon circulating hormones and metabolites, conception rates and early fetal growth was evaluated in yearling nulliparous heifers. Heifers were individually fed high or low protein diets (HPeri and LPeri) for 60 d before conception. At 23 d post-conception (dpc), half of each treatment group changed to an alternative post-conception high or low protein diet (HPost and LPost) yielding four groups in a 2 x 2 factorial design. A subset of 46 heifers was necropsied at 98 dpc; the remaining heifers (n = 64) were fed a common diet to meet requirements until parturition. Protein restriction was associated with lower maternal BW subsequent to reduced (but positive) ADG during the PERI and POST periods ($P < 0.05$). LPeri heifers had greater ADG than HPeri during the POST diet period ($P < 0.05$) and LPost heifers tended to have greater ADG than HPost during the second and third trimester ($P = 0.05$). Subsequently, BW were similar between dietary treatments at term. Pregnancy rate did not differ but embryonic loss between 23 and 36 dpc tended to be greater in LPeri heifers ($P = 0.09$). Overall, a greater proportion of male fetuses was detected at 60 dpc (63.3 vs. 36.7%; $P = 0.009$). Protein restriction decreased maternal plasma urea ($P < 0.05$ on -19, 23, 36, 60 and 95 dpc), NEFA ($P < 0.05$ on -19 dpc), progesterone ($P < 0.05$ on 23 and 36 dpc) and leptin ($P < 0.05$ on 95 dpc), and increased IGF1 ($P < 0.05$ on 60 dpc). Hormone and metabolite profiles, however, varied dependent upon the sex of the conceptus at critical stages of fetal development. In heifers carrying a male fetus, protein restriction decreased progesterone ($P < 0.05$ on 36 and 95 dpc), increased IGF1 ($P < 0.05$ on 36 dpc) and decreased NEFA *($P < 0.05$ on -19 and 256 dpc)*. At 95 dpc, LPost decreased leptin although LPeri diet had a carryover effect increasing leptin, in consort with increased ADG in this cohort ($P < 0.05$). In males, fetal crown-rump length was negatively correlated with IGF2 at 36 dpc ($P < 0.05$). In heifers carrying a female fetus, protein restriction decreased progesterone ($P < 0.05$ on 23 dpc), leptin ($P < 0.05$ on -19 and 95 dpc) and IGF2 ($P < 0.05$ on 36 dpc). In females, bPL and IGF1 were positively correlated at 128 and 190 dpc ($P < 0.05$). The results of this study demonstrate that protein restriction depresses maternal ADG, is

associated with maternal endocrine and metabolic perturbations dependent on fetal sex and may contribute to early fetal loss.

Key Words: beef, protein, fetal programming

INTRODUCTION

Maternal nutrition has been reported to influence maternal reproductive performance, fetal development and postnatal performance of the offspring in ruminants (Edwards and McMillen, 2002; MacLaughlin et al., 2005; Sullivan et al., 2010b; Micke et al., 2011b, a; Mossa et al., 2013). Wide variations in natural feed resources in extensive farming systems are common. In the northern Australian rangelands, protein, rather than energy, is the major limiting nutrient (Norman, 1963) with protein supplementation of replacement heifers a common management practice (Bortolussi et al., 2005; Burns et al., 2010). Embryo mortality in early gestation [fertilisation to 45 d post-conception (**dpc**)] accounts for the majority of reproductive inefficiency in beef herds between conception and weaning (Burns et al., 2010). Consequently, investigations into the effects of protein supplementation upon embryo survival and subsequent fetal development are critically important to the beef industry.

Growth of the fetus and the placenta are dependent on the hormonal and metabolic status of the cow. We have previously reported in preliminary communications that peri-conception restriction of protein reduced fetal growth in a sex-specific manner as early as 36 dpc in heifers (Copping et al., 2014). Dietary protein and energy are known to affect the function of the corpus luteum, which is integral to the production of sufficient progesterone to enable elongation of the embryo (Fair and Lonergan, 2012) before implantation (Chew et al., 1979). Furthermore, normal placental differentiation, development and function is progesterone dependent (Hoffmann and Schuler, 2002) with the placenta itself effecting fetal growth and development via both endocrine and metabolic mechanisms including IGF

(Sferruzzi-Perri et al., 2011), leptin (Fowden and Forhead, 2009), bovine pregnancy associated glycoprotein (**bPAG**), bovine placental lactogen (**bPL**) (Wooding, 1992) and glucocorticoids (Gardner et al., 2007; Fowden and Forhead, 2009).

In this paper we present the effects of protein restriction upon the physiology of the developing yearling heifer, subsequent conception rates and early *in utero* fetal development. We hypothesize that low maternal dietary protein during the peri-conception period and early gestation alters metabolic and endocrine profiles of the dams, increases early embryonic loss and reduces fetal development in a sex-dependent manner.

MATERIALS AND METHODS

Use of animals and the procedures performed in this project were in compliance with Australian code for the care and use of animals for scientific purposes (NHMRC, 2004) and were approved by the University of South Australia IMVS Animal Ethics Committee (Approval number: 18/11) and The University of Adelaide, Australia (Approval numbers: S2012-249).

Animals, Experimental Design and Treatments

The overall aim of this 5-yr study was to evaluate the long term impact of dietary protein restriction during the peri-conception (**PERI**; -60 to 23 dpc [implantation being 18 to 22 dpc; Wathes and Wooding (1980); Spencer et al. (2007)] and first trimester (**POST**; 24 to 98 dpc) periods upon maternal performance, fetal development and offspring physiology, growth and reproduction. The study was a two-by-two factorial design (Fig. S1). The animals were the heifers and their progeny that have previously been described in brief (Copping et al., 2014). Three hundred and sixty nulliparous Santa Gertrudis (*Bos taurus* x *Bos indicus*) heifers were selected from S Kidman and Co herds located at 'Glengyle' and 'Morney

Plains', south western Queensland, Australia. All heifers were vaccinated on two occasions 4 wk apart against viral and bacterial diseases (Websters Bovine Ephemeral Fever Vaccine (Living), Zoetis Australia, West Ryde, NSW, Australia; Bovilis MH+IBR, Coopers Animal Health, South Granville, NSW, Australia; Pestigard, Zoetis Australia,West Ryde, NSW, Australia; Ultravac Botulinum, Zoetis Australia, West Ryde, NSW, Australia; Ultravac 7 in 1, Zoetis Australia, West Ryde, NSW, Australia; Vibrovax, Zoetis Australia, West Ryde, NSW, Australia) before transport. Heifers were transported to 'Tungali', Sedan, South Australia, Australia (34º29´S, 139 º18´E) where they underwent a 60-d acclimatization period before commencement of the study. Heifers that did not acclimatise to feeding in individual stalls were removed.

At 12 mo of age, 60-d before AI, heifers were stratified by BW and randomly assigned to two equal PERI diet treatment groups, high (**H**) and low (**L**) protein (**HPeri** and **LPeri**; -60 to 23 dpc). Each heifer was fed a high (71 MJ ME and 1.18 kg CP·heifer^{-1}·day^{-1}) or low (63 MJ ME and 0.62 kg CP·heifer^{-1}·day^{-1}) protein diet consisting of a pelleted diet (Appendix 1) supplemented with a commercial vitamin and mineral concentrate (Minmix, Ridley Agriproducts, Toowong, Qld, Australia). The ration was measured and fed individually in stalls each day with straw (5% CP) available *ad libitum* in pens.

Heifers underwent a progesterone-based estrous synchronization program. Intravaginal progesterone-releasing devices were inserted sequentially on d -10 (progesterone 1.56 g, CUE-MATE , Bioniche Animal Health, Bayer Australia Ltd, Pymble, Australia) and heifers treated intramuscularly with 2 mg estradiol benzoate (Bomerol, Bayer Australia Ltd, Pymble, Australia). On d -2 intravaginal devices were removed and heifers treated intramuscularly with 2 ml PG (PGF2-alpha) (Ovuprost, Bayer Australia Ltd, Pymble, Australia) and 300 iu eCG (Pregnecol, Bayer Australia Ltd, Pymble, Australia). Twenty-four hours after device removal, heifers were treated intramuscularly with 1 mg estradiol benzoate. On d 0, the heifers were inseminated with semen from one Santa Gertrudis bull.

71

At 23 dpc, each nutritional treatment group was stratified by weight and half were randomly swapped to the alternative POST treatment (24 to 98 dpc), high (**HPost**: 102 MJ ME and 1.49 kg CP$\cdot$heifer$^{-1}\cdot$d^{-1}) or low (**LPost**: 98 MJ ME and 0.88 kg CP$\cdot$heifer$^{-1}\cdot$d^{-1}) (Table 1), giving rise to four groups: [HPeri-HPost (**HH**), HPeri-LPost (**HL**), LPeri-HPost (**LH**), LPeri-LPost (**LL**)]. Pregnancy was confirmed at 36 dpc and sex of the fetus determined at 60 dpc by rectal ultrasound. Fetuses were measured using trans-rectal ultrasonography (Sonosite M-Turbo; Sonosite Inc. - US, Bothell, Washington) at 36, 60 and 95 dpc as reported in preliminary communications (Copping et al., 2014). Briefly, measures taken at each respective time-point were dependent on the stage of development and included crown-rump length (**CRL**), abdominal diam. (**AD**), umbilical cord diam. (**UD**), biparietal diameter (**BPD**), Crown-nose length (**CNL**) and eye socket diam. (**ED**) as previously defined (Micke et al., 2010b).

A subset of forty six heifers (singletons) were humanely slaughtered in a commercial abattoir at 98 dpc (Copping et al., 2014) and fetuses of both sexes collected [singleton pregnancy: HH (6 male, 6 female); HL (10 male, 5 female); LH (5 male, 5 female); LL (4 male, 5 female)]. The fetal organ weights, fetal morphology and gross placenta parameters are reported in an accompanying paper. From the end of the first trimester of gestation (98 dpc), the remaining heifers (n = 64) were fed the same diet, which was formulated to provide additional growth of 0.5 kg/heifer$\cdot$d^{-1} until parturition (99 dpc to term; 79 MJ ME and 0.92 kg CP/heifer$\cdot$d^{-1}; Table 1). Heifers continued to receive the measured pellet portion of their diet individually on a daily basis with straw (5% CP) provided *ad libitum* in pens until parturition. One heifer (LL) gave birth to twins, and was subsequently excluded, leaving sixty-three heifers, singleton calves [HH (10 male, 8 female); HL (14 male, 4 female); LH (11 male, 4 female); LL (9 male, 3 female)] reported herein.

Heifers were weighed at approximately monthly intervals and immediately after birth. Average daily gain (ADG) was calculated by dividing the difference in BW between the start

and the end of each diet period (PERI, POST, 2nd and 3rd trimester) by the number of days after the start of that period.

Blood Sampling

Blood samples were collected by tail venipuncture at approximately monthly intervals beginning 1 mo before conception. Samples of whole blood were collected directly into 10 ml Vacutainer tubes containing 17 international units of lithium-heparin/ml of blood (Becton, Dickinson and Company, Plymouth PL6 7BP, UK). Tubes were gently inverted by hand for 5 to 10 s and placed on ice before centrifugation (Eppendorf 5702R, Eppendorf Zentrifugen GMBH, Leipzig, Germany) at 3,000 x g at 4ºC. Plasma was harvested and stored at -80ºC until assayed.

Metabolite and Hormone Assays

Bovine Pregnancy Associated Glycoproteins. A monoclonal based bPAG ELISA modified from Green et al. (2005) and as described by Pohler et al. (2016) was used to measure bPAG in plasma at 36, 60, 95, 128, 190, 256 dpc and at birth. The assay sensitivity was 0.28 ng/mL. The intra-and inter-assay co-efficients of variation (CV) were 10.5% and 14.5%, respectively.

Bovine Placental Lactogen. Plasma bPL concentrations at 128, 190, 256 dpc and at birth were determined in duplicate samples by RIA (Wallace, 1993). The assay sensitivity was 0.05 ng/mL. The intra-assay CV was 9.7%, and interassay CV was 10.0%.

Cortisol. Plasma cortisol concentrations at -19, 23, 36, 60, 95, 128, 190, 256 dpc and at birth were determined using a commercial RIA kit (Clinical Assays™, GammaCoat™, Cortisol 125I RIA Kit, DiaSorin, USA). The samples were assayed in duplicate 50 µL

aliquots. The limit of detection was 1.0 ng/ml. The intra-assay CVs for quality control samples containing 8.4 and 32.9 ng/mL were 5.0 and 7.1%, respectively.

Leptin. Plasma leptin concentrations at -19, 23, 36, 60, 95, 128, 190, 256 dpc and at birth were measured in duplicate in a single assay by a double-antibody RIA (Blache et al., 2000). The limit of detection was 0.05 ng/ml. The assay included six replicates of three control samples containing 0.37, 0.99 and 1.73 ng/ml, which were used to estimate the intra-assay CV of 2.54, 6.05 and 6.34%, respectively.

IGF1. Plasma IGF1 at 36, 60, 95, 128, 190, 256 dpc and at birth was measured in duplicate in a single assay by double-antibody RIA with human recombinant IGF1 (ARM4050, Amersham-Pharmacia Biotech, Buckinghamshire, England) and antihuman IGF1 antiserum (AFP4892898, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, CA, USA) after acid-ethanol extraction and cryoprecipitation (Breier et al., 1991). The limit of detection was 0.05 ng/mL and the intra-assay CVs for quality control samples containing 0.20 and 1.79 ng/mL were 7.2 and 3.9%, respectively.

IGF2. Plasma concentrations of IGF2 at 36 dpc were measured in duplicate in a single assay according to the method previously described (Forhead et al., 2011). Briefly, interference by binding proteins was minimised by the acid–ethanol cryoprecipitation method validated for ruminants (Breier et al., 1991). The reagents used were a highly purified human IGF2 (cat no. 031-30, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), a rabbit antiserum to hIGF2 (PAC1, GroPep, Adelaide, SA, Australia, 1:5000), and a 1:5 mixture of secondary antibody (Donkey anti rabbit, G4004, Jackson ImmunoResearch, West Grove, PA, USA, 1:60) and normal rabbit serum (1:500). The assay was validated for use in cattle by checking for parallelism using a serial dilution of pooled ovine plasma samples. The limit of detection was 0.05 ng/mL and the intra-assay CVs for quality control samples containing 1.23 and 2.58 ng/mL were 4.6 and 8.1%, respectively.

Progesterone. Plasma concentrations of progesterone at -19, 23, 36, 60, 95, 128, 190, 256 dpc and at birth were determined by RIA using anti-progesterone antibody-coated tubes (RIA Progesterone #IM1188, Beckman Coulter Australia Pty Ltd, Yeerongpilly, Qld, Australia). The sensitivity of the assay was 0.10 ng/mL. The intra-assay CV for quality control samples containing 1.11 ng/ mL was 1.29%, and the interassay CV was 4.78%.

Metabolites. The metabolites, plasma urea and NEFA were measured in plasma at -19, 23, 36, 60, 95, 128, 190, 256 dpc and at birth. Samples were measured by enzymatic colorimetric analysis on an Olympus AU400 Auto analyser in singlicate using commercially available kits (UREA/BUN, Beckman-Coulter, Gladesville, NSW, Australia for urea; and Wako NEFA-C, Novachem, Collingwood, Vic, Australia for NEFA). Samples were measured in 6 assays. The interassay CV for quality control samples containing 6.46 and 21.93 mmol/L urea and 0.871 and 1.129 mEq/L NEFA were 2.18 and 2.54% and 2.43 and 2.06% for urea and NEFA, respectively.

Statistical Analysis

Statistical analyses were performed using STATA 13.1 (Stata Corp College Station, Texas, USA). Data were checked for normality and transformed before analysis as required. The Fisher Exact χ^2 test was used to analyse data on conception and embryonic loss. Data for maternal BW and ADG for each of the PERI, POST and 2nd and 3rd trimester periods, were analysed using two-way ANOVA to determine the effects of PERI and POST maternal diet and their interaction. Factorial ANOVA was used to interpret the effects of PERI and POST maternal diet, fetal sex and interactions on ultrasonography measures of fetal body dimensions at 36, 60 and 95 dpc in singleton progeny. In addition, factorial ANOVAs tested the effects of PERI and POST maternal diet, fetal sex and interactions on cortisol, leptin, progesterone, urea and NEFA at individual periods of pregnancy (-19, 23, 36, 60, 95, 128, 190, 256 dpc and at birth), IGF1(36, 60, 95, 128, 190, 256 dpc and at birth), IGF2 (36 dpc),

bPAG (36, 60, 95, 128, 190, 256 dpc and at birth) and bPL (128, 190, 256 dpc and at birth). Separate analyses were applied at each time-point instead of a repeated measures approach because maternal diet treatment and number of animals were different over time (i.e., -19 and 23 dpc had only PERI diet treatments and the remainder PERI and POST diet treatments and a sub-set of heifers (n = 46) was euthanised at 98 dpc). Significant interactions were explored with one-way ANOVA and Tukey Kramer post-hoc test as required. Interactions were not significant unless expressly stated in the results. For clarity, in the absence of significant interaction terms the data are presented according to the main effects of PERI and POST maternal diet. Ultrasonography measures and maternal hormone and metabolite data for male and female conceptus were analysed together and separately. All data presented as unadjusted mean ± SEM unless otherwise stated. A P value of < 0.05 was considered statistically significant and P < 0.10 was considered a trend.

RESULTS

Maternal BW and ADG

Heifers were stratified according to BW and randomly allocated into maternal dietary treatment groups. Mean BW at the start and end of each diet (PERI and POST) treatment period, along with ADG, are shown in Tables 1A and 1B, respectively. Neither BW nor ADG were influenced by fetal sex throughout any period of gestation (-60 to 23 dpc; 24 to 98 dpc; 99 to 256 dpc and immediately post-calving; all P > 0.10 respectively).

At the commencement of the experiment, the BW of heifers allocated to the LPeri and HPeri diet groups were similar (P > 0.10). Body weight at the end of the PERI diet period (-60 to 23 dpc) was less in LPeri heifers vs. HPeri (P = 0.04), consequent to the moderately reduced ADG in LPeri heifers during the PERI diet period (P = 0.0001).

At the start of the POST diet period (24 to 98 dpc), BW of LPost and HPost heifers were similar ($P > 0.10$) reflecting the reallocation of heifers from the LPeri and HPeri diets as part of the cross-over experimental design. At the end of the POST period, BW was less in LPost heifers vs. HPost ($P = 0.02$). There was no interaction effect of the PERI and POST diets ($P > 0.10$) at the end of the POST diet period.

ADG during the POST diet period was reduced in LPost heifers ($P = 0.0003$). There was no diet interaction effect for ADG ($P > 0.10$); however, heifers that had been fed LPeri diet had a greater ADG during the POST diet period (24 to 98 dpc) compared to HPeri heifers (0.38 ± 0.03 vs. 0.13 ± 0.02, kg/head· d^{-1}; $P < 0.0001$).

From the end of the POST diet period to term, the remaining heifers received the same individually-fed diet. At 256 dpc, BW did not differ due to dietary treatment (LPeri: 506.4 ± 5.8 vs. HPeri: 502.7 ± 5.2, kg and LPost: 505.8 ± 5.2 vs. HPost: 502.8 ± 5.7, kg, respectively; $P > 0.10$). ADG between 99 and 256 dpc tended to be greater in heifers fed the LPost diet compared to HPost (0.65 ± 0.02 vs. 0.60 ± 0.02, kg·head^{-1}·d^{-1}, respectively, $P = 0.06$). ADG did not differ due to PERI diet (LPeri: 0.62 ± 0.02 vs. HPeri: 0.62 ± 0.02, kg·head^{-1}·d^{-1}), nor was there a diet interaction (both $P > 0.10$). Similarly, immediately post-calving, BW did not differ due to dietary treatment (LPeri: 466.5 ± 6.9 vs. HPeri: 456.0 ± 5.2 and LPost: 458.8 ± 5.2 vs. HPost: 462.2 ± 6.6, kg, respectively; $P > 0.10$). .

Table 1. Maternal BW and ADG at start and end of exposure to diets low or high in protein during the (A) peri-conception (PERI; -60 to 23 d post-conception) and (B) post-conception (POST; 24 to 98 d post-conception) periods of gestation.

A

Item	Diet[1]	
	LPeri (LL+LH)	HPeri (HH+HL)
n	46	63
Start BW, kg	347.7 ± 3.4	342.0 ± 3.3
End BW, kg	384.6 ± 4.1^a	396.6 ± 3.7^b
ADG, kg/d	0.40 ± 0.02^a	0.59 ± 0.02^b

B

Item	Diet[1]	
	LPost (LL+HL)	HPost (HH+LH)
n	54	55
Start BW, kg	390.4 ± 3.7	392.7 ± 4.2
End BW, kg	402.5 ± 3.7^a	414.2 ± 3.8^b
ADG, kg/d	0.17 ± 0.03^a	0.30 ± 0.03^b

[1]LPeri = low protein diet in the PERI period; HPeri = high protein diet in the PERI period. LPost = low protein diet in the POST period; HPost = high protein diet in the POST period. LL = low protein diet in the PERI and POST period; HL = high protein diet during PERI and low protein diet in the POST period; HH = high protein diet in the PERI and POST period; LH = low protein diet during PERI and high protein diet in the POST period

[a,b] Within a row, mean $\pm$ SEM with different superscripts differ ($P < 0.05$).

Pregnancy Rates and Progeny Sex Ratio

The overall pregnancy rate, defined as the number of heifers in calf at 36 dpc (singletons and twins) as identified by ultrasonography, was 36.3%. Conception rates for the heifers who received LPeri diet during the peri-conception period (-60 to 23 dpc) were 7% less than in those fed HPeri diet, but did not differ (33.0% vs. 39.6%; $P > 0.10$). From the laboratory assay used, 7 ng/ml of progesterone in maternal plasma was considered indicative of pregnancy at this point (Starbuck et al., 2004). By this measure, 26 heifers lost the embryo between 23 and 36 dpc; 19 of these had received LPeri diet during the PERI diet period ($P = 0.088$).

Heifers with singleton fetuses that were either necropsied at 98 dpc (n = 46) or calved at term (n = 63), had a greater proportion of male fetuses than females overall (63.3 vs. 36.7%, $P = 0.009$). The proportion of males, however, was similar between the two PERI diet groups (LPeri: 63.0% vs. HPeri: 63.5%, $P > 0.10$).

Maternal Hormones and Metabolites

Progesterone. Overall, heifers fed the LPeri diet had reduced circulating maternal plasma progesterone concentrations at 23 and 36 dpc compared to HPeri (Fig. 1; $P < 0.05$). At 95 dpc, progesterone tended to be greater in those heifers that had received LPeri diet ($P = 0.09$) and also in those receiving HPost diet ($P = 0.08$). At 128 dpc, progesterone varied with an interaction between POST diet and fetal sex ($P = 0.04$) and tended to vary with an interaction between PERI diet and fetal sex ($P = 0.06$). These apparently discordant effects were contingent upon fetal sex as illustrated below.

When analysed separately, within heifers carrying male fetuses (Fig. 2), progesterone was reduced at 36 dpc in heifers that had received the LPeri diet vs. HPeri ($P = 0.02$). By 95 dpc, POST diet influenced progesterone, with concentration reduced in LPost heifers vs.

HPost ($P = 0.004$). At 128 dpc, a carryover effect of the PERI diet was observed with progesterone tending to be greater ($P = 0.06$) in LPeri heifers vs. HPeri.

Within heifers with female fetuses (Fig. 2), progesterone concentration was reduced at 23 dpc in LPeri heifers vs. HPeri ($P < 0.05$). At 128 and 190 dpc, progesterone was elevated in LPost heifers vs. HPost ($P < 0.05$).
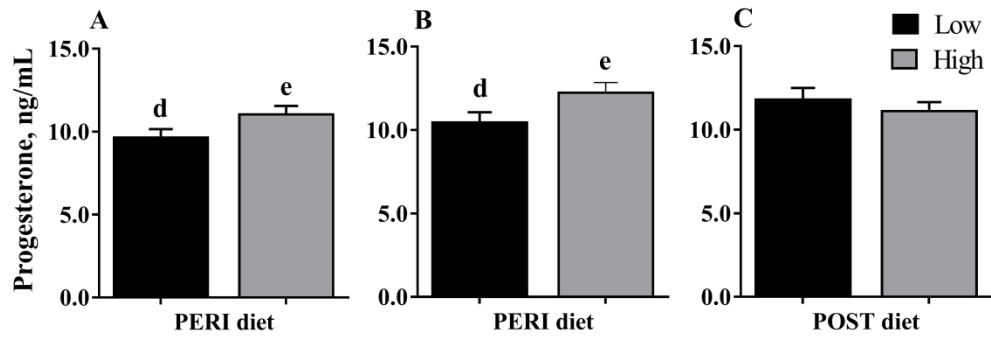
Figure 1. Differences in maternal plasma progesterone concentration in pregnant heifers fed diets low or high in protein during the PERI (-60 to 23 days post-conception) and POST (24 to 98 dpc) periods of gestation for PERI diet groups at 23 dpc (A) and 36 dpc (B) and POST diet groups at 36 dpc (C).

[d,e] Within a graph, mean ± SEM with different superscripts differ ($P < 0.05$).
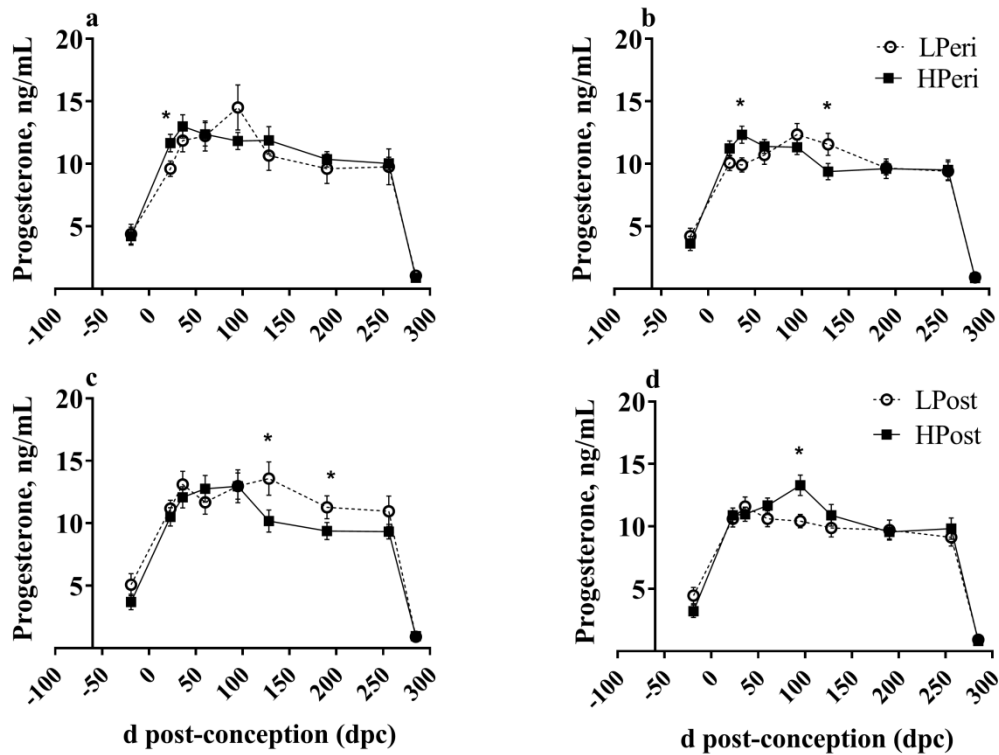
Figure 2. Maternal plasma progesterone concentration profiles from 19 d before conception (-19 dpc) through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a,b; PERI: -60 to 23 dpc) or post-conception (c,d; POST: 24 to 98 dpc) periods of gestation. Data for heifers carrying female fetuses are presented in left panel and in right panel for heifers carrying male fetuses.

[1]Total number of heifers until 98 dpc (n = 109), after 98 dpc (n = 63).

* Denotes mean ± SEM within a time point differ (*P* < 0.05).

Leptin. Overall, at 19 d before conception, at 23 dpc and at 95 dpc maternal plasma leptin concentration varied with an interaction between PERI maternal diet and subsequent fetal sex ($P < 0.05$) as explored below. Also at 95 dpc, LPost heifers had lower leptin concentrations than HPost ($P = 0.0132$). At 128 and 190 dpc, leptin tended to be reduced overall in LPost heifers vs. HPost (both $P < 0.10$).

When sexes were analysed separately (Fig. 3), leptin concentrations in those heifers subsequently determined to be carrying male fetuses did not differ due to PERI diet at either -19 or 23 dpc. At 95 dpc, leptin was less in LPost heifers vs. HPost and there was a carryover effect of PERI diet with leptin concentrations greater in LPeri heifers vs. HPeri ($P < 0.05$). This effect was still observed later in pregnancy with leptin concentrations at both 128 dpc and birth tending to be greater in LPeri heifers vs. HPeri ($P < 0.10$).

Within heifers pregnant with females, leptin was less at 19 d before conception in LPeri heifers vs. HPeri ($P < 0.05$) but was similar at 23 dpc (P= 0.1087). By 95 dpc, POST diet affected leptin with reduced levels in LPost heifers compared to HPost ($P = 0.04$). At 128 dpc, leptin tended to less in heifers that had received the LPost diet ($P = 0.06$).

IGF1. Overall, at 36 dpc maternal plasma IGF1 concentration tended to be increased by LPeri diet compared to HPeri ($P = 0.09$). At 60 dpc, LPost diet increased plasma IGF1 ($P = 0.04$). When analysed separately, within heifers with male fetuses, but not female, (Fig. 4), plasma IGF1 at 36 dpc was elevated by both LPeri ($P = 0.01$) and LPost ($P = 0.04$) diets and at 60 dpc, IGF1 tended to be greater in LPost heifers vs. HPost ($P = 0.09$).

IGF2. Maternal plasma IGF2 concentration at 36 dpc varied with an interaction between PERI diet and fetal sex ($P = 0.02$). When analysed separately, in heifers with male fetuses (Fig. 5), IGF2 was not altered by maternal diet ($P > 0.10$). In heifers with female fetuses, IGF2 was less in heifers that had received the LPeri diet compared to HPeri ($P = 0.02$) but did not differ due to POST diet ($P > 0.10$). Overall, heifers carrying male fetuses tended to have reduced IGF2 at 36 dpc compared to those with females (60.7 ± 1.6 vs. 66.7 ± 2.1 ng/mL; $P = 0.07$).
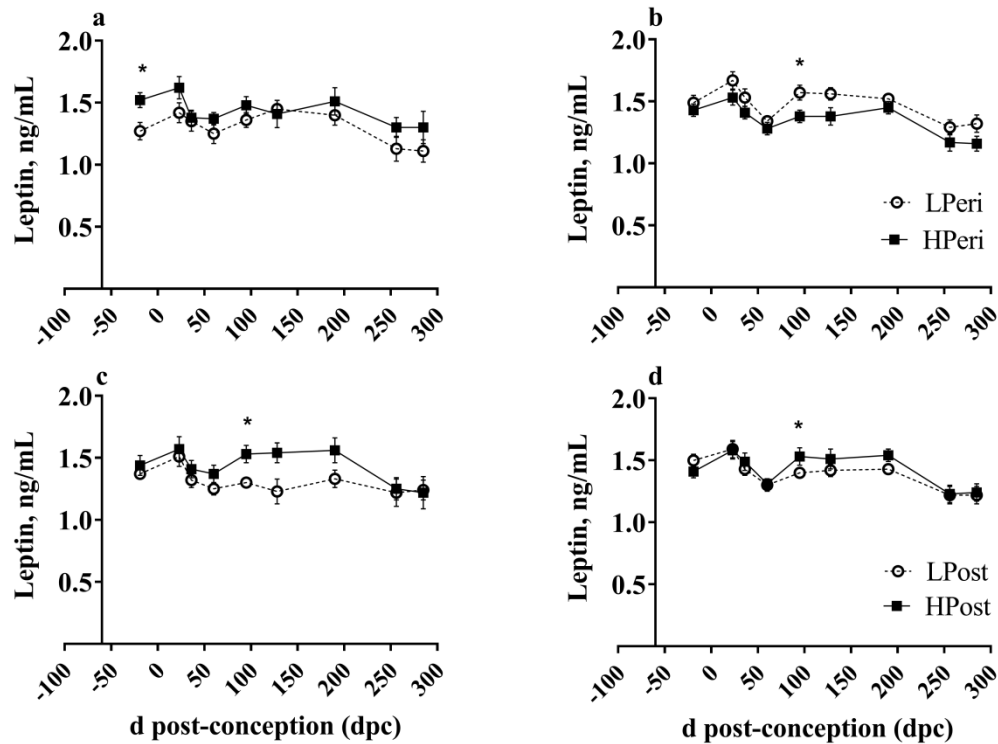
Figure 3. Maternal plasma leptin concentration profiles from 19 d before conception (-19 dpc) through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a, b; PERI: -60 to 23 dpc) and post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers with male fetuses.

[1]Total number of heifers until 98 dpc (n = 109), after 98 dpc (n = 63).

* Denotes mean ± SEM within a time point differ ($P < 0.05$).
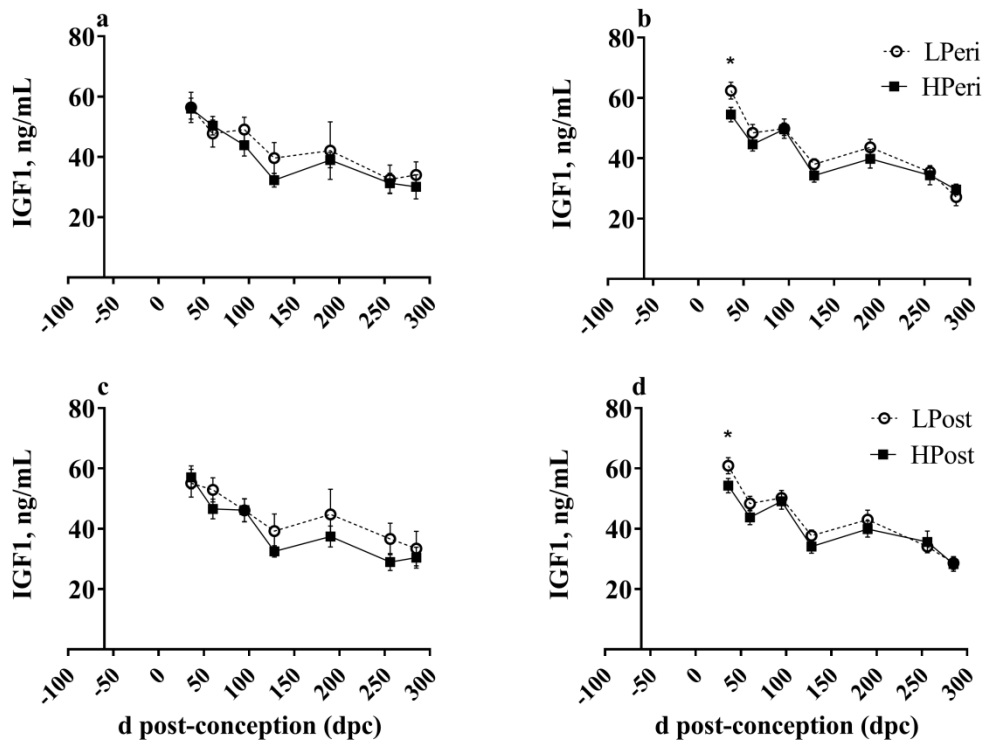
Figure 4. Maternal plasma IGF1 concentration profiles from 36 dpc through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers with male fetuses.

[1]Total number of heifers until to 98 dpc (n = 109), after 98 dpc (n = 63).

* Denotes mean ± SEM within a time point differ *(P < 0.05)*.
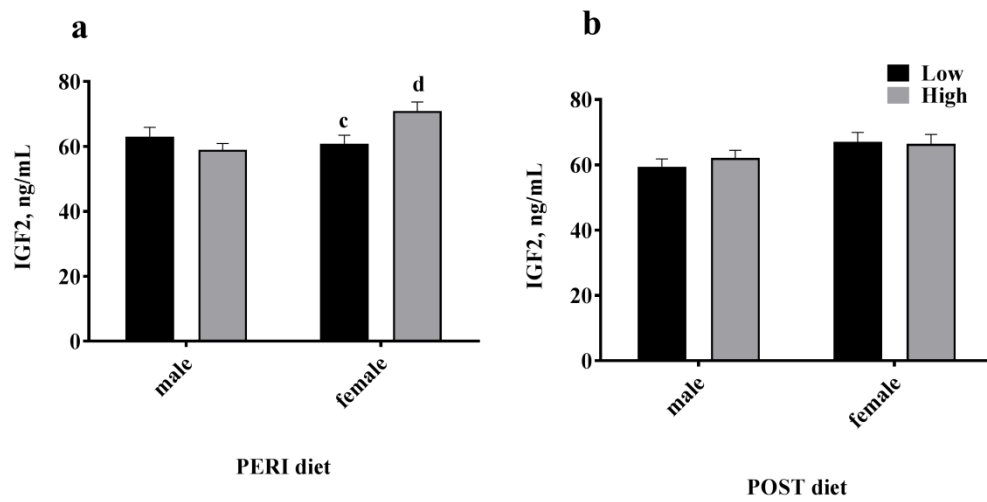
Figure 5. Maternal plasma IGF2 concentrations at 36 d post-conception by fetal sex in heifers fed diets Low or High in protein during the (a) the peri-conception (PERI; -60 to 23 dpc) or (b) post-conception (POST; 24 to 98 dpc) periods of gestation. [c,d]Mean ± SEM differ ($P < 0.05$).

Cortisol, bPL and bPAG. Maternal diet and fetal sex did not affect maternal plasma cortisol, bPAG or bPL concentrations when analysed overall with sexes combined or separately (Fig. 6, 7 and 8; $P > 0.10$) except within heifers carrying male fetuses at 190 dpc, with bPL tending to be reduced in LPost heifers vs. HPost ($P = 0.06$) and in heifers carrying females, cortisol concentration tended to be elevated at birth in LPeri heifers vs. HPeri ($P = 0.06$).

NEFA. Overall, at 19 d before conception maternal plasma NEFA concentrations were less in LPeri heifers vs. HPeri ($P = 0.006$). At 36 dpc, POST diet tended to reduce NEFA in LPost heifers compared to HPost ($P = 0.06$). At 95 dpc, this pattern was reversed with NEFA tending to be greater in LPost heifers ($P = 0.06$). At 23 dpc, NEFA was less overall in heifers with male fetuses compared to those carrying females ($P = 0.04$; 0.11 ± 0.01 vs. 0.15 ± 0.02 nmol/L, respectively). Maternal diet and fetal sex did not affect NEFA concentrations at any other point (all $P > 0.10$).

When analysed separately within heifers carrying male fetuses at 19 d before conception, NEFA was reduced in LPeri heifers compared to HPeri (Fig. 9; $P = 0.0178$). At 23 dpc, NEFA tended to be greater in LPeri heifers ($P = 0.09$). Within heifers carrying females, maternal diet did not influence NEFA concentrations at any point (Fig. 9; $P > 0.10$).

Plasma urea. Circulating plasma urea concentrations reflected the protein level of the maternal diet throughout the PERI and POST diet periods (Fig. 10). LPeri diet reduced urea concentrations compared to HPeri at -19, 23 and 36 dpc (all $P < 0.05$). At 36 dpc, POST diet also influenced plasma urea concentrations which were reduced in LPost heifers compared to HPost ($P < 0.0001$) following the start of the POST diet at 23 dpc. At 60 dpc, urea concentrations varied with an interaction between PERI and POST diets (Fig. 11; $P = 0.0007$). Heifers fed a low protein diet in both PERI and POST periods (LL) had reduced plasma urea concentrations compared to those that changed diet from HPeri to LPost (HL). Both LL and HL groups had reduced plasma urea concentrations ($P < 0.05$) than heifers being fed the HPost diet (LH and HH groups), which did not differ ($P > 1.0$). At 95 dpc, PERI diet

no longer influenced plasma urea ($P > 0.10$) but LPost diet decreased plasma urea concentrations compared to HPost ($P < 0.05$). Overall, plasma urea concentrations did not differ between males and females at any point ($P < 0.05$).

When analysed separately, in heifers carrying males, maternal plasma urea concentrations were reduced by LPeri diet compared to HPeri at 19 d before conception, 23 and 36 dpc (all $P < 0.05$) and tended to be reduced at 60 dpc ($P = 0.10$). After the diet change at 23 dpc, LPost diet was associated with reduced plasma urea concentrations at 36, 60 and 95 dpc (all $P < 0.05$), a tendency to be reduced at 190 dpc *($P = 0.08$)* and reduced concentrations at 256 dpc ($P = 0.02$) compared to HPost. Within heifers carrying females, urea concentrations were reduced in LPeri heifers vs. HPeri at 19 d before conception and 23 dpc ($P < 0.05$), and tended to be reduced at 60 dpc ($P = 0.052$). Urea was reduced by LPost diet at 36, 60 and 95 dpc (all $P < 0.05$).
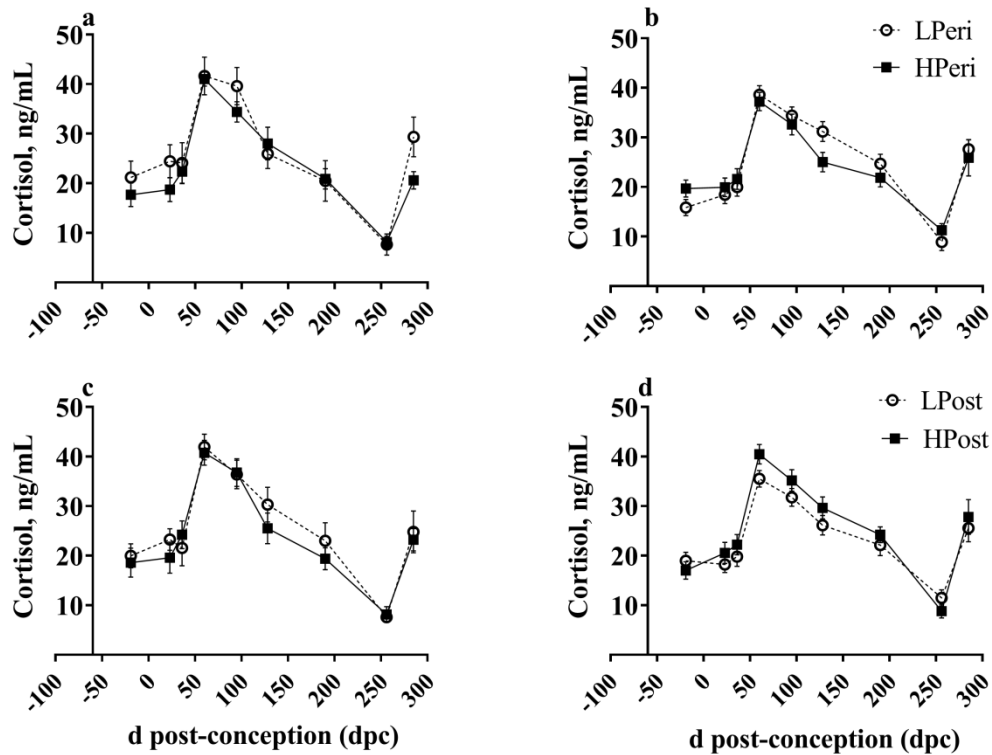
Figure 6. Maternal plasma cortisol concentration profiles from 19 d before conception (-19 dpc) through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during the peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers with male fetuses.

[1]Total number of heifers until 98 dpc (n = 109), after 98 dpc (n = 63).

No differences within any time point ($P > 0.50$).
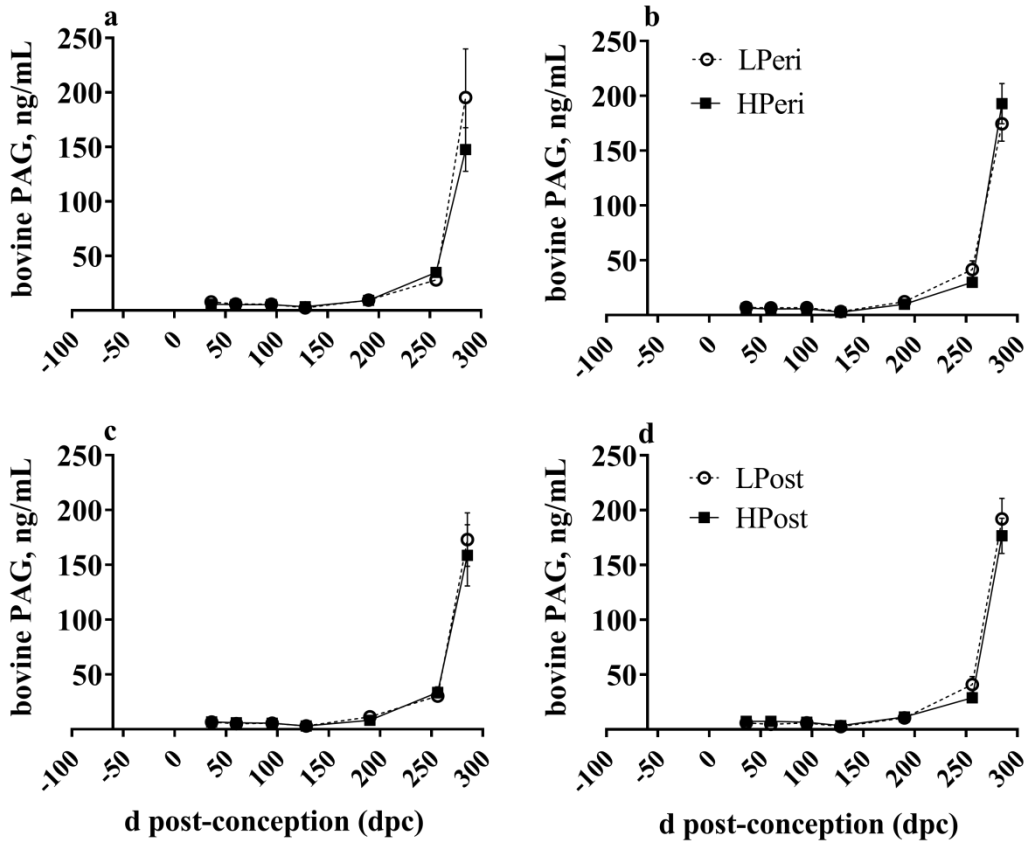
Figure 7. Maternal plasma bovine pregnancy associated glycoprotein (bPAG) concentration profiles from 36 dpc to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during the peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers carrying male fetuses.

[1]Total number of heifers until 98 dpc (n = 109), after 98 dpc (n = 63).

No differences within any time point ($P > 0.50$).
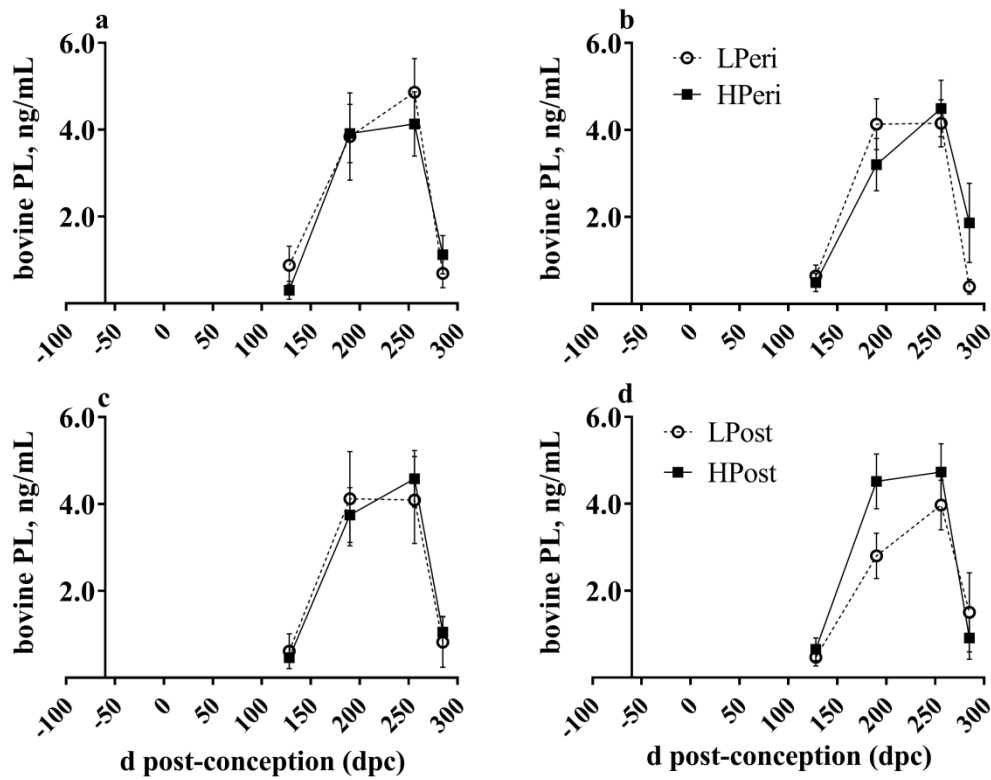
Figure 8. Maternal plasma bovine placental lactogen (bPL) concentration profiles from 128 dpc to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers carrying male fetuses.

[1]Total number of heifers after 98 dpc (n = 63).

No differences within any time point ($P > 0.50$).
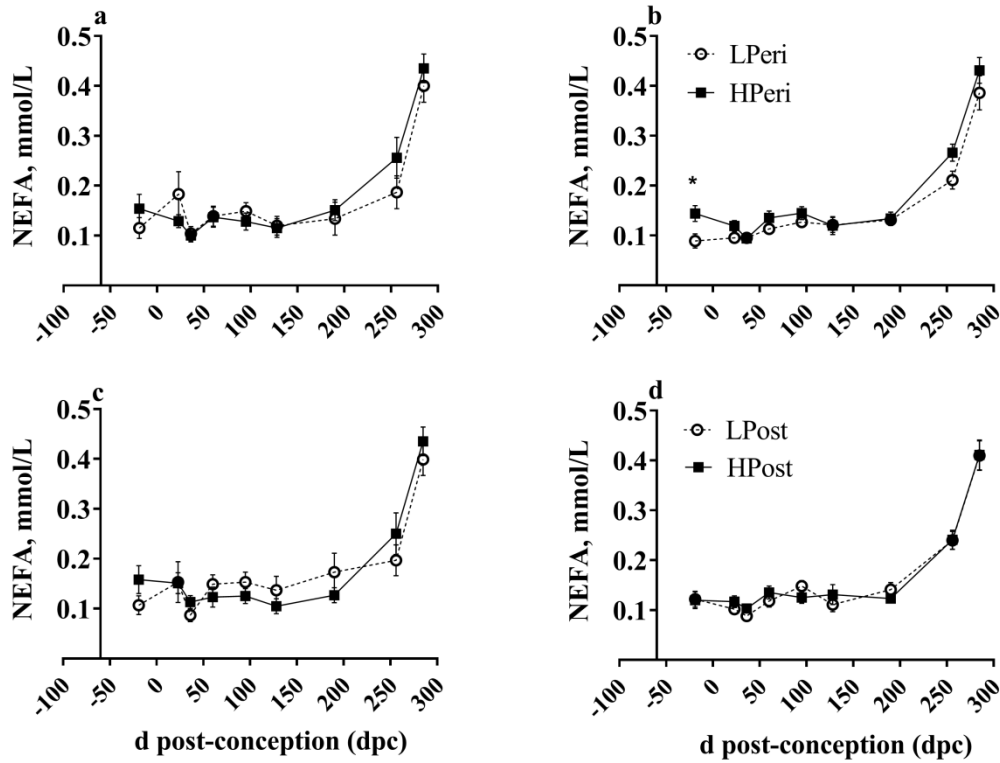
Figure 9. Maternal plasma NEFA concentration profiles from 19 d before conception (-19 dpc) through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers carrying male fetuses.

[1]Total number of heifers until to 98 dpc (n = 109), after 98 dpc (n = 63).

* Denotes mean ± SEM within a time point differ (*P* < 0.05).
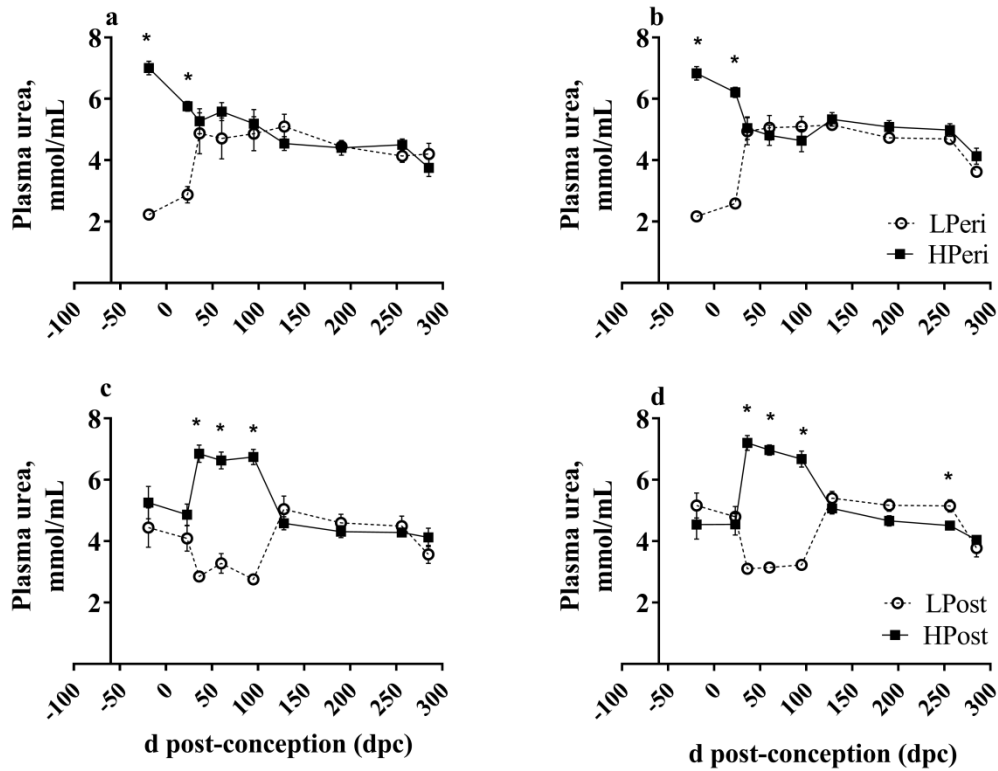
Figure 10. Maternal plasma urea concentration profiles from 19 d before conception (-19 dpc) through to term by fetal sex in heifers[1] fed diets low (L) or high (H) in protein during peri-conception (a, b; PERI: -60 to 23 dpc) or post-conception (c, d; POST: 24 to 98 dpc) periods of gestation. Data from heifers carrying female fetuses (a, c) are presented in the left panel and in right panel (b, d) for heifers carrying male fetuses.

[1]Total number of heifers until 98 dpc (n = 109), after 98 dpc (n = 63).

* Denotes mean ± SEM within a time point differ (*P* < 0.05).
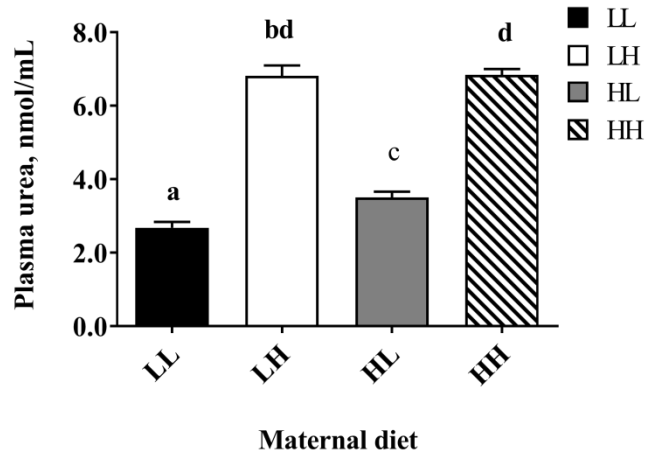
Figure 11. Maternal plasma urea concentration at 60 d post-conception (dpc) in heifers fed

diets low (L) or high (H) in protein during peri-conception (PERI; -60 to 23 dpc) and/or post-

conception (POST; 24 to 98 dpc) periods of gestation.

[a,b,c,d] Mean ± SEM with different superscripts differ ($P < 0.05$); LL = Low level of dietary

protein in the PERI and POST periods; LH = Low level of dietary protein in the PERI and

high in the POST period; HL = High level of dietary protein in the PERI and low in the POST

period; HH = High level of dietary protein in the PERI and POST periods.

Fetal Ultrasound Measures

Ultrasound measures of fetal development at 36, 60 and 95 dpc are presented in Table 2. Ultrasonography measures of CRL at 36 dpc and BPD at 60 dpc respectively, have been reported previously (Copping et al., 2014) in preliminary communications. Briefly, at 36 dpc, LPeri diet decreased fetal CRL overall compared with HPeri ($P < 0.05$) and male fetuses were longer than females overall ($P < 0.05$). When each sex was analysed separately, CRL was reduced by the LPeri diet in the female fetus only (1.39 ± 0.03 vs. 1.49 ± 0.03 cm; $P = 0.047$). By 60 dpc, overall BPD tended to be decreased by the LPost vs. HPost diet ($P < 0.10$); the stronger influence on BPD though was still the LPeri diet ($P = 0.043$). When analysed separately, BPD at 60 dpc was reduced by LPeri vs. HPeri diet in the male fetus only (1.39 ± 0.02 vs. 1.44 ± 0.01 cm; $P = 0.017$). Due to increasing fetal size, ultrasonography measures of BPD at 95 dpc, and CRL at 60 and 95 dpc could not be obtained.

At 60 dpc, AD and CNL did not differ due to maternal diet overall and within each sex ($P > 0.10$). Male fetuses had greater AD than females overall (1.7 ± 0.01 vs. 1.7 ± 0.02, cm; $P = 0.048$) and tended to have a larger CNL than females ($P = 0.07$). At 95 dpc, maternal diet and fetal sex did not affect measures of AD, CNL, ED or UD, either overall or within in each sex (all $P > 1.0$).

Table 2. Ultrasound measurements[1] of fetuses at 36, 60 and 95 d post-conception following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[2].

	Treatment								
PERI	Low		High		*SEM*	*P* value[3]			
POST	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
Item									
Total n	21	25	33	30					
36 dpc									
CRL, cm[4]	1.49	1.49	1.53	1.54	0.014	0.0001	0.049	0.732	0.457
n	21	25	32	30					
60 dpc									
AD, cm	1.73	1.73	1.72	1.71	0.011	0.033	0.142	0.320	0.327
n	21	25	33	28					
CNL, cm	2.53	2.50	2.50	2.49	0.016	0.073	0.558	0.686	0.545
n	21	24	33	28					
BPD, cm[4]	1.42	1.37	1.44	1.42	0.009	0.133	0.043	0.096	0.347
n	21	25	33	30					
95 dpc									
AD, cm	4.51	4.40	4.46	4.49	0.030	0.273	0.510	0.770	0.020
n	21	25	33	30					
CNL, cm	5.57	5.64	5.66	5.58	0.030	0.364	0.537	0.901	0.080
n	21	24	31	26					
ED, cm	1.11	1.12	1.11	1.12	0.008	0.534	0.8362	0.385	0.843
n	21	25	33	29					
UD, cm	1.04	1.03	1.03	1.07	0.011	0.445	0.573	0.497	0.301
n	21	25	33	29					

[1]Ultrasound measurements: CRL = crown-rump length; AD = abdominal diam.; CNL = crown-nose length; BPD = biparietal diam.; Eye D. = eye socket diam.; UD = umbilical cord diam." n" refers to no. of fetuses measured at each ultrasound time point for each measurement taken.

Values are unadjusted mean ± SEM.

[2]Dams were individually fed diets Low or High in protein during the peri-conception (PERI; -60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[3]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

[4]Reported in brief in preliminary communications (Copping et al., 2014) and presented here in entirety for comparative purposes only.

DISCUSSION

This paper reports the conception results, the maternal metabolic and endocrine patterns and early *in utero* fetal measures in a large 5-yr study exploring the effects of maternal protein diet during the peri-conception and first trimester periods in beef heifers upon feto-placental development and offspring production parameters. It is the first study we believe to report on sex-specific effects of peri-conception diet in the bovine upon fetal development. Briefly, varying levels of maternal dietary protein during the peri-conception (-60 to 23 dpc) and first trimester (24 to 98 dpc) periods of gestation in beef cattle resulted in altered fetal development and maternal hormonal and metabolite adaptations in the first trimester. Intriguingly, many of these changes were sex-specific as previously reported in bovines (Copping et al., 2014; Hernandez-Medrano et al., 2015; Micke et al., 2015) and as reported in ovines (Jaquiery et al., 2012).

Nutrition

Wide variations in natural feed resources in extensive farming systems are common in many countries. In the northern Australian rangelands, protein, rather than energy, is often the major limiting nutrient (Norman 1963) with protein supplementation of replacement heifers a common management practice (Bortolussi et al., 2005; Burns et al., 2010). The dietary protein levels used in the current study therefore reflected pasture conditions in Australian rangelands without (low), and with (high), protein supplement. The ration was as isocaloric as possible for ruminants fed the forage component of the diet under group housing and energy and CP content between the low and high diets varied by a 1.1-fold difference and a 1.9 to 2.1-fold difference, respectively. The lipid content of the diet was similar and although there were differences in starch content, levels in both low and high diets were moderately low. Moreover, daily energy (and protein) intakes have greater effects than the substrate (starch,

sugar, fat) from a ruminant nutrition perspective when livestock are at low to moderate levels of production, as was the case in this experiment (Horn et al., 2005). Protein intake was restricted during both the PERI and POST period in the low group whilst both groups received similar energy intake. As the variation in CP content between the low and high diets was substantially more than the difference in energy, we therefore consider the differences observed in the current study are more likely to be attributable to the effects of dietary CP intake during the PERI and POST periods rather than energy intake.

Heifer Performance

The nutritional treatments altered heifer BW and ADG, with protein restriction during the PERI period resulting in small, but significant, differences in BW and ADG by 23 dpc. A similar pattern was observed in heifers fed the low protein diet during the POST period. Interestingly, the influence of the low protein diet extended beyond the PERI and POST maternal diet periods with increased ADG observed subsequent to ceasing nutritional treatments. This suggests that compensatory gain occurred when these heifers received levels of protein in the diet that met or exceeded requirements. This contrasts with the results reported by Sullivan et al. (2009b) where carryover effects of depressed ADG in heifers continued until term following maternal dietary protein restriction during the first and second trimester. However, our results are similar to the catch up growth observed by Mossa et al. (2013). The study by Sullivan et al. (2009b) was in two-year old heifers, as distinct from the adolescent yearling heifers reported herein, and furthermore dietary treatments in the Sullivan et al. (2009b) study were not isocaloric and were applied at different stages of gestation (0 to 180 dpc). This may partly explain the difference in observed effects upon ADG as nutritional effects upon maternal weight are affected by age of the dam (Hennessy et al., 2002). Additionally, although BW in the current study and in that of Sullivan et al. (2009b) was affected by nutritional treatment, it was not associated with weight loss with ADG being

positive throughout. This contrasts with many studies on maternal nutrient restriction in sheep and cattle where more severe dietary treatments were imposed, often associated with periods of maternal weight loss followed by realimentation (Long et al., 2009; Long et al., 2012; Mossa et al., 2013). The heifers in our study were individually fed throughout the study until term [as in the study by (Sullivan et al., 2009b)] to enable observation of effects in animals with limited ability for realimentation until term; a scenario that may be observed under range conditions.

Overall, the pregnancy rate observed in the current study was lower than anticipated but similar to that commonly reported following fixed time AI (FTAI) programs in nulliparous heifers of *Bos indicus* type that have undergone treatment to synchronise ovulation [36.3 vs. 31 to 40 %; (Cavalieri et al., 2002; Butler et al., 2011a; Edwards et al., 2015)]. This relatively poor conception rate following FTAI has been reported to be associated with a high level of ovarian dysfunction including persistent CL's, failure to re-ovulate and a shortened luteal phase (Butler et al., 2011b). Reduced oocyte developmental competence in adolescent heifers (as used in the current study) has also been reported in multiple animal species (Armstrong, 2001) and may be a contributing factor.

Hormones and Metabolites

Protein levels contained in the diet are positively associated with circulating plasma urea concentrations in ruminant species (Anthony et al., 1986; Elrod and Butler, 1993; Butler et al., 1996; Kusina et al., 1999; Wallace et al., 2006b; Swanson et al., 2015). The increased plasma urea concentrations in the heifers fed the high protein diet (HPeri and HPost) were therefore expected as excess dietary protein increases ammonia production in the rumen, which is metabolised to urea by the liver (Huntington and Archibeque, 2000). The concentrations, whilst within an acceptable physiological range (2.8 – 8.8 mmol/L) (Gath et al., 2012), reached levels previously reported as associated with impaired fertility in dairy

cows (Canfield et al., 1990; Butler et al., 1996; Larson et al., 1996). In studies in beef heifers, supplementation with dietary protein elevated plasma urea at the time of conception and in early gestation but had no effect on embryo survival (Kenny et al., 2002; Gath et al., 2012). Kenny et al. (2002) concluded that the previously reported adverse effects of urea upon early oocyte development in lactating dairy cattle may result from the interaction of negative energy balance and excess dietary protein intake rather than a direct outcome of elevated blood urea concentrations, whilst Gath et al. (2012) hypothesized that the negative effects of urea may occur earlier during oocyte development in the ovary rather than during fertilization or post-fertilization. Similarly, in the current study the elevated urea concentration in heifers receiving the high protein diet did not reduce embryo survival.

In contrast, the effect of low maternal plasma urea concentrations upon embryo survival is less clear but has also been linked to poor fertility in dairy cattle (Wathes et al., 2007). Embryo loss between 23 and 36 dpc in the heifers receiving the low protein diet tended to be increased, with plasma urea concentrations in this group below 4.5 mmol/L; a physiological level previously defined as low by Wathes et al. (2007). The low plasma urea concentration may be due to the protein-deficient diet providing fewer precursors for nitrogen supply (Moore and Varga, 1996). This decreased urea concentration occurred in combination with reduced progesterone, the effect of which is discussed below.

Elevated plasma NEFA concentration in cattle is often associated with negative energy balance and is inversely related to ADG and nutrient intake (Lucy et al., 1991; Konigsson et al., 2008). Based on their ADG, heifers in the current study were in a positive energy status irrespective of treatment. Therefore, the reduced NEFA concentration at -19 dpc and the tendency to be reduced at 36 dpc in heifers receiving the low protein diet were unexpected as NEFA concentrations in beef cattle have been reported to be similar when managed to achieve differing, but positive, ADG (Ellenberger et al., 1989; Bossis et al., 2000). There is an energetic cost associated with protein metabolism of diets with increased CP levels (Hales et al., 2013) and this may have mildly altered the energy balance in the

heifers receiving the high protein diet. However, the NEFA concentrations reported, whilst different at several points during the periods of maternal dietary treatment, were relatively low and within the normal physiological range (< 0.05 mmol/L; (Wathes et al., 2007). The subsequent overall pattern of elevation of NEFA concentrations observed in late gestation in the heifers taken to term is as expected (Adewuyi et al., 2005). Collectively, the measures of circulating urea and NEFA as well as leptin (discussed below) indicate that the experimental design was sufficient to alter the metabolic status of the heifers during the periods of dietary intervention despite the heifers being in positive weight gain throughout.

Leptin concentrations are modulated by body fatness and nutrient intake (Houseknecht et al., 1988). The reduction in plasma leptin concentrations observed in the heifers receiving the low protein diet was therefore anticipated. The elevation of leptin at 95 dpc in male-carrying LPeri heifers was unexpected but may be an artefact of the higher ADG also seen in the LPeri heifers during the POST maternal diet period. Interestingly, leptin was decreased 19 d before conception only in the heifers that subsequently carried a female but not a male conceptus. Leptin regulates the hypothalamic-pituitary-thyroid axis via positive regulation of thyrotropin releasing hormone (TRH) production and release (Bianco, 2011). This axis has a significant role in fetal and postnatal growth due to the diverse mechanisms of thyroid hormones in stimulating growth, development and differentiation, and modulating energy homeostasis (Fowden, 1995; Thrift et al., 1999; Micke et al., 2015). The previously reported sex- specific effect of low dietary protein lowering maternal leptin concentrations at pre-term in heifers carrying male fetuses was not observed (Micke et al., 2015); the heifers in that study, however, exhibited a carryover effect of maternal diet with depressed ADG in the third trimester after dietary protein restriction that was not a characteristic of the current study.

Hormones are able to modulate directly or indirectly the maternal and fetal metabolism and nutrient transport in the placenta (Fowden and Forhead, 2009). Indeed the placenta itself also acts as an endocrine organ regulating fetal growth (Chavatte-Palmer et al., 2014) and maternal metabolism (Tarrade et al., 2015). Bovine placental lactogen and bPAG

are glycoproteins produced by placental binucleate cells (Wooding, 1992). Concentrations of these proteins are indicators of placental development and function (Breukelman et al., 2005) and stimulate reallocation of maternal nutrients to the fetus (Bertolini et al., 2006). Bovine placental lactogen was not measured until the second trimester as bPL is known to be only detectable in maternal circulation after 110 dpc (Guilbault et al., 1988). In our study, reduced maternal dietary protein in the peri-conception period and first trimester (LPeri and LPost) did not influence placental function as indicated by bPL and bPAG concentrations. This is contrary to a previous study (Sullivan et al., 2009b; Micke et al., 2015) which showed that bPAG was increased in heifers fed low dietary protein, these authors ascribing this as hormonal signalling by the fetus for enhanced nutrient requirement. The concentrations of bPAG in the current study followed a similar pattern to previously reported profiles in bovine gestation (Green et al., 2005; Wallace et al., 2015) and the lack of effect may be due to the difference in the timing of the nutritional perturbation.

Progesterone has a pivotal role in the establishment and maintenance of pregnancy (Lonergan, 2011; Lonergan and Forde, 2015) through modulation of the luteolytic signal, regulation of conceptus growth and interferon-tau production (Chagas e Silva and Lopes da Costa, 2005). It is established that circulating progesterone levels depend on the quality of the corpus luteum and may be impacted by dietary intake and energy balance (O'Callaghan and Boland, 1999). Inadequate circulating progesterone has been associated with increased levels of early embryonic loss (Lonergan, 2011; Lonergan et al., 2016). Elevated progesterone early post-estrus leads to an altered uterine environment favouring elongation of the conceptus (Lonergan and Forde, 2015) whilst progesterone also directly affects conceptus growth and development (Garrett et al., 1988). In the current study, protein restriction during the peri-conception period was associated with lower circulating progesterone concentrations at several time points. In heifers carrying a female fetus, progesterone measures at 23 dpc [implantation; Wathes and Wooding (1980)] were reduced by the low protein peri-conception diet. This may have contributed to the trend of increased embryonic loss in the LPeri cohort.

The sampling regimen employed, however, did not allow us to fully explore the relationship between progesterone concentrations and embryo loss.

Previous studies show the maternal IGF system has a role in modulating placental growth and development, influencing nutrient partitioning between conceptus and maternal tissues (Sferruzzi-Perri et al., 2006; Sferruzzi-Perri et al., 2011). Moreover, the maternal IGF system itself has been reported to be modulated by factors including endocrine signals (Osgerby et al., 2002) produced by the placenta (Guilbault et al., 1988; Bertolini et al., 2006; Weber et al., 2007) and dietary protein (Perry et al., 2002; Sullivan et al., 2009d). The decline in circulating IGF1 concentration through pregnancy is in agreement with profiles previously reported in beef cattle (Hossner et al., 1997; Sullivan et al., 2009d). The association of maternal dietary protein restriction with small, but significant, elevation in circulating maternal IGF1 levels at several time points in the first trimester is contrary to our previous study (Sullivan et al., 2009d), that showed consistent positive circulating IGF1 response to increased maternal dietary protein. The diet in that study, however, was not isocaloric. Previous studies in cattle suggest that the IGF1 response to dietary protein may be increased when fed in conjunction with increased energy (Elsasser et al., 1989). This may indicate that the response observed by Sullivan et al. (2009d) was modulated, in part, by the energy level of the diet and may also explain the similar effect previously reported in mated yearling beef heifers (Perry et al., 2002). Additionally, in the current study the maternal IGF1 response to dietary protein restriction in early gestation was seen in the heifers carrying male fetuses but not those pregnant with females. The sex of the fetus was not explored individually in the analyses reported by Perry et al. (2002) or Sullivan et al. (2009d) which may have masked differences.

IGF2 is the major fetal growth factor (Constancia et al., 2002; Sferruzzi-Perri et al., 2006). A reduction in maternal IGF2 at 36 dpc in the LPeri cohort carrying females, but not males, may indicate a reduced rate of growth in these embryos as observed in their reduced CRL as exogenous IGF2 is known to induce enhanced fetal growth in the guinea pig

(Sferruzzi-Perri et al., 2007). The IGF2 response to protein restriction is in agreement with Perry et al. (2002) who reported that maternal IGF2 in the second trimester was reduced by maternal dietary protein restriction in *Bos taurus* yearling heifers whilst others have reported that IGF2 is not sensitive to nutrition in cattle (McGuire et al., 1992).

Correlations between IGF1, IGF2 and measures of fetal growth such as weight and CRL at different points in pregnancy, vary between species and dietary regimens (Lok et al., 1996; Osgerby et al., 2003; Sullivan et al., 2009d). In this study maternal IGF2 was negatively correlated with fetal CRL at 36 dpc in heifers carrying males only ($r = -0.29$; $P < 0.05$). This finding is contrary to that reported by Micke et al. (2015) who observed a positive correlation between IGF2 and male, but not female, fetal CRL at 39 dpc.

It has been established that differences exist in growth rate, metabolism and gene expression level between female and male embryos (Gutierrez-Adan et al., 2006). Consequently it has been suggested that environmental conditions may affect embryos differently dependent on their sex (Kwong et al., 2000; Bermejo-Alvarez et al., 2011) and furthermore, that bovine male blastocysts may be more responsive pre-implantation than females under stress conditions (Gutierrez-Adan et al., 2006). Recent evidence suggests the placenta too may differ between sexes in its ability to respond and adapt to adverse environmental conditions (Rosenfeld, 2015). When metabolic, hormonal and fetal development parameters were explored separately by fetal sex in the current study, some of the nutritional treatment effects were dependent upon the sex of the conceptus. In the LPeri cohort, heifers carrying female fetuses displayed reduced levels of progesterone at 23 dpc. This period is immediately post-embryo elongation when adequate progesterone levels are essential (Clemente et al., 2009) before implantation (Wathes and Wooding, 1980) with full adhesion of the embryo occurring by 35 dpc (Senger, 2005). This effect was not observed in the male carrying heifers. Additionally, despite no sex-related differences in circulating plasma urea and NEFA, leptin concentrations were reduced only in the female, but not male, cohort at this point in pregnancy. Interestingly at 36 dpc, as previously reported (Copping et

al., 2014), CRL was decreased by the LPeri diet. When each sex was considered separately, this was observed only in the female concomitant with the reduction in maternal IGF2 concentration. Conversely, by 60 dpc there was a sex-specific growth effect with the male fetus showing a greater reduction in BPD measures from the LPeri diet compared to the female (Copping et al., 2014). Furthermore, a significant correlation between bPL and IGF1 at 128 (r = 0.54; $P < 0.05$) and 190 dpc (r = 0.62; $P < 0.05$) occurred only in heifers carrying a female fetus and may suggest a mechanism whereby the female fetus was signalling enhanced requirement for growth (Bertolini et al., 2006) after ceasing dietary restriction. Collectively, this suggests that the perturbations in endocrine and metabolite status observed in these heifers in response to dietary treatments occurred in consort with modifications in the early growth of their fetus in a sex-specific manner.

Conclusion

The results of this study demonstrate that protein restriction during the peri-conception period (-60 to 23 dpc) and first trimester (24 to 98 dpc) decrease early fetal growth in the bovine in a sex-specific manner in association with maternal endocrine perturbations, and may contribute to embryonic loss. Both outcomes may have implications for the producer of range cattle. In an extensive pasture-based production system, where variation in feed quality through breeding and gestation occurs, quantification of the effects upon long-term productivity of the progeny remains an important area of study.

Acclimatisation	Peri-conception Diet Period	Post-conception Diet Period	2nd and 3rd trimesters
Common diet	Low (L)	Low/Low (LL)	Common diet
		High/Low (HL)	
	High (H)	Low/High (LH)	
		High/High (HH)	

Days post-conception

- 120   - 60   -10   0   23   36   60   98   Calving
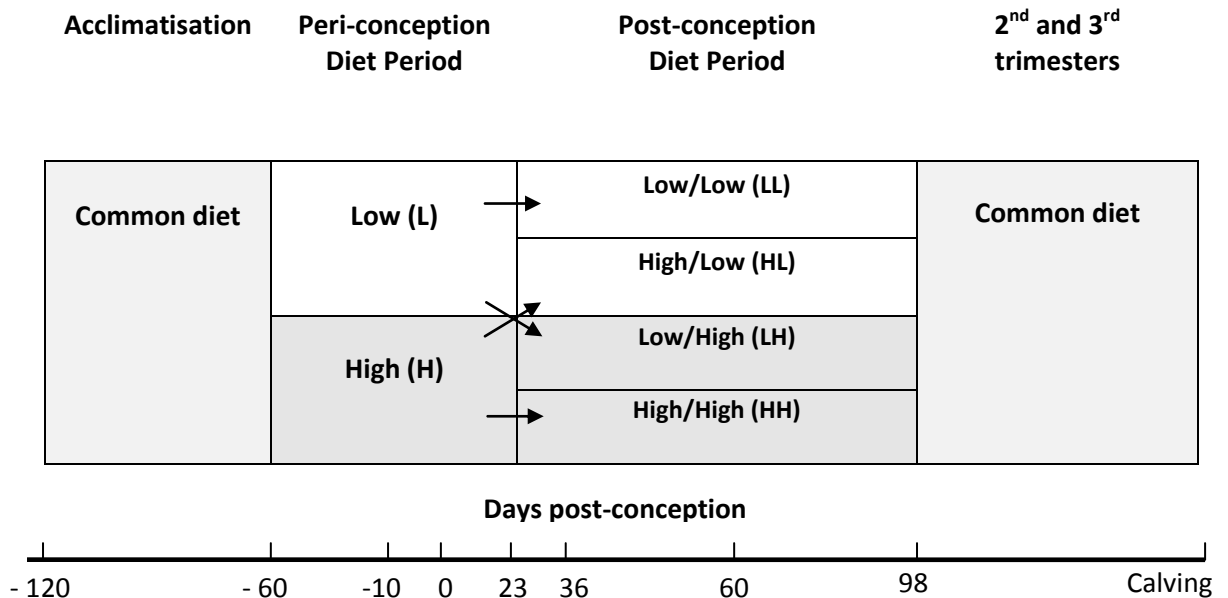
Animal husbandry time-points

- -10 dpc   Estrus synchronisation (CUE-MATES inserted)
- 0 dpc   Artificial Insemination
- 36 dpc   Pregnancy status determined by ultrasound. Non-pregnant heifers removed
- 60 dpc   Fetal sex determined by ultrasound
- 98 dpc   Subset of heifers euthanised (n = 46)

Supplementary Figure 1. Diagrammatic representation of 2 x 2 factorial experimental design (not to scale).

[1]*Abbreviations:* PERI = Peri-conception diet period (-60 to 23 dpc); POST = Post-conception diet period (24 to 98 dpc); Low (L) = low protein diet; High (H) = high protein diet; Low/Low (LL) = low protein diet in the peri- and post-conception period; High/Low (HL) = high protein diet during peri-conception and low protein diet in post-conception period; Low/High (LH) = low protein diet during peri-conception and high protein diet in post-conception period; High/High (HH) = high protein diet in the peri- and post-conception period.

Chapter 4

Maternal peri-conceptional and first trimester protein restriction in beef heifers:

2. Impacts upon placental parameters, fetal and neonatal calf development.

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Name of Principal Author (Candidate)	Katrina Copping
Contribution to the Paper	Contributed to experimental design. Collected cow/calf and placenta samples and data, performed statistical analysis and interpreted data, wrote the manuscript.
Overall percentage 70 (%)	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 30/10/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii.   permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Collected fetal ultrasonography data
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Signature	Date 14/10/16

Contribution to the Paper	Co-supervisor. Contributed to initial funding application. Contributions to sample collection and manuscript evaluation.
Signature	Date 12/9/2016

Name of Co-Author	Viv Perry
Contribution to the Paper	Principal supervisor, advisory role, overall project design and co-ordination, contributions to experimental design, procedures, animal monitoring, sample collection, evaluation and editing of the manuscript.
Signature	Date 20/11/2016

Maternal peri-conceptional and first trimester protein restriction in beef heifers: 2. Impacts upon placental parameters, and fetal and neonatal calf development[1]

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ABSTRACT: The influence of maternal dietary protein during the periconception and first trimester upon fetal and placental development, calf weight and morphology were determined in yearling nulliparous heifers (n = 360). Heifers were individually fed a diet high or low in protein [1.18 g CP/d (**HPeri)** or 0.62 g CP/d (**LPeri**) beginning 60 d before conception. From 24 to 98 d post-conception, half of each treatment group changed to the alternative post-conception diet and were fed 1.49 g CP/d (**HPost**) or 0.88 g CP/d (**LPost**) yielding four treatment groups in a 2 x 2 factorial design. A subset of heifers (n = 46) was necropsied at 98 d post-conception, and placental parameters, fetal morphology, organ weights and fetal hepatic gene expression assessed. From 98 d post-conception, the remaining heifers (n = 64) were individually fed a common diet until parturition when measures of the placenta and neonate were assessed. In the 98 dpc fetus, LPost diet decreased fetal BW in males and females, absolute pancreas weight, septum and ventricle weights and lung weight while increasing relative brain weight ($P < 0.05$). In the male feto-placental unit, but not the female, LPost diet decreased placentome volume ($P < 0.05$) and tended to reduce placentome number, reduced absolute pancreas and liver weights, and brain weight relative to fetal weight ($P < 0.05$). In females, LPost increased relative liver weight ($P < 0.05$). Indicative of brain sparing in the IUGR fetus; LPost diet increased the ratio of crown-nose length to crown-rump length, a measure of fetal adaptation to placental insufficiency ($P < 0.05$) at 98 dpc. Accordingly, in LPost males, although not females, the ratio of brain to liver weight was increased ($P < 0.05$). At term neonatal calf and placental measures were not different ($P > 0.10$). The results of this study demonstrate that protein restriction of adolescent heifers during the periconception period and early gestation decreases fetal growth, alters placenta parameters and produces asynchronous organ development at the 98 d fetus in a sex-specific manner. These changes may contribute to functional consequences for progeny productivity traits; however, these may not be apparent from gross morphometry at birth.

Key Words: beef cattle, protein, fetal programming

INTRODUCTION

Maternal nutrition during gestation has been reported by many studies to influence fetal development, post-natal growth, metabolism and reproduction in species including humans, laboratory and domestic animals of agricultural importance (McMillen and Robinson, 2005; McMillen et al., 2008; Long et al., 2009; Sullivan et al., 2009a; Micke et al., 2010a; Mossa et al., 2013). The effects of episodes of maternal undernutrition and/or over nutrition have been reported to vary depending on the timing, severity and length of nutrient restriction as well as on the sex of the fetus (Hernandez-Medrano et al., 2015; Micke et al., 2015). Critical windows of vulnerability during fetal development differ for specific tissues and organs (McMillen et al., 2001). These periods of vulnerability include; oogenesis and preimplantation period (early embryo development) (Velazquez, 2015), placental development (Perry et al., 1999; Fowden et al., 2006; Vonnahme et al., 2013), organogenesis, and the perinatal period (McMillen et al., 2001; Nathanielsz, 2006). During these critical windows, perturbations such as altered maternal nutrition can result in long-term consequences for the progeny.

Nutritional restriction during the peri-conception period has been reported in sheep to result in impaired blastocyst formation (Borowczyk et al., 2006), altered development of the hypothalamic-pituitary-adrenal axis (Edwards and McMillen, 2002; Bloomfield et al., 2004), changes in pathways that regulate myogenesis and muscle growth and differentiation (Quigley et al., 2005b; Costello et al., 2008), and recently altered hepatic insulin signalling and glucocorticoid regulation of hepatic glucose output, as well as hepatic fatty acid metabolism (Nicholas et al., 2013; Lie et al., 2014b). The effects of maternal nutrition during the peri-conception period and early gestation on feto-placental development in cattle are, however, poorly understood (Mossa et al., 2015; Sinclair et al., 2016).

113

The aim of this experiment was to evaluate the impact of maternal dietary protein during the peri-conception and first trimester periods upon fetal and placental development in the offspring of nulliparous heifers. We hypothesize that restricted maternal protein during the peri-conception period and early gestation reduces fetal and placental development with subsequent deleterious effects upon fetal morphometry at 98 dpc and at term.

MATERIALS AND METHODS

Use of animals and the procedures performed in this project were in compliance with Australian code for the care and use of animals for scientific purposes (NHMRC, 2004) and were approved by the University of South Australia IMVS Animal Ethics Committee (Approval number: 18/11) and The University of Adelaide, Australia. (Approval numbers: S2012-249).

Animals, Experimental Design, and Treatments

The purpose of this study was to evaluate the impact of maternal dietary protein during the peri-conception (**PERI**; -60 to 23 dpc (implantation being 18 to 22 dpc (Spencer et al., 2007; Spencer and Hansen, 2015b) and first trimester (**POST**; 24 to 98 dpc) periods upon fetal and placental development in nulliparous heifers. The maternal dietary protein levels reflected pasture conditions in Australian rangelands without (Low), and with (High), protein supplement.

The study was a two-by-two factorial design. The animals were the heifers and their progeny that have previously been described in brief (Copping et al., 2014). Experimental design and heifer management has been reported in detail in the companion paper (Copping, unpublished data: Chapter 3). In short, three-hundred and sixty nulliparous Santa Gertrudis

114

(*Bos taurus* x *Bos indicus*) heifers underwent a 60-d acclimatisation period prior to commencement of the study at 'Tungali', Sedan, South Australia (34º29´S, 139 º18´E).

At 12 mo of age, 60-d prior to AI, heifers were stratified by bodyweight and randomly assigned to two equal peri-conception (PERI; -60 to 23 dpc) treatment groups, high and low protein (**HPeri** and **LPeri**). Each heifer was fed a high (71 MJ ME and 1.18 kg CP·heifer^{-1}·d^{-1}) or low (63 MJ ME and 0.62 kg CP·heifer^{-1}·d^{-1}) protein diet consisting of a pelleted diet (Appendix 1) that was measured and fed individually in stalls each day. Straw (5% crude protein) was available *ad libitum* in pens. The diet was as isocaloric as possible for ruminants fed the forage component of the diet under group housing and was supplemented with a commercial vitamin and mineral concentrate (Minmix, Ridley Agriproducts, Toowong, Qld).

Heifers underwent a progesterone-based estrous synchronization program and were inseminated with frozen semen from one Santa Gertrudis bull on d 0. At 23 dpc half of each nutritional treatment group was swapped to the alternative post-conception treatment (POST; 24 to 98 dpc), high (HPost: 102 MJ ME and 1.49 kg CP·heifer^{-1}·d^{-1}) or low (LPost: 98 MJ ME and 0.88 kg CP·heifer^{-1}·d^{-1}) (Table 1), giving rise in total to four treatment groups: [HPeri-HPost (**HH**), HPeri-LPost (**HL**), LPeri-HPost (**LH**), LPeri-LPost (**LL**)].

Pregnancy was confirmed at 36 dpc and sex of the fetus determined at 60 dpc by rectal ultrasound. At the end of the first trimester (98 dpc), a sub-set [forty six heifers, singleton pregnancy: HH (6 male, 6 female); HL (10 male, 5 female); LH (5 male, 5 female); LL (4 male, 5 female)] were necropsied. This cohort was randomly selected based on maternal weight and sex of the fetus, however, the HL group had a disproportionate number of male fetuses so a larger number of these animals were available at this point. Remaining heifers (n = 64) were individually fed a common diet until parturition (99 dpc to term; 79 MJ ME and 0.92 kg CP/heifer· d^{-1}; Table 1). The diet was formulated to provide additional growth of 0.5 kg/d. Heifers continued to receive the measured pellet portion of their ration daily until term with straw (5% CP) provided *ad libitum* in pens until parturition. One heifer (LL) gave birth to twins, and was subsequently excluded, leaving sixty-three heifers, singleton calves [HH (10

male, 8 female); HL (14 male, 4 female); LH (11 male, 4 female); LL (9 male, 3 female)]
reported herein.

Heifers were weighed at approximately monthly intervals with this performance reported in a companion paper (Copping, unpublished data: Chapter 3).

Fetal Necropsy

Heifers were humanely killed in a commercial abattoir at 98 dpc and used for human consumption. The gravid uterus was immediately removed and total weight of the uterus and fetus taken. The fetus was excised, weighed, measured and then dissected. Similar to the earlier ultrasonography measures taken of the fetus (Copping, unpublished data: Chapter 3) measures of fetal biparietal diam. (**BPD**), crown-nose length (**CNL**), crown-rump length (**CRL**) and umbilical cord diam. (**UD**) were obtained using sliding vernier callipers. A measure of abdominal circumference (**AC**) was taken at the level of the umbilical cord using a flexible tape–measure.

Fetal brain, heart, kidneys, liver, lung and pancreas were collected and weighed. Due to time constraints in handling the fetal tissue, only the left kidney was weighed. The heart was dissected into the atrial cap, septum, and left and right ventricles with each individually weighed. All other fetal organs were weighed complete. Placentas were dissected from the uterus and all placentomes were counted and weighed. Placentome volume was calculated using saline displacement (Kannekens et al., 2006).

Neonatal Calf Measurements

At calving, heifers were visually monitored 24 h. Calves were collected within 15 min of birth and prior to sucking. Calf sex was recorded and the whole body and trunk measures (Micke et al., 2010b) that were recorded included; calf birth BW, CRL and AC. Cranial

measures recorded were BPD and CNL. Trunk and cranial measures were measured to the nearest 0.5 cm and birth BW to the nearest 0.1 kg. Height was measured from the base of the hoof to the top of the wither. Placentas were collected immediately upon expulsion, checked for completeness and weighed. If the expulsion time of the placenta exceeded 12 h, the placenta was classified as retained fetal membranes (**RFM**) (Peter, 2013). Seven partially eaten placentas and two RFM were excluded from the analyses of all placental measures and calculations (Sullivan et al., 2009c). Cotyledons were dissected away from membranes of each placenta, counted and weighed. Cotyledon volume was calculated using water displacement (Kannekens et al., 2006). Placental efficiency at birth was calculated as a ratio between calf birth BW and placental weight (Leiser et al., 1997).

Statistical Analysis

Data were checked for normality and transformed before further analysis if required. Factorial ANOVA (STATA13.1/IC, StataCorp, College Station, TX, USA) was used to analyse the effects of PERI and POST maternal diet, fetal sex and interactions on fetal morphology, placental measures and organ weights at 98 dpc, calf and placental measures at term and indices of disproportionate growth in singleton progeny only. Gestation length was included as a co-variate for birth measures to account for the length of exposure to the 2nd and 3rd trimester maternal diet. Significant interactions were explored with posthoc comparisons between different diet groups using the Tukey-Kramer test as required. Data for male and female offspring were analysed together and independently. Correlation coefficients were calculated between placental parameters, fetal or birth weight and metabolic hormones at 98 dpc and at term. All data are presented as unadjusted mean ± SEM unless otherwise stated. A P value of < 0.05 was considered statistically significant and $P < 0.10$ was considered a trend.

RESULTS

Fetal Measurements at 98 dpc

Overall measures of fetal and placental parameters at 98 dpc are presented in Table 1. Placentome number was decreased ($P = 0.03$) and placentome volume tended to be reduced by LPeri vs. HPeri diet ($P = 0.06$) whilst LPost vs. HPost diet reduced placentome volume ($P = 0.01$) and weight ($P = 0.047$). Placental efficiency (fetal weight/placentomal weight) did not differ ($P > 0.10$).

Male fetuses were heavier than females overall ($P < 0.001$). As reported by Copping et al. (2014), LPost fetuses were lighter than HPost. Within males and within females, LPost diet decreased fetal weight by 8% and 10%, respectively ($P < 0.05$).

Males had a larger BPD and AC overall at 98 dpc compared to females ($P < 0.05$) and BPD tended to be decreased in LPeri vs. HPeri fetuses (Copping et al., 2014). Abdominal circumference varied with the interaction between PERI and POST diet, and fetal sex ($P < 0.05$). In females only, AC was reduced in fetuses from heifers fed HPeri and LPost (HL) diet compared to those fed a constant high protein diet during both during the PERI and POST periods (HH) (Fig. 1; $P = 0.03$).

Absolute organ and relative weights for fetuses at 98 dpc are reported in Table 1 and Table 2, respectively. Compared with males overall, females tended to have reduced absolute fetal brain weight ($P = 0.051$) but greater when assessed relative to fetal weight ($P = 0.005$). Females also had reduced absolute weights of fetal heart, atrial cap, lung and pancreas ($P < 0.05$), and tended to have reduced absolute liver ($P = 0.07$) and kidney weight ($P = 0.09$), but similar to males when assessed relative to fetal BW (all $P > 0.10$).

Absolute brain weight tended ($P = 0.09$) to be reduced in LPeri vs. LPost fetuses. The PERI diet did not influence the absolute or relative weights of any other fetal organ (all $P > 0.10$). In contrast, POST diet affected the absolute weights of the fetal heart and its structures

along with the lung and pancreas. Heart weight was reduced in LPost vs. HPost fetuses as previously reported (Hernandez-Medrano et al., 2015) along with septum and left and right ventricle weights (all $P < 0.05$). Furthermore, atrial cap weight varied with an interaction between maternal nutrition during PERI and POST with atrial cap weight greater in LH vs. LL fetuses (0.75 ± 0.05 vs. 0.62 ± 0.04, g, respectively; $P = 0.03$). Absolute lung ($P = 0.03$) and pancreas weights ($P = 0.04$) were also reduced in LPost fetuses compared to HPost. Overall, absolute liver weight was unaffected by diet but was reduced in LPost vs. HPost males (Fig. 2; $P = 0.01$) when sexes were analyzed separately. Absolute weights of the fetal kidney did not differ ($P > 0.10$).

Relative fetal brain weight was greater ($P = 0.007$) in LPost compared with HPost fetuses, and also within males (27.2 ± 0.6 vs. 24.9 ± 0.8, g/kg, respectively; $P = 0.045$), but not females. Relative liver weight varied with an interaction between POST diet and fetal sex ($P = 0.02$). Within females, but not males, relative liver weight was increased in LPost vs. HPost fetuses (Fig.2; $P < 0.05$). Relative heart, lung, kidney and pancreas weights were unaffected by maternal diet treatment ($P > 0.10$).

Table 1. Measurements and absolute organ weights of fetuses at 98 d post-conception following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[1].

	Treatment					P value[2]			
PERI	Low		High		SEM				
POST	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
Item									
N	9	10	15	10					
Gravid uteru wt, g	2.71	2.87	2.71	2.83	0.04	0.394	0.768	0.132	0.922
Fetal wt[3], g	294.4	321.3	305.2	327.7	5.36	0.0001	0.695	0.004	0.761
AC, cm	14.39	14.80	14.48	14.92	0.11	0.007	0.862	0.036	0.709
Heart wt[4], g	2.15	2.45	2.31	2.44	0.04	0.007	0.599	0.004	0.512
Left ventricle, g	0.50	0.53	0.51	0.59	0.01	0.073	0.326	0.040	0.376
Right ventricle, g	0.39	0.49	0.44	0.47	0.01	0.340	0.532	0.016	0.210
Septum, g	0.50	0.58	0.54	0.58	0.01	0.202	0.620	0.037	0.568
Atrial cap[5], g	0.61	0.75	0.69	0.65	0.02	0.007	0.539	0.115	0.031
Brain wt, g	8.02	8.32	8.59	8.51	0.01	0.065	0.088	0.502	0.433
Left kidney wt, g	1.17	1.19	1.21	1.24	0.03	0.087	0.637	0.667	0.773
Lung wt, g	8.90	9.26	9.23	10.06	0.17	0.002	0.150	0.040	0.269
Pancreas wt, g	0.17	0.20	0.19	0.21	0.007	0.047	0.660	0.044	0.831
Liver wt, g	10.63	11.55	11.32	11.57	0.23	0.080	0.575	0.182	0.610
Placentome, no.	47.4	46.5	57.5	65.0	3.1	0.095	0.020	0.653	0.609
Placentome wt, g	140.65	146.05	146.95	169.78	5.21	0.843	0.158	0.192	0.428
Placentome	146.33	151.10	149.13	185.42	4.60	0.746	0.062	0.010	0.105

volume, mL

Values are unadjusted mean ± SEM.

[1]Dams were individually fed diets Low or High in protein during the peri-conception (PERI; -60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[2]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

[3] Reported in brief in preliminary communications (Copping et al., 2014) and presented here in entirety for comparative purposes.

[4]Reported in Hernandez-Medrano et al. (2015) and presented here for comparative purposes.

[5]PERI x POST x SEX interaction (P < 0.05).

Table 2. Relative organ weight in fetuses on 98 d post-conception following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[1].

	Treatment				*SEM*	*P* value[2]			
	Low		High						
PERI	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
POST									
Item									
N	9	10	15	10					
Heart wt, g / fetal BW, kg	7.37	7.66	7.57	7.46	0.09	0.167	0.871	0.683	0.211
Brain wt, g / fetal BW, kg	27.44	26.19	28.48	26.01	0.42	0.036	0.374	0.013	0.263
Liver wt, g / fetal BW[3], kg	36.09	35.95	37.32	35.28	0.56	0.076	0.657	0.294	0.305
Lung wt, g / fetal BW, kg	30.44	28.85	30.34	30.75	0.42	0.519	0.274	0.475	0.287
Pancreas wt, g / fetal BW, kg	0.59	0.63	0.62	0.63	0.04	0.779	0.712	0.486	0.730

Values are unadjusted mean ± SEM.

[1]Dams were individually fed diets Low or High in protein during the peri-conception (PERI; -60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[2]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

[3] POST x fetal sex interaction (*P* = 0.02).
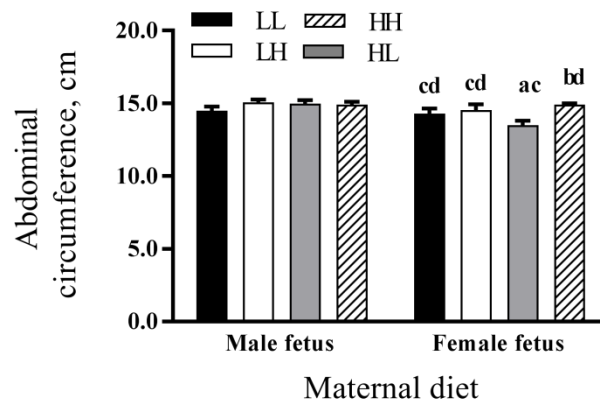
Figure 1. Abdominal circumference in male and female fetuses at 98 dpc from heifers fed diets[1] low (L) or high (H) in protein during peri-conception (PERI; -60 to 23 dpc) and/or post-conception (POST; 24 to 98 dpc) periods of gestation. [a,b,c,d]Mean ± SEM with different superscripts differ ($P < 0.05$)

[1]LL = Low level of dietary protein in PERI and POST periods;

LH = Low level of dietary protein in PERI and high in POST period;

HL = High level of dietary protein in PERI and low in POST period;

HH = High level of dietary protein in PERI and POST periods.
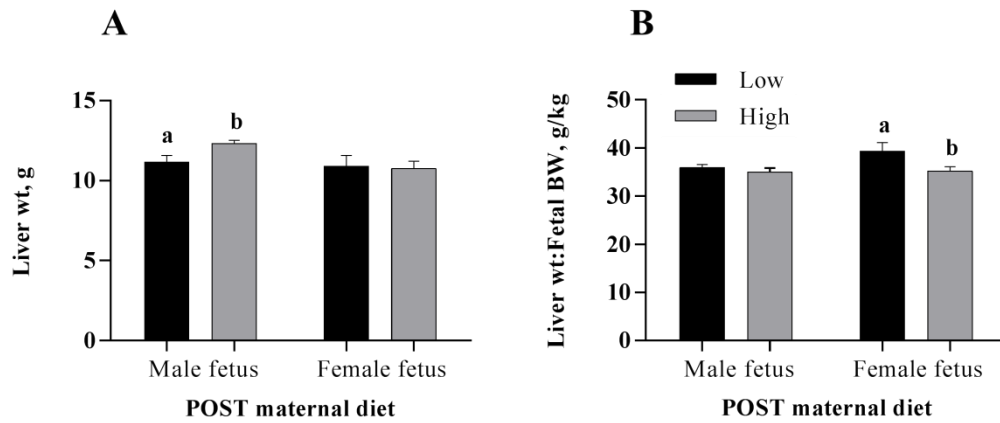
Figure 2. Absolute (A) and relative liver weight (B) in male and female fetuses at 98 dpc from heifers fed diets low (L) or high (H) in protein during the post-conception (POST; 24 to 98 dpc) period of gestation.

[a,b]Denotes mean ± SEM differ ($P < 0.05$)

Indices of Disproportionate Growth at 98 dpc

To investigate the effects of maternal diet on proportional growth, ratios between BPD and AC (**BPD:AC**), CNL and CRL (**CNL:CRL**), CRL and fetal BW (**CRL:fetal BW**) as well as brain and liver weight (**Brain:Liver**) were calculated on the 98 dpc fetal measures (Table 3).

Overall, CRL: fetal BW ratio ($P < 0.001$) was less in males than females but BPD:AC and CNL:CRL were not different between sexes ($P > 0.05$), nor did PERI diet influence any measure of proportional growth ($P > 0.05$). LPost diet increased fetal CNL:CRL ($P = 0.044$) and CRL:fetal BW ($P = 0.003$) and tended to increase BPD: AC compared to HPost ($P = 0.08$). There was a trend for Brain:Liver ratio to vary with an interaction between POST diet and fetal sex ($P = 0.07$). When sexes were analysed separately, Brain:Liver was increased by LPost diet but only in males (0.78 ± 0.03 vs. 0.69 ± 0.03, g/g, respectively; $P = 0.03$) and CNL:CRL also tended to be increased in LPost males (data not presented, $P = 0.06$).

Term Parameters in Neonatal Calf and Placenta

Mean placenta weight across all treatment groups was 4.89 ± 0.17 kg and mean placental efficiency value was 7.0 ± 2.9, kg/kg. Overall, and within males and females (data not presented), placental parameters and placental efficiency at term did not differ due to maternal diet, calf sex or gestation length (Table 4; $P > 0.10$).

Gestation length and morphology measures at birth are presented in Table 4. Mean gestation length was 281.3 ± 0.6 d (range 271 to 293 d). Gestation length tended to be decreased by LPost compared to HPost diet ($P = 0.09$) and was longer for male compared to female calves overall ($P = 0.03$). Mean calf birth BW was 32.1 ± 0.6, kg (range 20.0 to 42.8, kg). Overall, calf birth BW and morphology measures were similar between male and female calves ($P > 0.10$). Maternal diet did not influence calf birth BW overall or within each sex (P

> 0.10) as previously reported (Copping et al., 2014). This was the case either with, or without, the inclusion of maternal BW as a covariate in the statistical model. Calf birth BW was positively correlated overall with gestation length ($r = 0.5416$; $P < 0.05$) and placenta weight ($r = 0.6034$; $P < 0.0001$), irrespective of diet.

Indices of Disproportionate Growth at Birth.

Overall, and within each sex, there were no effects of maternal diet on proportional growth, as measured by ratios between BPD and AC, CNL and CRL, CRL and calf birth BW (Table 5; $P > 0.10$). Ponderal index varied with an interaction between PERI and POST diet ($P = 0.01$) but there was no difference between the four diet groups ($P = 0.119$).

Correlation Coefficients at 98 dpc and Term.

Correlation coefficients between fetal weights, placental measures and metabolic hormones (IGF1 and leptin) at 98 dpc and term are reported in Tables 7 and 8, respectively.

At 98 dpc fetal weight was moderately positively correlated with placentome weight and volume. Placentome weight was strongly positively correlated with placentome number and volume and placentome number was moderately positively correlated with placentome volume. IGF1 had a weak negative correlation with placentome weight and volume.

At term, birth weight was strongly positively correlated with placenta weight and moderately positively correlated with cotyledon weight and volume. Placental weight was strongly positively correlated with cotyledon weight and volume and cotyledon weight was very strongly correlated with cotyledon volume. There were weak negative correlations between leptin and birth weight and placenta weight, and IGF1 and cotyledon number (all P <0.05). Similar correlations between fetal or birth weights and placental measures existed in both male and female feto-placental units at 98 dpc and at term (data not presented, P < 0.05).

However, correlations between IGF1 and placentome weight and volume at 98 dpc and cotyledon number at term occurred only in males whilst the correlation between leptin and birth weight and placenta weight occurred only in females (data not presented, P < 0.05). Additionally, leptin was weakly associated with placentome weight at 98 dpc in the female, but not the male

Table 3. Indices of disproportionate growth[1] in fetuses on 98 d post-conception following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[2]

	Treatment				SEM	P value[3]			
PERI	Low		High						
POST	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
Item									
N	9	10	15	10					
BPD, mm : AC, cm	2.47	2.44	2.55	2.46	0.02	0.475	0.290	0.206	0.477
CNL, mm : CRL, mm	3.17	3.04	3.23	3.13	0.03	0.143	0.168	0.39	0.940
Brain wt, g : Liver wt[4], g	0.77	0.73	0.77	0.73	0.01	0.373	0.833	0.199	0.900
CRL, mm : Fetal BW, g	0.06	0.06	0.06	0.06	0.001	<0.001	0.759	0.005	0.776

Values are unadjusted mean ± SEM.

[1]BPD: AC – Biparietal diameter: Abdominal circumference ratio

CNL: CRL – Crown-nose length : Crown-rump length ratio

Brain : Liver – Brain weight : Liver weight ratio

CRL: Fetal - Crown-rump length : Fetal BW ratio

[2]Dams were individually fed diets Low or High in protein during the periconception (PERI;- 60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[3]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

[4] POST x SEX interaction (*P* = 0.07).

Table 4. Gestation length, neonatal calf morphology measures and measurements of placental parameters at term[1] from heifers exposed to diets low or high in protein during the peri-conception and first trimester periods of gestation[2].

	Treatment				SEM	*P* value[3]			
PERI	Low		High						
POST	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
Item									
N	12	15	18	18					
Gestation length, d	281.1	282.3	279.9	282.4	0.7	0.026	0.820	0.091	0.447
Bi-parietal Diam, cm	14.7	14.3	15.6	14.2	0.3	0.448	0.504	0.195	0.699
Crown-Rump length, cm	79.8	9.9	82.1	79.4	0.6	0.595	0.522	0.234	0.105
Crown-Nose length, cm	19.7	20.2	20.6	19.4	0.3	0.673	0.974	0.643	0.186
Wither height, cm	76.7	77.8	78.1	75.9	0.5	0.528	0.975	0.185	0.031
Abdominal circumference, cm	70.8	74.3	71.9	71.8	0.5	0.816	0.585	0.242	0.062
Placenta[1]									
N	11	12	15	16					
Placenta wt, kg	4.95	4.88	5.08	4.66	0.17	0.388	0.906	0.454	0.608
Cotyledon, no.	74.5	87.2	79.1	76.4	2.6	0.787	0.549	0.406	0.149
Cotyledon wt, g	1660.0	1699.0	1662.5	1645.4	54.9	0.420	0.821	0.975	0.788
Cotyledon volume, mL	1610.7	1660.8	1639.0	1637.2	53.8	0.249	0.976	0.876	0.804
Placental efficiency[4]	6.32	7.71	6.68	7.21	0.29	0.642	0.863	0.157	0.409

Values are unadjusted mean ± SEM

[1]Only placental data for the 54 heifers with complete measurements are presented.

[2]Dams were individually fed diets Low or High in protein during the periconception (PERI;-60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[3]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

[4]Placental efficiency = calf birth BW: placenta weight, kg/kg

Table 5. Indices of disproportionate growth in calves at birth from heifers exposed to diets low or high in protein during the peri-conception and first trimester periods of gestation[2]. Values are mean ± SEM.

	Treatment								
PERI	Low		High		*SEM*	*P* value[3]			
POST	Low	High	Low	High		SEX	PERI	POST	PERIxPOST
Item									
N	12	15	18	18					
BPD, cm : AC, cm	0.21	0.19	0.22	0.20	0.004	0.373	0.465	0.153	0.767
CNL, cm : CRL, cm	0.25	0.25	0.25	0.25	0.004	0.601	0.817	0.790	0.781
CRL, cm : Birth wt, kg	2.60	2.45	2.54	2.59	0.041	0.314	0.784	0.897	0.041
Ponderal Index, kg/m^3	31.2	33.1	32.7	31.2	0.55	0.505	0.572	0.625	0.010

[1]BPD: AC = Biparietal diam.: Abdominal circumference ratio

CNL: CRL = Crown-nose length: Crown-rump length ratio

CRL: Fetal = Crown–rump length: Birth WT ratio

Ponderal index = Fetal BW/CRL^3

[2]Dams were individually fed diets Low or High in protein during the periconception (PERI;- 60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[3]Full model analysed was PERI x POST x SEX. The *P* value for main effects and PERI x POST interaction shown for all variables. All other interactions were not significant (*P* > 0.05) unless expressly stated.

Table 6. Correlation coefficients[a] between fetal weight, placental measures and plasma concentrations of IGF1 and leptin in heifers with singleton calves at 98 dpc (n = 46) following exposure to maternal diets low (L) or high (H) in protein during PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) periods of gestation.

	Fetus wt, kg	Placentome wt, kg	Placentome number, no	Placentome volume, mL	IGF1, ng/mL	Leptin, ng/mL
Fetus wt, kg	-					
Placentome wt, kg	**0.45**	-	-			
Placentome number, no	0.17	**0.64**	-	-		
Placentome volume, mL	**0.45**	**0.82**	**0.55**	-		
IGF1, ng/mL	-0.27	**-0.30**	-0.21	**- 0.37**	-	
Leptin, ng/mL	0.02	0.14	-0.13	0.07	-	-

[a] Significant correlations are shown in **bold** ($P < 0.05$)

Table 7. Correlation coefficients[a] between birth weight, placental measures and plasma concentrations of IGF1 and leptin in heifers with singleton calves at birth (n = 63) following exposure to maternal diets low (L) or high (H) in protein during PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) periods of gestation.

	Birth wt, kg	Placenta wt, kg	Cotyledon wt, kg	Cotyledon number, no	Cotyledon volume, mL	IGF1, ng/mL	Leptin, ng/mL
Birth wt, kg	-						
Placenta wt, kg	**0.61**	**-**					
Cotyledon wt, kg	**0.49**	**0.61**	**-**				
Cotyledon number, no	0.05	0.11	0.24	**-**			
Cotyledon volume, mL	**0.55**	**0.64**	**0.96**	0.22	-		
IGF1, ng/mL	0.04	-0.11	-0.03	**- 0.36**	-0.6	-	
Leptin, ng/mL	**-0.28**	**-0.32**	**-0.11**	-0.18	-0.12	0.18	-

[a] Significant correlations are shown in **bold** ($P < 0.05$)

DISCUSSION

This 5-yr study explores the effects of maternal protein diets during the peri-conception and first trimester periods in nulliparous beef heifers upon feto-placental development and offspring production parameters. It is the first study we believe to report sex-specific effects of the peri-conception diet in the bovine on feto-placental development to term. Varying levels of maternal dietary protein during the peri-conception (-60 to 23 dpc) and first trimester (24 to 98 dpc) periods resulted in altered fetal size and body proportions at 98 dpc but similar birth BW at term. This dietary perturbation was associated with sex-specific asymmetric organ development at 98 dpc; a characteristic of intrauterine growth restriction (IUGR) that has been previously reported in the bovine (Micke et al., 2010a; Copping et al., 2014; Hernandez-Medrano et al., 2015; Micke et al., 2015) and in the ovine (Jaquiery et al., 2012). Maternal dietary protein restriction, at the level used in this study, is commonly observed in commercial cattle operations (Bortolussi et al., 2005) and we have previously shown that such restriction alters productivity traits in the progeny (Sullivan et al., 2009e; Micke et al., 2010a; Micke et al., 2011b).

Fetal Organ Development

The 'thrifty phenotype' hypothesis (Hales and Barker, 1991) suggests that gestational undernutrition induces nutrients and energy to be diverted to favour the development of organs such as the brain, at the expense of organs and systems that may be less critical for the immediate survival of the fetus (e.g. kidney, liver) (McMillen et al., 2001; Reynolds and Caton, 2012). These adaptations have been shown in both human and animal studies to be a contributing factor to the development of metabolic diseases in adult life (Redmer et al., 2004; McMillen and Robinson, 2005; Owens et al., 2007; McMillen et al., 2008; Symonds et al.,

2012), and in production animals, to affect economically important reproductive (Sullivan et al., 2009a; Mossa et al., 2013) and carcass traits (Long et al., 2010b; Micke et al., 2010a).

In the current study, the LPost diet affected the absolute weights of the heart (Hernandez-Medrano et al., 2015) and its structures in 98 dpc fetuses, along with the absolute weights of the liver, pancreas and lung. These data are consistent with earlier female bovine fetal studies (Long et al., 2009; Mossa et al., 2013). In the current study, when each fetal sex was analysed separately, the developing cardiovascular system was affected in the female only, associated with altered fetal haemodynamics (Hernandez-Medrano et al., 2015) and cardiovascular development which persisted to the neonatal and adolescent period. These findings correspond with an earlier study (Mossa et al., 2013).

Absolute liver weight at 98 dpc was reduced by the LPost diet but only in the male fetus. Relative liver weight, however, was unaffected in the males but interestingly, was increased in the LPost female fetuses. In sheep, there is a direct relationship between the weight of the fetal liver and fetal body weight, however, the variation in relative liver weight is reported to be less strongly related to fetal body weight than is the brain (McMillen et al., 2001). Such relationships have not been well explored in the IUGR bovine fetus. Additionally, maternal nutrient restriction in sheep has been shown to not only alter fetal liver growth but has also been associated with changes in hepatic function (Lie et al., 2014b). Further study is now underway to explore the effects of maternal protein restriction in the bovine during the peri-conception period and the first trimester upon hepatic gene expression in both the 98 dpc fetal liver and in the liver of the adult offspring. This may provide insight into the underlying molecular pathways which promote susceptibility to increased fat deposition in growing animals subsequent to protein restriction during early oocyte and/or embryonic development (Nicholas et al., 2013; Lie et al., 2014a; Lie et al., 2014b).

Protein restriction also influenced the development of the fetal pancreas, lung and brain at 98 dpc. Pancreas and lung weight were reduced by the LPost diet. Altered structure and function of pancreatic islets has also been reported in rodents following maternal

nutritional perturbation (Fowden and Hill, 2001) whilst dietary protein deficiency has been reported to alter glucose metabolism (Desai et al., 1995). The functional outcomes of these fetal changes are unknown in the bovine as understanding of nutritional programming of the pancreas in ruminants is comparatively less well established than in the rodent (Vonnahme et al., 2007). Preliminary communications from a previous study in nutrient restricted cows (60% NRC, 30 to 140 dpc) showed fetuses had decreased pancreatic islet size at 140 dpc but insulin levels in cord blood were not different to that in control animals (Doscher et al., 2014). As the endocrine component is only a small part of the total pancreatic mass (Limesand et al., 2006), further histology of both the fetal and adult pancreas in the bovine is required to facilitate understanding of the relationship between size and function. In humans, IUGR has been associated with altered lung development and the long-term impairment of lung function (Greenough et al., 2004). In sheep, experimentally induced IUGR has also been reported to alter lung weight but not morphology in the fetus (Sutherland et al., 2012). Postnatally, however, morphological changes in lung structure have been reported and may underlie impaired respiratory function in adulthood (Joyce et al., 2001; Maritz et al., 2001). The post-natal implication of the reduced fetal lung weight in the bovine is unknown, however, bovine respiratory disease in feedlots is recognised as the major cause of illness and death and impaired respiratory function may have potentially negative consequences under feedlot conditions (Snowder et al., 2006).

Protein restriction during the PERI or POST periods increased brain weight assessed relative to fetal weight in male, but not female, fetuses at 98 dpc indicative of a brain sparing effect in this cohort. Previous studies in sheep have reported similar effects in IUGR fetuses and attributed this to resources being reallocated to spare brain metabolism to the detriment of the development of other organs i.e. trunk and abdominal viscera (McMillen et al., 2001; Osgerby et al., 2002). These studies, however, did not differentiate fetuses by sex. Ratios of CNL:CRL and Brain:Liver ratio were higher in the LPost male fetuses, providing further evidence of brain sparing (Dawes, 1968) as previously explored in IUGR models in sheep

(Wallace et al., 2002a; Field et al., 2015). Such asymmetric IUGR is reported to be related to utero-placental insufficiency and redistribution of fetal blood flow concomitant with reduction in fetal cell growth (Platz and Newman, 2008). Together, the observed differences in fetal measures and indices of growth are indicative of both sex-specific IUGR and asymmetric development in the protein restricted fetuses (McMillen et al., 2001; Platz and Newman, 2008).

In contrast to the differences observed by ultrasonography (Copping et al., 2014) and in the gross measures of the fetuses assessed at 98 dpc, in animals taken to term there were few effects of maternal diet on either gross morphological measures of the neonatal calf or upon indices of disproportionate growth. This suggests compensatory growth of the fetus occurred in the second and third trimester, a scenario demonstrated by studies both in sheep (Todd et al., 2009; Jaquiery et al., 2011; Jaquiery et al., 2012; Kleemann et al., 2015) and cattle (Summers et al., 2015a). Dietary protein restriction in the first trimester did not reduce birth BW in agreement with previous studies investigating nutritional restriction in early gestation (Long et al., 2009; Micke et al., 2010b; Mossa et al., 2013). This contrasts to the reduction in birth BW observed following second trimester dietary protein restriction (Micke et al., 2010b; Miguel-Pacheco et al., 2016). Interestingly, the male fetus was heavier and longer in comparison to the female at 98 dpc as expected (Eley et al., 1978), yet the normal sex-related birth BW difference (Bellows and Short, 1978; Holland and Odde, 1992) was disrupted at term (Copping et al., 2014). Perhaps placental adaptations after restriction ceased were sufficient to meet the lower nutritional demands for catch-up growth of the usually smaller female but not to completely mitigate the early suppression of growth in the male (Burton and Fowden, 2012; Rosenfeld, 2015). The sex-specific effects upon the gross placental measures reported below, allow some interpretation of this role.

Placental Parameters

Altered placental development has previously been linked with IUGR in sheep (Wallace et al., 1996; Wallace et al., 2002a) and cattle (Perry et al., 1999; Long et al., 2009; Miguel-Pacheco et al., 2016). As distinct from the ewe (Borowczyk et al., 2006; Funston et al., 2010), cotyledonary growth increases progressively throughout gestation in the cow (Reynolds et al., 1990; Vonnahme et al., 2007) but changes in capillary area density during mid to late gestation are comparatively modest (Vonnahme et al., 2007). The current study reported a similar average placental weight (4.9 kg) to that previously reported in dairy cattle [5 kg; Laven and Peters (2001)] but higher than the 4.1 kg and 3.9 kg reported in two (Miguel-Pacheco et al., 2016) and three yr old calving beef heifers (Sullivan et al., 2009c). Placental efficiency, a measure of the capacity of the placenta to support fetal growth (Fowden et al., 2009), was lower (7.0:1) than the 8.5:1 reported by Sullivan et al. (2009c). This apparent reduction in efficiency may be attributed to the age difference of the heifers as placental efficiency in beef heifers increases with age (Sullivan et al., 2009c); the current study representing an adolescent pregnancy with competing requirements between the dam and the conceptus for nutrition (Wallace et al., 2004). Placenta weight, cotyledon weight and placentome weight were positively correlated with birth and fetal BW at 98 dpc, in agreement with previous studies (Prior and Laster, 1979; Perry et al., 1999; Sullivan et al., 2009c). The PERI and POST diets were associated with altered placental parameters at 98 dpc but not at term, analogous to the observed effects in the fetus and term neonate. It is well established that the placenta has considerable adaptive plasticity (Perry et al., 1999); the reduced number of placentomes at 98 dpc in LPeri heifers and reduction in placentome weight and volume in LPost heifers suggests an adaptation of the placental phenotype in response to maternal protein restriction and consequent metabolic status of the heifers (as discussed below). Previous studies on placental development have suggested the number of cotyledons at term did not alter from those present at 75 dpc in *Bos indicus* zebu (Stickland and Purton, 1977), or 90 dpc in *Bos taurus* cattle (Prior and Laster, 1979). Sullivan et al. (2009c), however, reported

that restricted maternal dietary protein intake between 93 and 180 dpc in heifers was associated with a reduction in cotyledon number at parturition suggesting that there may be capacity for the placental unit in the bovine to adjust beyond the first trimester period. This concept is consistent with the reduction in cotyledon weights and caruncular weights seen at 125 dpc in placentas from heifers (with female fetuses) that were restricted (50 vs. 100%) between 30 and 125 dpc (Zhu et al., 2007). Following realimentation, only cotyledon weights remained affected at 250 dpc; alterations in placental vascularity, however, became apparent by 250 dpc (Vonnahme et al., 2007). Doppler measurements of blood flow and uterine artery parameters from 120 dpc (Hernandez-Medrano et al., 2015) suggest that vascular supply to the placenta in the present study was similarly altered once maternal diet treatment ceased; a scenario also reported in cows realimented following nutritional restriction in early gestation whereby blood flow to the ipsilateral horn was increased following realimentation, but not during the period of restriction (Camacho et al., 2014).

Interestingly, in parallel to our observations in the fetal organs at 98 dpc, the effects of maternal protein restriction on placentome volume and weight at 98 dpc were also seen predominately within the male placenta. We observed multiple effects in male organ weights at 98 dpc compared to the more limited effects in the females with the exception of the heart. This is concomitant with the reported sex-specific effects (Hernandez-Medrano et al., 2015; Micke et al., 2015) of peri-conception and first trimester diets upon bovine fetal development whereby dietary perturbation in very early gestation affected the male feto-placental unit more significantly. In contrast, perturbations occurring during the second and third trimester in human studies (Clifton, 2010) report that the female is more responsive; displaying growth reduction enabled by altered placental signalling (Hodyl et al., 2010). Similarly in the bovine, a high protein maternal diet in the second trimester increased BPD at birth with the effect being greater in the female (Miguel-Pacheco et al., 2016). As reported in the companion paper (Copping, unpublished data: Chapter 3), bPL in the heifers carrying female fetuses (and not male) at 128 and 190 dpc was correlated with IGF1 in this cohort. Bovine placental lactogen

(bPL) is known to upregulate IGF1 which may act in both the placenta and fetus to enhance growth (Anthony et al., 1995). Additionally, correlations of placental parameters with maternal leptin and IGF1 concentrations varied dependent on sex. Sullivan et al. (2009c) reported negative correlations between maternal leptin in late gestation and placental weight as observed in this study, a relationship also documented previously in sheep (Thomas et al., 2001). These relationships were ascribed to leptin playing a role in regulating nutrient partitioning; however, sex of the fetus was not considered. In the current study, placental correlations with leptin occurred only in females. We have previously reported sex-specific altered blood flow in response to nutrient restriction in the heifers in the present study with enhanced uterine artery flow to the male feto-placental unit compared to females at 120 dpc (Hernandez-Medrano et al., 2015) and altered feto-placental Doppler perfusion indices in mid to late gestation in all heifers associated with earlier dietary protein restriction. LPeri diet was associated with increased blood flow to the fetus later in gestation once dietary interventions had ceased, with the change in blood flow volume over time being greater in females than males (Hernandez-Medrano et al., 2015). This may suggest that, in the bovine at least, the male may be responsive to perturbation in the peri-conception period and first trimester. In the subsequent trimesters, however, these responses are reversed and the female fetus in the second trimester was able to respond to the enhanced nutrient availability via endocrine signals from the feto-placental unit, which the male was not.

Collectively, this suggests that the maternal dietary treatments between -60 to 98 dpc, followed by feeding to meet nutritional requirement (but not realimentation), altered the fetal growth trajectory in a sex-specific manner. We propose that the female feto-placental unit affected nutrient partitioning, which the male feto-placental unit was unable to fully replicate. Whilst the mechanisms behind this effect are not clear, we have previously reported the sex-specific differences in both Doppler feto-placental perfusion indices and response of these indices following the maternal dietary interventions (Hernandez-Medrano et al., 2015): perhaps the positive correlation of bPL in the second trimester with maternal circulating IGF1

in only the female cohort (Copping, unpublished data: Chapter 3), combined with the negative correlation between maternal leptin, and birth and placenta weight, also only in females, potentially indicate a signalling mechanism enabling enhanced growth of the female feto-placental unit at this stage in gestation. The role of the placenta in this interaction may be further illuminated by histology and gene expression studies as in other species (Hodyl et al., 2010; O'Connell et al., 2013).

Conclusion

The results of this study demonstrate that protein restriction during the peri-conception period (-60 to 23 dpc) and first trimester (24 to 98 dpc) decreases early fetal growth, alters placenta parameters and produces asynchronous organ development in the bovine by 98 dpc. Intriguingly, the sex-related differences normally observed in birth BW and apparent at 98 dpc were disrupted, suggesting that sex-specific catch up growth to term had occurred, but it was dependent upon early gestational dietary treatment.

We propose that an accelerated growth response in restricted fetuses, subsequent to the return to 100% of nutrient requirement in the 2nd and 3rd trimesters, reduced the differences in fetal BW and morphology seen at 98 dpc and thereby the normal disparity between heifer and bull calf birth BW. As distinct IUGR was observed at 98 dpc but was not evident from the gross measures at birth, the challenge for producers will be to recognise that birth BW alone is not a reliable indicator of IUGR. In extensive pasture-based production systems, where variation in feed quality through breeding and gestation occurs, quantification of the effects upon long-term productivity of the progeny thus remains an important area of study.

Chapter 5

Reproductive development in bull progeny is modified by peri-conception and first trimester dietary protein in the heifer.

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Contribution to the Paper	Experimental Design. Collected birth data, maternal data, post – natal progeny growth and scrotal/semen data, samples for hormone assays and abattoir tissues/data. Performed statistical analysis and interpreted data (excluding histology) with assistance from statistician. Wrote the manuscript as joint co-author (introduction / materials and methods (excluding histology)/results (excluding histology) / discussion (excluding histology) / conclusion).
Overall percentage (%) 60%	
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 30/10/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Principal supervisor, advisory role, overall project design and co-ordination, contributions to experimental design, procedures, animal monitoring, sample collection, evaluation and editing of the manuscript.		
Signature		Date	15/11/16

144

Peri-conception and first trimester diet modifies reproductive development in bulls.

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Abstract

Nutritional perturbation during early gestation alters male reproductive development in laboratory species and sheep. In the bovine, both the developmental trajectory of the fetoplacental unit and its concurrent response to dietary perturbations is dissimilar. Nulliparous beef heifers were used to evaluate the effects of gestational dietary protein restriction during the peri-conception [PERI; -60 to 23 days post-conception (dpc)] and first trimester periods (POST; 24 to 98 dpc) on the reproductive development of the male progeny. Heifers ($n = 360$) were individually fed a high or low protein diet (HPeri and LPeri) for 60 days before conception. At 23 dpc, half of each treatment group changed to an alternative post-conception high or low protein diet (HPOST and LPOST) yielding four treatment groups in a 2 x 2 factorial design. A subset of heifers ($n = 46$) were humanely slaughtered at 98 days post-conception, and fetal testis ($n = 25$) development assessed. Reproductive development of the singleton male progeny ($n = 40$) was assessed post-weaning until slaughter at 598 days of age, when adult testicular cytology was evaluated. In the fetus, low protein peri-conception maternal diet increased the proportion of seminiferous tubules and decreased blood vessel area in the testis and within the parenchyma. Low protein first trimester maternal diet decreased blood vessel number within the capsule in the fetus and increased blood vessel number in the adult testis. In the developing bull, the low protein peri-conception maternal diet lowered sperm quality during pubertal development with a concomitant delay in age of puberty. These effects were subsequent to lower FSH concentrations in this low peri-conception group at both 330 and 438 days of age.

Introduction

Fetal developmental programming of physiological systems is a well-established concept (McMillen and Robinson, 2005). Maternal peri- and post-conception nutrition influences fetal development, which in turn can affect post-natal growth, adiposity, gonad development, gamete quality and hormonal status of the offspring (Sullivan et al., 2009a; Sullivan et al., 2010b; Dupont et al., 2012; Mossa et al., 2013). Seasonal variation in the nutritional value and the quantity of pasture available to pregnant ruminants can occur in grass-fed production systems (Burns et al., 2010). Such variation in pre-natal nutrition has been shown to affect both testis development and circulating gonadotrophin levels in the pre-pubertal bull (Sullivan et al., 2010b). However, the implications for adult reproductive performance in cattle progeny are not known: this paucity of research on the direct effects of *in utero* nutrition on male progeny post-natal reproductive function and fertility is widely acknowledged (Dupont et al., 2012; Chadio and Kotsampasi, 2014; Chavatte-Palmer et al., 2014; Mossa et al., 2015; Sinclair et al., 2016). Comparable studies in rams, have reported effects upon age at puberty (Da Silva et al., 2001), testicular weight (Bielli et al., 2002), testicular volume (Da Silva et al., 2001), Sertoli cell numbers, the diameter of seminiferous tubules (Kotsampasi et al., 2009), prepubertal testosterone (Da Silva et al., 2001) and pituitary response to GnRH (Kotsampasi et al., 2009). Age of puberty in cattle (as in sheep) is considered a driver of efficiency; shortening the generation interval, increasing genetic gain and thereby overall lifetime productivity (Barth and Ominski, 2000; Yilmaz et al., 2006).

Many studies have shown that folliculogenesis (Fair, 2010) and the following period of early embryo development are sensitive to perturbations in the maternal environment (Edwards and McMillen, 2002; Ashworth et al., 2009; Mossa et al., 2013). The response to such perturbations is orchestrated via the developing placenta (Sullivan et al., 2009c). As the growth trajectory of the bovine placenta differs from the ovine and rodent models, and is in fact more similar to the human (Wooding and Flint, 1994), the resultant response of the fetoplacental unit to such perturbation also differs (Hernandez-Medrano et al., 2015).

Correspondingly, unlike altricial or small ruminant models, bovine embryo development occurs at similar developmental time points to the human; organogenesis is complete by 42 days post-conception (dpc) (Hopper, 2014), with the genital ridges, forming at 27 to 30 dpc (Ross et al., 2009). Sertoli cells begin to proliferate between 40 to 50 dpc and play a crucial role in gonad development during fetal life and in post-natal spermatogenesis (Bielli et al., 2001). Disruptions to the proliferation of fetal Sertoli cells may occur through modifications in the development of the hypothalamic-pituitary-gonadal axis in early fetal life (Klonisch et al., 2004; O'Shaughnessy and Fowler, 2011) and associated changes in the concentration of hormones including follicle-stimulating hormone (FSH), triiodothyronine (T3), thyroxine (T4) and growth hormone (GH) (Dupont et al., 2012). Consequently, this may affect development of other testicular cells, leading to altered testicular function in post-natal life (Sharpe et al., 2003; Dupont et al., 2012).

Spermatogenesis in the post-natal bull is a complex dynamic process of cellular replication and differentiation (Barth and Oko, 1989; Wrobel, 2000). A suite of molecular pathways is essentially regulated by an interdependent complement of hormones including testosterone, FSH, inhibin and activin which rise and fall in a specified sequence during pre-puberty and peri-puberty to result in functional sperm in the adult bull (Evans et al., 1996; Matsuzaki et al., 2000; Kaneko et al., 2001). This sequence is known to be disrupted by nutritional intervention during the pre-weaning period (Brito et al., 2007b; Brito et al., 2007c) possibly mediated by metabolic hormones (i.e. IGF1) (Brito et al., 2007a; Brito et al., 2007c; Barth et al., 2008) with consequent effects upon the development of spermatogenesis. It is noteworthy that previously reported effects of pre-natal nutrition also include changes in concentrations of many of the aforementioned hormones.

The aim of this experiment was to examine the effects of dietary protein intake in heifers during the peri-conception period and the first trimester on the reproductive development of their male progeny. We hypothesised that the peri-conception and first trimester low protein diet would delay puberty with deleterious effects upon testis development and sperm

production. Furthermore, this would be associated with alterations to the hormonal milieu in the developing bull.

Material and Methods

Ethics Approvals

All procedures were performed with the prior approval of University of South Australia IMVS Animal Ethics Committee (Approval number: 18/11), The University of Adelaide, Australia (Approval number: S2012-249), The University of New England, Australia (Approval number AEC14-037) and the University of Nottingham, U.K. (Approval number 1117 140320).

Experimental Design and Animal Management

The purpose of this experiment was to evaluate the impact of maternal dietary protein during the peri-conception (PERI; -60 to 23 days post-conception (dpc) (implantation being 18 to 22 dpc (Wathes and Wooding, 1980; Spencer and Hansen, 2015a) and first trimester (POST; 24 to 98 dpc) periods in nulliparous beef heifers upon fetal and post-natal reproductive development in the male progeny. The dietary protein levels reflected pasture conditions in Australian rangelands without (low), and with (high), protein supplement.

The study was a two-by-two factorial design. The animals and fetuses studied were singleton male progeny of two-year old heifers that have previously been described (Copping et al., 2014). Briefly, 360 nulliparous Santa Gertrudis (*Bos taurus* x *Bos indicus*) heifers were selected on the basis of weight and age from S Kidman and Co herds located at 'Glengyle' and 'Morney Plains', south western Queensland, Australia. Heifers were transported to 'Tungali', Sedan, South Australia (34º29´S, 139 º18´E) where they underwent a 60-day acclimatisation prior to commencement of the study. Heifers that did not acclimatise to the individual feeding were removed from the study.

At 12 months of age, 60 days prior to artificial insemination, heifers were stratified by bodyweight and randomly assigned to two equal peri-conception (PERI) treatment groups, high and low protein (HPeri and LPeri). Each heifer was fed a high (71 MJ ME and 1.18 kg crude protein/head/day) or low (63 MJ ME and 0.62 kg crude protein/head/day) protein diet (Appendix 1) consisting of a pelleted diet that was individually fed in stalls. Straw (5% crude protein) was available *ad libitum* in pens. The ration was as isocaloric as possible for ruminants fed the forage component of the diet under group housing and it was supplemented with a commercial vitamin and mineral preparation (Minmix, Ridley Agriproducts, Toowong, Qld).

Heifers underwent a progesterone based oestrous synchronisation program prior to artificial insemination on Day 0 with frozen semen from one Santa Gertrudis bull. At 23 dpc, half of each nutritional treatment group was swapped to the alternative post-conception (POST) treatment, high (HPost: 102 MJ ME and 1.49 kg crude protein//head/day) or low (LPost: 98 MJ ME and 0.88 kg crude protein/head/day) (Appendix 1), giving rise to four groups: [HPeri-HPost (HH), HPeri-LPost (HL), LPeri-HPost (LH), LPeri-LPost (LL)]. There was a 1.9 to 2.1-fold difference in CP content and 1.1-fold difference in energy content between the high and low diets. Protein intake was restricted (Freer, 2007) during both the peri-conception period and first trimester in the low group whilst both groups received similar energy intake. As the variation in CP content between the high and low diets was much greater than that in energy, the differences observed in the current study are more likely to be attributable to the effects of maternal protein intake during the peri-conception period and first trimester of gestation rather than energy intake.

Pregnancy was confirmed in 124 heifers at 36 dpc and fetal sex was determined at 60 dpc by transrectal ultrasonography. At 98 dpc, a sub-set of heifers was humanely slaughtered at a commercial abattoir and fetuses of both sexes ($n = 46$; singletons) collected as described (Copping et al., 2014), with the 25 singleton male fetuses reported herein (n: HH = 6, HL = 10, LH = 5, LL = 4). Fetal gonads were dissected, weighed and collected for histological processing.

From the end of the first trimester of gestation (98 dpc), all heifers were fed the same diet, which was formulated to provide additional growth of 0.5 kg/head/day until parturition (79 MJ ME and 0.92 kg crude protein/head/day; Table 1). Heifers received the pellet portion of their diet individually on a daily basis with straw (5% crude protein) provided *ad libitum* in pens until the animals reached parturition.

Sixty-three heifers (singleton pregnancy) completed the study and gave birth to 18 live female and 43 live bull calves. Progeny remained with their mothers as one group grazing on improved and native pastures until weaning at 183.3 ± 0.8 days of age. After weaning, progeny were segregated according to sex and grazed improved and native pasture until 507.3 ± 0.8 days of age. Non-castrated male progeny were transported from Sedan, South Australia to the 'Tulimba' Research Feedlot, Kingstown, NSW (30º28´S,151 º11´E) prior to slaughter at a commercial abattoir on 598.3 ± 0.8 days of age with a final liveweight of 652.3 ± 11.4 (HH), 677.0 ± 10.0 (HL) 678.6 ± 19.1 (LH) and 647.4 ± 15.5 (LL) kg. At slaughter, gonads were dissected, weighed and collected for histological processing. Two progeny were removed from the study after birth, due to poor mothering. An additional animal (LL) was a cryptorchid and was excluded from the analysis leaving 40 singleton male progeny that completed the study reported herein (n: HH = 10, HL = 14, LH = 8, LL = 8).

Tissue Fixation and Processing

The complete left testis in the fetus and a 1cm^3 piece from each testis (same for every sample) in the adult were dissected and fixed overnight in 4% paraformaldehyde diluted in 0.1 M PBS

in a ratio of 1:5 (tissue volume: fixative solution volume). Samples were washed 3 times, for 24 hours each, in PBS (0.14 M NaCl, 0.03 M NaH_2PO_4, 0.05 M Na_2HPO_4). Tissues were processed on an automated tissue processor in the following solutions, 30-min in the case of fetal testis and one hour in the adult testis per solution: 70% ethanol, 80% ethanol, 95% ethanol, 3 times in 100% ethanol, 2 times in 100% xylene and 2 times in paraffin wax at 60°C under vacuum. Following processing, the testes were orientated and embedded into paraffin wax.

All samples, both adult and fetus, were sectioned at a thickness of 10µm using a microtome (Leica 5M 2255, Germany). The sections were dried onto polysilinated slides (Thermo Scientific, Germany) on a hot plate at 42ºC for one hour and then for 24 hours at room temperature prior to histological staining.

Cell Counts and Proportions

The development of the testis was assessed by the measurement of the following structures: testicular cell number (Sertoli, germ and interstitial cells), seminiferous tubules and blood vessels. These were distinguished within the testis by staining with two techniques: immunohistochemistry using a Novolink Leica immunostaining kit (Leica Microsystems, UK, Novolink Polymer Detection Kit, REF# RE7150-k) with Mis-C20 primary antibody (1:1000 dilution, Santa Cruz Biotechnology, USA, sc-6886) and Picrosirius staining (Polysciences, Inc., USA). Following tissue staining, sections were photomicrographed using a DM5000B microscope (Leica Microsystems Inc, Mannheim, Germany) with a Leica CTR5000 light box and Leica DFC420 colour capture camera. The magnification of the eyepiece and lens is stated below for each count using systematic random sampling and stereology methods previously described (Mayhew, 1991). In brief, sections were selected using systematic random sampling (ensuring a minimum of 200 µm distance between samples to avoid double cell counting). Photomicrographs were captured from each section in a systematic random manner prior to stereological counting/measurements being undertaken ($n = 5$ sections per

sample for cell counts/proportions, and $n = 3$ sections per sample for seminiferous tubules and BV measurement). This technique ensured unbiased measurements throughout the tissue. In the fetal testis every testicular cell was identified and manually counted on a total of 420 photomicrographs, seminiferous tubule numbers and dimensions ($n = 315$ photomicrographs, 20X magnification) and capsular and parenchymal blood vessels ($n = 5670$, photomicrographs at 10X and 40X magnification, respectively) were measured manually using an image analysis program (Image-Pro Plus (version 6.3), Media Cybernetics, USA) (n: HH = 5, HL = 7, LH = 5, LL = 4). In the adult testis, seminiferous tubules and blood vessels were measured using the same image analysis program ($n = 800$, micrographs, 10X magnification and $n = 1200$ photomicrographs, 20X magnification respectively) (n: HH = 10, HL = 14, LH = 8, LL = 8). Following calibration of the imaging software, tubules and vessels were manually circumscribed and the average number of tubules and blood vessels per tissue area (referred to throughout as 'number' of BV or seminiferous tubules) was calculated. In addition, the BV and seminiferous tubule areas occupied per tissue section were calculated, this is referred to as BV or tubule 'area' throughout.

Animal Measures

Liveweight and scrotal circumference

Heifers were visually monitored 24-hours a day throughout calving. Progeny birthweight was recorded within 15-min of birth and prior to first suckling. Liveweight was recorded monthly from birth using Ruddweigh electronic weigh scales. Scrotal circumference was assessed monthly from 214.3 ± 0.8 days of age (post-weaning) using the Australian Cattle Veterinarians recommended procedure (Beggs et al., 2013) with a Reliabull scrotal measuring tape (Lane Manufacturing Inc., Denver CO).

Blood sampling

Progeny blood samples were collected approximately monthly from weaning until slaughter at 20 months of age. Prior to the commencement of other procedures, samples of whole blood were collected by venipuncture directly into Vacutainer tubes containing lithium-heparin (Becton, Dickinson and Company, Plymouth PL6 7BP, UK) using 1.2 mm x 38 mm needles. Tubes were rotated by hand for 5 to 10s and stored on ice prior to centrifugation (Eppendorf 5702R, Eppendorf Zentrifugen GMBH, Leipzig, Germany) at 3,000 x g at 4ºC. Plasma was harvested then stored frozen at -80ºC until analysis.

Assays

Plasma concentrations of follicle stimulating hormone (FSH), leptin, insulin-like growth factor 1 (IGF1), testosterone, anti-Müllerian hormone (AMH), inhibin and activin A were assayed as detailed.

   Plasma FSH was measured in duplicate by a double-antibody radioimmunoassay (Atkinson and Adams, 1988) validated for use in cattle using NIAMDD-oFSH-RP-1 (biopotency 75 x NIH-FSH-S1) and NIADDK-anti-oFSH-1 serum. The intra assay coefficients of variation were 5.7%, 2.7% and 4.4% for control plasma with means of 1.27 ng/mL, 2.25 ng/mL and 3.15 ng/mL, respectively. The limit of detection was 0.15 ng/mL. As the sample levels were 3-4 times higher than the limit of detection they were read in the linear part of the standard curve.

   Plasma was assayed for leptin in duplicate by a double-antibody radioimmunoassay (Blache et al., 2000) previously validated for bovine samples (Kadokawa et al., 2001). Samples processed in a single assay and the assay included six replicates of three control samples containing 0.29, 0.71 and 1.68 ng/mL, which were used to estimate the intra-assay coefficients of variation of 5.4%, 4.4% and 6.6%. The limit of detection was 0.05 ng/mL.

   Plasma testosterone was assayed in duplicate using the reagents of the Immunochem® double antibody testosterone RIA kit (MP Biomedical Australia, Seven Hills, NSW,

Australia) following the manufacturer's protocol. The assay was validated using a serial dilution of two bovine samples. The intra-assay coefficient of variation for quality control samples containing 0.26 ng/mL and 2.3 ng/mL was 6.5% and 2.9%, respectively. The lowest and highest limits of detection were 0.07 ng/mL and 6.5 ng/mL, respectively.

Plasma was assayed for IGF1 in duplicate by double-antibody radioimmunoassay with human recombinant IGF1 (ARM4050, Amersham-Pharmacia Biotech, Buckinghamshire, England) and antihuman IGF1 antiserum (AFP4892898, National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases, California, USA) following acid-ethanol extraction and cryoprecipitation (Breier et al., 1991). The assay was previously validated for bovine samples (Chagas et al., 2007). Samples were processed in a single assay. The intra-assay coefficient of variation for control samples containing 51.6 ng/mL and 253.6 ng/mL was 6.2% and 5.9%, respectively. The limit of detection was 0.1 ng/mL.

AMH levels were determined using Bovine AMH ELISA kit AL-114 (Ansh Labs, Webster, Texas, USA) following the manufacturer's protocol. Samples were diluted 15 times using the sample diluents provided in the kit as recommended by the manufacturer. The intra-assay coefficient of variation for quality control samples containing 290.8 pg/mL and 874.98 pg/mL was 2.6% and 3.7%, respectively. The limit of detection was 28.4 pg/mL.

Bovine inhibin levels were measured at the Hudson Institute of Medical Research using a radioimmunoassay employing a rabbit antiserum raised against the alpha-subunit of bovine inhibin (McLachlan R.I. et al., 1986), which detects both inhibin A and B proteins and free inhibin alpha-subunit (including pro-alpha-C) in multiple species. Iodinated human recombinant 31 kDa inhibin was used as tracer, and 31 kDa human recombinant inhibin was used as standard. Goat anti-rabbit IgG (GAR#12) was used as second antibody. The assay has been validated for measurement of inhibin in bovine serum samples, and values (in ng/ml) are expressed relative to the purified human inhibin standard. The intra-assay coefficient of

variation was 6.2% and the lowest and highest limits of detection were 0.26 ng/mL and 8.73 ng/mL, respectively (based on ED90 and ED10 values).

Total bovine serum activin A concentrations were measured at the Hudson Institute of Medical Research employing a two-site enzyme immunoassay specific for activin A (Knight et al., 1996) modified and validated for measurement of bovine serum samples. Human recombinant activin A, which is identical in sequence to bovine activin A, purified as described previously from material provided by Biotech Australia Pty Ltd (East Roseville, NSW, Australia) (Robertson et al., 1992), was used as the standard. Values (in pg/ml) are expressed relative to the purified activin A standard. The mean intra- and inter-assay coefficients of variation for three Plates were 5.4% and 7.3%, respectively. The lowest and highest limits of detection were 8.84 pg/mL and 1984 pg/mL (2 SD) respectively

Semen Collection

Semen collection commenced in spring at approximately monthly intervals from 10 months of age until slaughter at 20 months of age. After preliminary stimulation of the ampulla via rectal massage, semen was collected using standard electroejaculation technique (Lane Pulsator IV, Lane Manufacturing Inc. Denver CO, USA) as previously described (McAuliffe et al., 2010; Beggs et al., 2013). If an animal did not produce a satisfactory sample within several minutes following electrostimulation, the animal was released and a single further attempt was made after a 10-min interval (Callaghan et al., 2016).

Assessment of semen traits was undertaken immediately following collection using established methodology and standards (Entwistle and Fordyce, 2003; Fordyce et al., 2006) by the same technician blinded to treatment. Briefly, ejaculate density was scored immediately following collection using a 1 (clear to cloudy), to 5 (creamy), scale. A drop of semen was placed on a pre-warmed glass slide (37ºC) with a plastic transfer pipette (1mL) and assessments made of motility (%) and mass motility (wave motion) using a phase contrast microscope. Motility was estimated as the percentage of sperm that were progressively motile under their own propulsion (viewed at 400X magnification). Mass motility was assessed

under 40X magnification on a 1 (no swirl) to 5 (fast distinct swirl with continuous dark waves) scale (Burns et al., 2013; Corbet et al., 2013). Animals that did not produce an ejaculate were assigned a value of zero for density, motility and mass motility (Corbet et al., 2013). Semen (0.1 mL) was diluted with phosphate-buffered formal saline (4.9 mL) for sperm concentration assessment, with sperm counted in a haemocytometer.

Semen (1 to 2 drops) was placed into phosphate-buffered formal saline (1.0 mL) for assessment of sperm morphology. The morphology of 100 individual spermatozoa in each sample considered to contain sufficient sperm for examination (Burns et al., 2013) was assessed using 1000X magnification under differential interference contrast microscopy by an Australian Cattle Veterinarians accredited sperm morphologist blinded to treatment at a commercial third-party pathology laboratory. Morphology traits were individually recorded based on the sperm abnormality format as described (Fordyce et al., 2006). The sperm abnormality categories included midpiece abnormalities, knobbed acrosomes, proximal cytoplasmic droplets, abnormal tails and loose heads, pyriform heads, vacuoles and teratoid sperm and swollen acrosomes (Fordyce et al., 2006) as shown in Supplementary Fig. 1. Total remaining normal sperm were noted as percent normal sperm per ejaculate at each time point.

Determination of Pubertal Age and Sexual Maturity

The threshold used for age at puberty is defined as: the first time an ejaculate contained semen concentration ≥ 50 x 10^6 spermatozoa per mL with $\geq 10\%$ motile spermatozoa (Wolf et al., 1965). After attainment of puberty, sexual maturity was characterised by the first time an ejaculate contained $\geq 70\%$ morphologically-normal spermatozoa after attaining puberty (Brito et al., 2004).

Statistical Analysis

Data were checked for normality and transformed before analysis if required. Data for maternal liveweight, maternal average daily gain, fetal weight, fetal testis weight, testicular

cell development, gestation length paired testis weight, age of puberty, age of maturity, inhibin, activin A and AMH were analysed using two-way ANOVA (STATA 13.1, Stata Corp College Station, Texas, USA) to determine the effects of maternal diet during PERI and POST periods and their interaction term. Significant interactions were explored with Tukey-Kramer post hoc test as required.

To investigate the interactions between maternal diet (PERI and POST) and time, hormone concentrations (leptin, FSH, IGF1, testosterone), sperm traits and scrotal circumference linear mixed-effects models were performed, adjusting for repeated measures over time for each of the 40 calves. An autogressive (1) covariance structure was used as it provided the best fitting model compared with other structures. For sperm morphological abnormalities, Generalized Estimating Equation (GEE) models with a Poisson distribution were performed, adjusting for repeated measures over time for each of the 40 calves. Post-hoc comparisons were made for each model: differences of least squares means for the linear mixed-effects models and Incidence Rate Ratios (IRR) for the Poisson GEE models. The statistical software used was SAS 9.3 (SAS Institute Inc., Cary, NC, USA). There were no significant interactions between maternal diet during PERI and POST periods for the variables investigated unless expressly stated in the results. Thus, for clarity, the results have been presented as the main effects of PERI and POST maternal diet. Statistical significance is reported at $P < 0.05$ and tendency at $P < 0.10$.

Results

Maternal Liveweight

At both the commencement and end of the PERI maternal diet period (-60 to 23 dpc) and the POST maternal diet period (24 to 98 dpc), the liveweights of the heifers were similar (Table 1a and Table 1b; all $P > 0.10$). There was no interaction of the PERI and POST diet ($P = 0.475$) on liveweight at the end of the POST maternal diet period. Average daily weight gain (ADG) during the PERI diet period was lower ($P < 0.001$) for the LPeri (LL + LH) heifers

compared to the HPeri (HH + HL) heifers. ADG during the POST maternal diet period did not differ between LPost (LL + HL) and HPost (HH + LH) groups ($P = 0.164$), nor was there a diet interaction (P = 0.482). Heifers who had received the LPeri diet had higher ADG during the POST diet period compared to those that received the HPeri diet (0.36 ± 0.04 versus 0.17 ± 0.04 kg/head/day; $P = 0.002$).

From the end of the POST diet period to late gestation (99 dpc to 256 dpc), during which time all dams received the same diet, maternal liveweights did not differ due to PERI or POST diet, nor was there a diet interaction (all $P > 0.10$) (LPeri: 506.4 ± 7.3 versus HPeri: 507.2 ± 6.3 kg and LPost: 507.3 ± 6.1 versus HPost: 506.3 ± 7.6 kg, respectively) ADG also did not differ (all $P > 0.10$) (LPeri: 0.63 ± 0.02 versus HPeri: 0.64 ± 0.02 and LPost: 0.65 ± 0.02 versus HPost: 0.61 ± 0.02 kg/head/day, respectively). Immediately post-calving, a similar pattern was observed whereby maternal liveweights did not differ due to PERI or POST maternal diet, nor was there a diet interaction (all $P > 0.10$) (LPeri: 456.6 ± 8.3 versus HPeri: 464.2 ± 7.7 and LPost: 468.6 ± 7.1 versus HPost: 452.4 ± 8.5 kg, respectively).

Table 1. Maternal liveweight and average daily gain (ADG) at start and end of exposure to diets[1] low (L) or high (H) in protein during the (a) PERI (-60 to 23 dpc) and (b) POST-conception (24 to 98 dpc) period.

(a)

Diet	LPeri (LL + LH)	HPeri (HH + HL)
N	16	24
Start weight (kg)	345.6 ± 5.5	335.6 ± 5.3
End weight (kg)	382.9 ± 6.5	395.3 ± 5.5
ADG (kg/hd/day)	0.40 ± 0.03^a	0.64 ± 0.02^b

(b)

Diet	LPost (LL + HL)	HPost (HH + LH)
N	22	18
Start weight (kg)	391.4 ± 5.5	389.0 ± 6.9
End weight (kg)	405.7 ± 5.0	410.6 ± 5.8
ADG (kg/hd/day)	0.20 ± 0.04	0.30 ± 0.04

Data are mean $\pm$ SEM. Values with different superscripts differ significantly (P < 0.05).

[1]LL = Low protein maternal diets peri- and postconception, LH = Low protein maternal diet in the peri-conception period and high protein postconception, HL = High protein maternal diet in the peri-conception period and low protein post conception, HH = High protein maternal diets peri- and postconception

Fetal and Animal Measures

Fetal and gonad weight at 98 dpc

Male fetuses from LPost dams were lighter at 98 dpc ($P < 0.05$) compared to males from HPost dams. There was no effect of PERI diet, or the diet interaction term on male fetal weight at 98 dpc as reported (Copping et al., 2014). Maternal diet did not influence absolute gonad weight or relative gonad weight at 98 dpc (Supplementary Table 1).

Birthweight and post-weaning growth

At birth, there was no effect of maternal diet upon birthweight or gestation length (Supplementary Table 2; $P > 0.05$). Increased gestation length was associated with increased birthweight ($r = 0.475$; $P < 0.001$). From weaning until slaughter (600 days), liveweight increased with age ($P < 0.0001$) but did not vary due to maternal diet (Copping, unpublished data, Chapter 6; $P > 0.10$).

Scrotal circumference

Scrotal circumference in all treatment groups increased with age (Supplementary Fig. 2; $P < 0.0001$). There was no overall effect of neither maternal diet, nor of gestation length, upon progeny scrotal circumference measures between 214 to 554 days of age (Supplementary Fig. 2; all $P > 0.10$).

Semen Traits

Semen quality parameters

There were maternal nutrition and time effects on a range of semen quality parameters (Fig. 1). There were effects of time ($P < 0.0001$) and PERI maternal diet ($P = 0.0433$) on mass activity whereby, bulls from LPeri dams had lower semen mass motility scores compared to bulls from HPeri dams (Fig. 1a). There were interactions between POST maternal diet and

time for mass motility ($P = 0.0181$) such that bulls from LPost dams had increased mass motility compared to bulls from HPost dams at 554 days of age (Fig. 1b; $P = 0.0433$) and tended to be higher at 351 ($P = 0.08$) days of age. There were effects of time ($P < 0.001$) and PERI maternal diet on semen density (Fig. 1c) and sperm motility (Fig. 1e). Overall, bulls from LPeri dams had lower sperm density (Fig. 1c; $P = 0.04$) and motility (Fig. 1e; P = 0.0217) compared to bulls from HPeri dams. There was an interaction between PERI maternal diet and time for the motility parameter (Fig. 1e; $P = 0.0124$). Bulls from LPeri dams produced ejaculates with reduced motility at 351 ($P = 0.03$), 395 ($P = 0.024$) and 438 ($P = 0.0024$) days of age and tended to have reduced motility at 465 ($P = 0.08$) days of age compared to bulls from HPeri dams. Overall, there were effects of time ($P < 0.001$) on semen concentration and concentration tended to be lower in bulls from LPeri dams (Fig. 1g; $P = 0.058$) but there was no interaction of maternal diet and age. POST maternal diet did not influence density (Fig. 1d), motility (Fig. 1f) or concentration parameters (Fig. 1h), nor was there any difference in any semen quality parameters due to the diet interaction (all $P > 0.05$).

Sperm morphology

There were effects of time ($P < 0.0001$) and PERI maternal diet ($P = 0.0208$) on percent normal sperm (Figure 2). Overall, percent normal sperm was lower in bulls from LPeri dams (Fig. 2a) compared to HPeri. The reduction in percent normal sperm within LPeri bulls was consequent to increased levels of sperm abnormalities (Figure 3).

Specifically, a higher overall incidence of abnormal mid-pieces and knobbed acrosome defects (Fig. 3a and 3c; $P < 0.05$) were observed in ejaculates from LPeri bulls. There were interactions between PERI maternal diet and time (Fig. 3c; $P = 0.0043$) for knobbed acrosomes with a higher incidence of this defect in ejaculates from LPeri bulls at 438 (IRR = 4.27; $P = 0.0061$), 465 (IRR = 3.60; $P = 0.0156$) and 520 (IRR = 5.39; $P = 0.005$) days of age. There were also interactions between PERI maternal diet and time for abnormal tail and loose head defects (Fig. 3e; $P = 0.0024$) such that bulls from LPeri dams produced ejaculates with a

higher incidence of abnormal tails and loose heads compared to bulls from HPeri dams at 465 (IRR = 1.87; P = 0.0394), 520 (IRR = 2.62; P = 0.0039) and 554 days of age (IRR = 2.41; P = 0.0248). There was an interaction between PERI maternal diet and age for vacuole and teratoid defects (Fig. 3g; P = 0.0434), however, there were no significant differences at any individual age. There was also an interaction between POST maternal diet and time for proximal droplet defects (Fig. 3j; P = 0.0032), however, there were no significant differences at any individual age. Overall, POST maternal diet increased the incidence of swollen acrosome defects which was higher overall in ejaculates from bulls with LPost dams than bulls from HPost dam (P = 0.0352). POST diet did not influence the incidence of any other sperm defect, nor was there any difference in any sperm defect due to the diet interaction (all P > 0.05). There were effects of age overall (Fig. 3; all P < 0.0001) for all defects reported. (Data not shown for swollen acrosome and pyriform head defects).

Puberty

Puberty was first reached by a bull at 329 days of age with the final bull reaching the threshold by 521 days of age. Puberty was achieved later in bulls from LPeri dams compared to bulls from HPeri dams (Table 2; P = 0.049). There was no difference in puberty due to POST maternal diet or the diet interaction (Table 2; P > 0.05).

Sexual Maturity

Maturity as assessed using the minimum threshold of 70% normal sperm was not achieved by 17.5% (n = 7: LPeri = 4; HPeri = 3 and LPost = 4; HPost = 3) of the bulls in this study. The first bull reached the threshold at 330 days of age. Of those bulls that achieved maturity (n = 33), there was a tendency for bulls from LPeri dams to reach maturity later than bulls from HPeri dams (466.9 ± 19.0 versus 425.9 ± 12.2 days of age; P = 0.079). There were no differences due to POST maternal diet (LPost: 435.3 ± 15.6 versus HPost: 448.0 ± 15.2 days of age; P > 0.10), nor the diet interaction (P > 0.05).
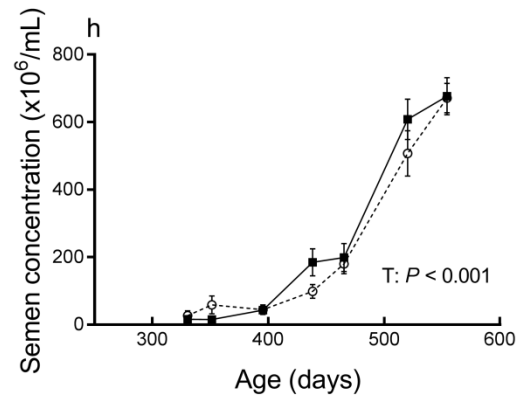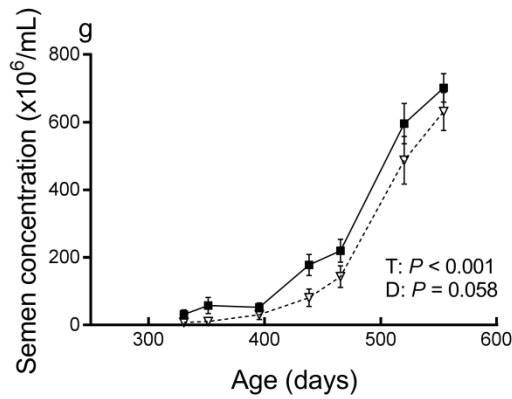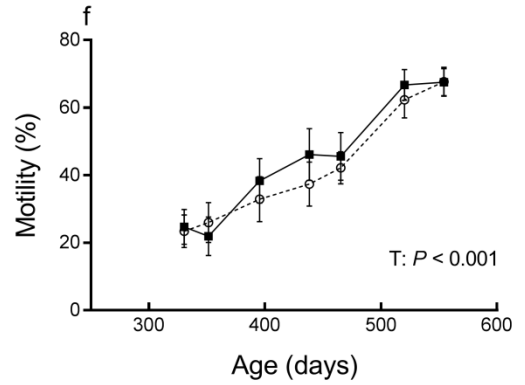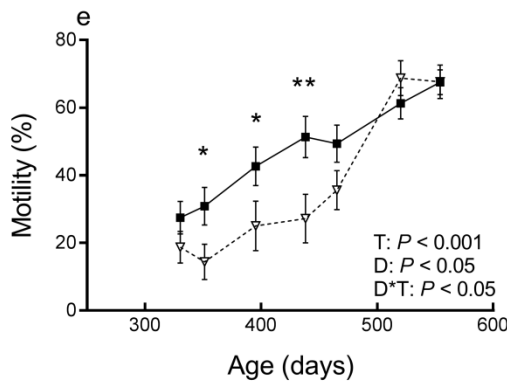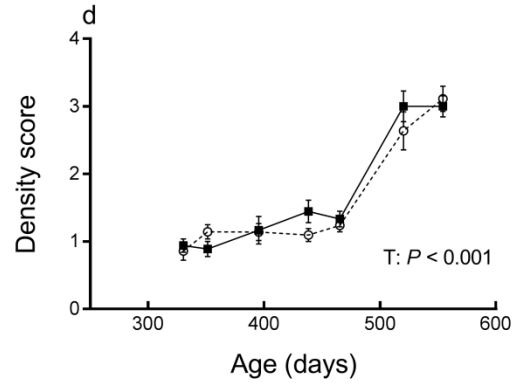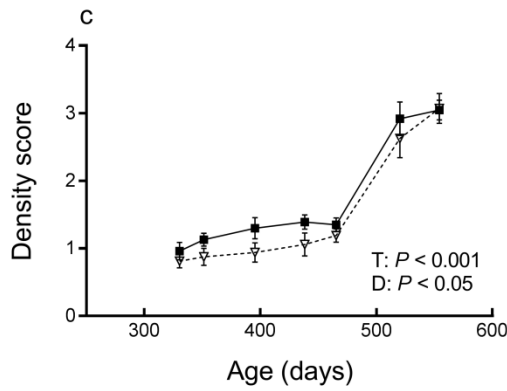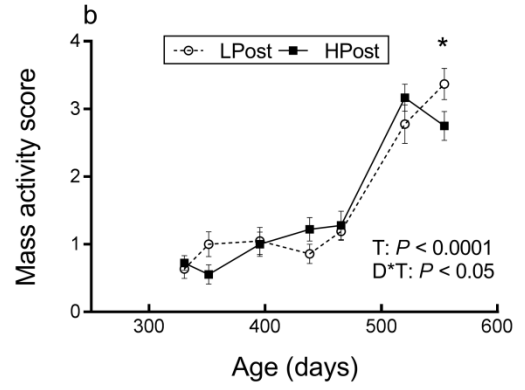
Figure 1. Semen quality parameters in male progeny (n = 40) between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (a,c,e,g) PERI-conception period (-60 to 23 dpc) or (b,d,f,h) or POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, T, D x T: Maternal diet, time and maternal diet-by-time interaction effects respectively. Means with superscripts indicate differences between groups within age.   * $P < 0.05$, ** $P < 0.01$

Figure 2. Percent Normal Sperm (PNS %) in male progeny (n = 40) between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (a) PERI-conception period (-60 to 23 dpc) or (b) POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, T, D x T: Maternal diet, time and maternal diet-by-time interaction effects respectively.
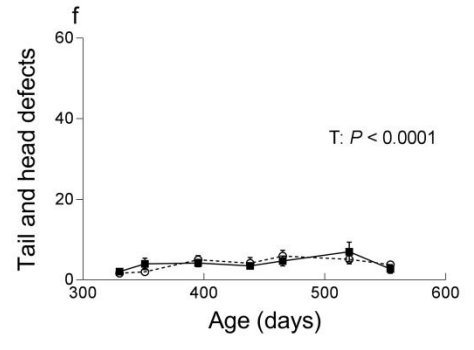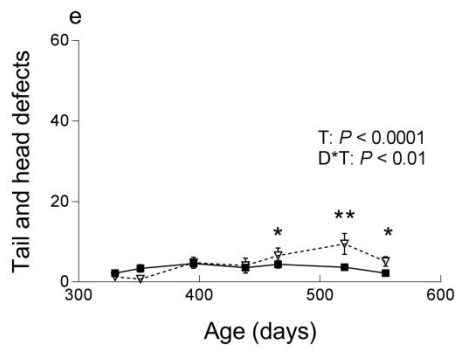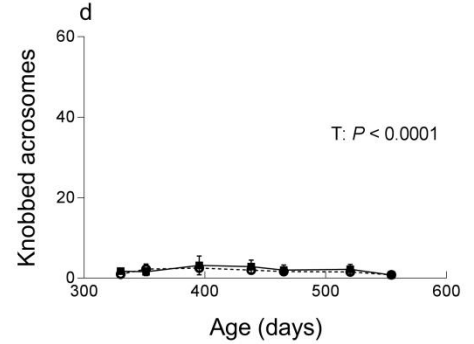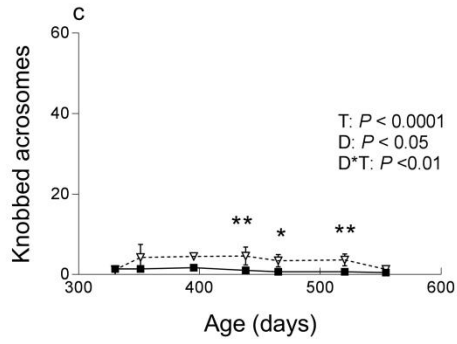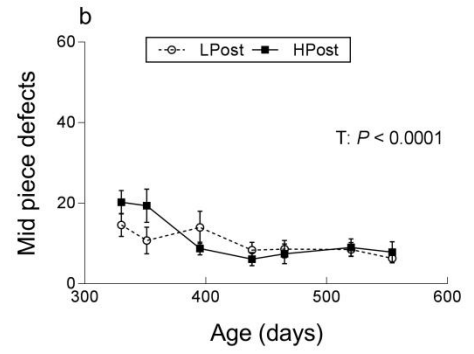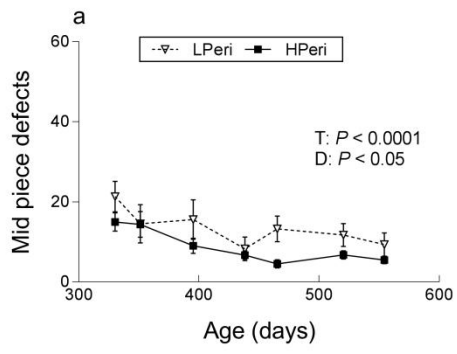
Figure 3. Sperm morphology defects (count per 100 sperm) in male progeny (n = 40) between 330 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (a,c,e,g,i) PERI-conception (-60 to 23 dpc) or (b,d,f,h,j) POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, T, D x T: Maternal diet, time and maternal diet-by-time interaction effects respectively. Means with superscripts indicate differences between groups within age.   * $P < 0.05$, ** $P < 0.01$

Table 2. Age of puberty in male progeny following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	*P*-value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	16	24	22	18			
Age (days)[1]	436.4 ± 10.8 [a]	403.3 ± 11.3 [b]	414.9 ± 10.9	419.2 ± 13.1	0.049	0.808	0.503

Data are mean ± SEM. Values with different superscripts differ significantly ($P < 0.05$).

[1] age at which sperm motility $\geq$ 10% and semen concentration $\geq$ 50 x10^6 /mL

Paired Testes Weight

The absolute and relative weights of the paired testes were similar between maternal diet groups at slaughter at 598.3 ± 0.8 days of age (Supplementary Table 2; $P > 0.05$). Total paired testis weight at slaughter was highly correlated with the final scrotal circumference (Supplementary Table 2) measured at 554.3 ± 0.8 days ($r = 0.82$; $P < 0.05$).

Hormone Concentrations

Circulating inhibin and activin A concentrations measured at 3 and 4 months of age were not influenced by neither PERI or POST maternal diet (Table 3; $P > 0.10$), nor the diet interaction ($P > 0.05$). However, circulating AMH concentration at 10 months of age were higher in bulls from LPeri dams (Table 4; $P = 0.04$) compared to HPeri bulls and tended to be higher in bulls from LPost dams compared to HPost bulls (Table 3; $P = 0.09$).

There were overall effects of time ($P < 0.001$) on plasma FSH (Figure 4), IGF1 (Figure 5) and leptin levels (Figure 5). Time also tended to influence plasma testosterone concentration (Figure 4; $P = 0.09$). There were interactions between PERI maternal diet and time for FSH (Fig. 4a; $P = 0.0435$) such that LPeri bulls had lower circulating FSH at 330 ($P = 0.0317$) and 438 ($P = 0.0147$) days of age, and tended to have lower levels at 273 ($P = 0.06$) and 302 ($P = 0.09$) days of age. There were also interactions between POST maternal diet and time for IGF1 (Fig. 5b; $P = 0.0127$) such that LPost bulls had higher circulating IGF1 at 465 days of age ($P = 0.004$) compared to HPost bulls. There were no main effects overall of PERI or POST maternal diet, or their interaction term on FSH, testosterone, IGF1 or leptin concentrations ($P > 0.10$).

Testis Development

The proportion of testicular cells (Sertoli, germ, interstitial cells/Leydig cells; Supplementary Fig. 3) in 98 dpc fetus was not altered either by the PERI or POST maternal diet, nor their interaction term (Supplementary Table 3; $P > 0.05$). Seminiferous tubule and blood

Table 3. Peripheral inhibin (ng/mL) and activin A (pg/mL) levels at 3 and 4 months of age, and anti-Müllerian hormone (AMH) (ng/mL) at 10 months of age in male progeny following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	P – value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	16	24	22	18			
3-months							
Age (days)	124.7 ± 1.4	125.7 ± 0.9	126.4 ± 1.1	124.0 ± 1.0			
Inhibin (ng/mL)	7.0 ± 0.3	7.2 ± 0.3	7.1 ± 0.3	7.0 ± 0.3	0.550	0.697	0.177
Activin A (pg/mL)	38.1 ± 1.0	43.3 ± 2.2	42.0 ± 2.4	40.3 ± 1.7	0.178	0.955	0.060
4-months							
Age (days)	153.7 ± 1.4	154.7 ± 0.9	155.4 ± 1.1	153.0 ± 1.0			
Inhibin (ng/mL)	7.7 ± 0.2	7.6 ± 0.2	7.7 ± 0.2	7.5 ± 0.2	0.720	0.618	0.534
Activin A (pg/mL)	36.8 ± 2.0	35.6 ± 1.4	37.2 ± 1.6	34.8 ± 1.8	0.741	0.238	0.110
10-months							
Age (days)	301.7 ± 1.4	302.7 ± 0.9	303.4 ± 1.1	301.0 ± 1.0			
AMH (ng/mL)	18.5 ± 0.4 [a]	17.3 ± 0.4 [b]	18.2 ± 0.4	17.3 ± 0.5	0.039	0.090	0.550

Data are mean ± SEM. Values with different superscripts differ significantly ($P < 0.05$).
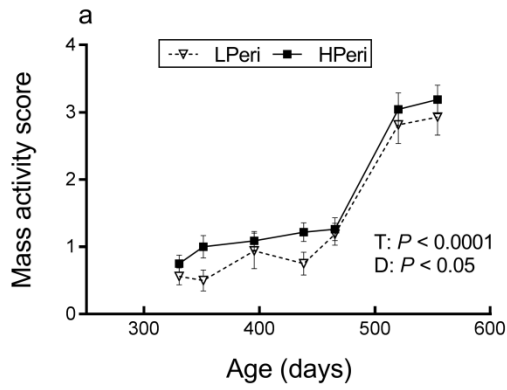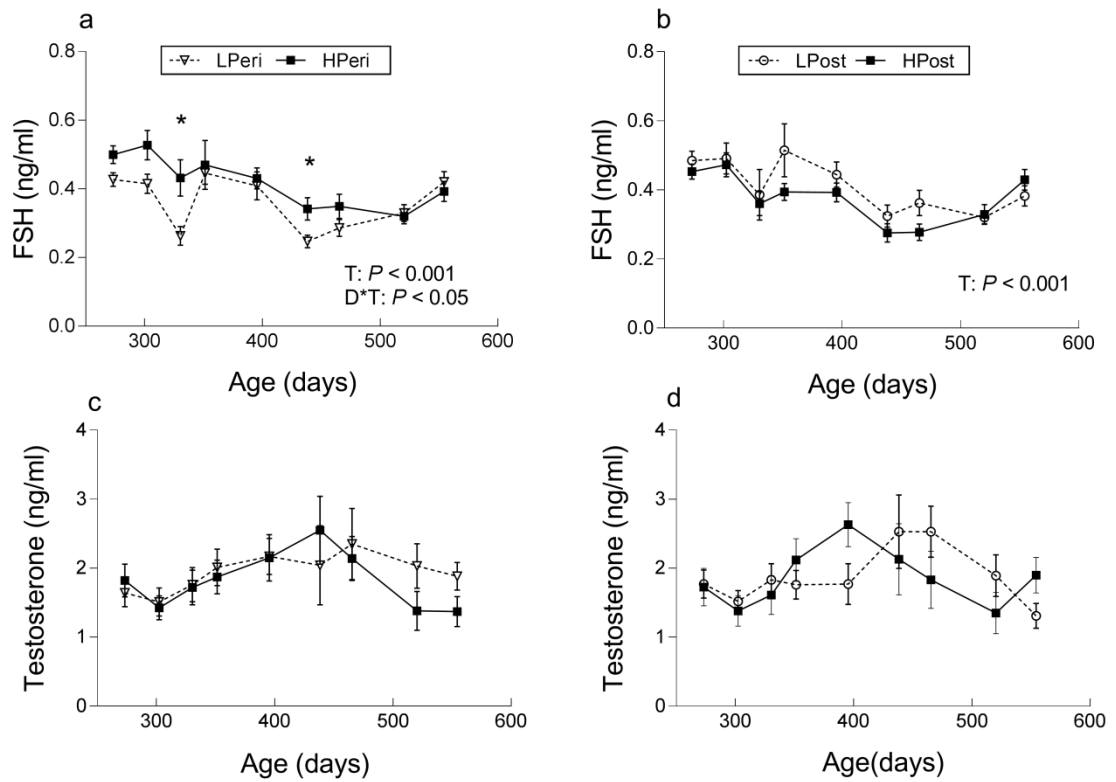
Figure 4. Circulating concentrations of FSH (ng/mL) and testosterone (ng/mL) in male
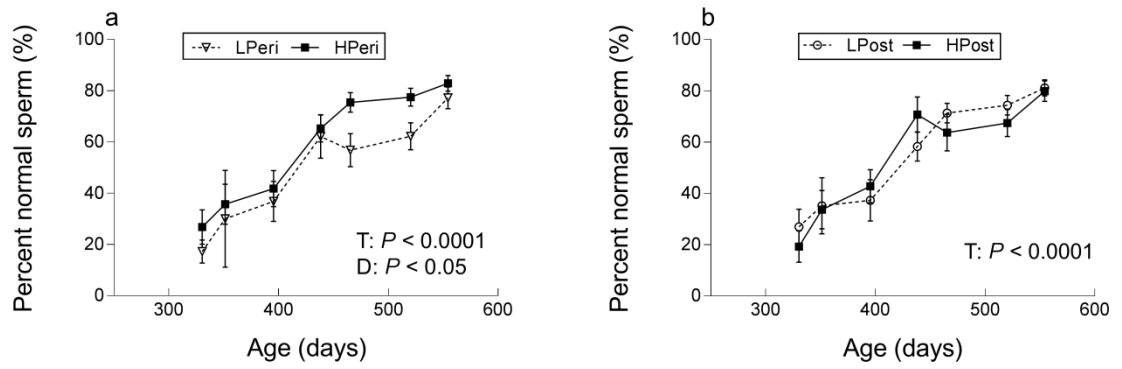
progeny (n = 40) between 273 and 554 days of age following exposure to maternal diets low

(L) or high (H) in protein during the (a,c) PERI-conception period (-60 to 23 dpc) or (b,d)

POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, T, D x T: Maternal diet,

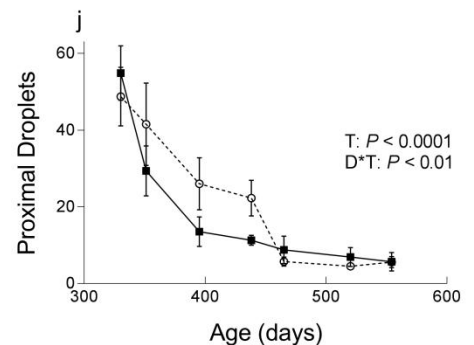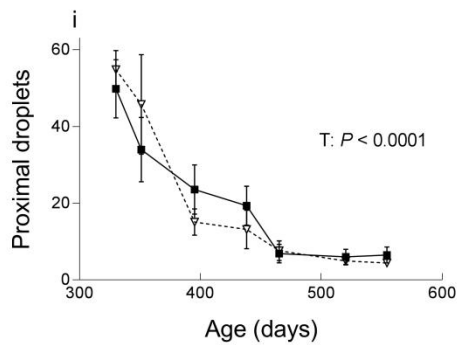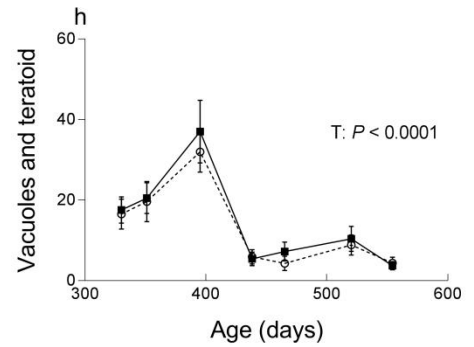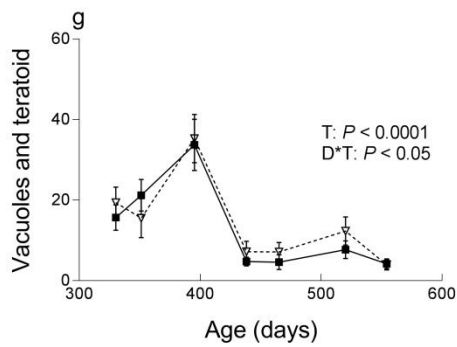time and maternal diet-by-time interaction effects respectively. Means with superscripts

indicate differences between groups within age.   * $P < 0.05$
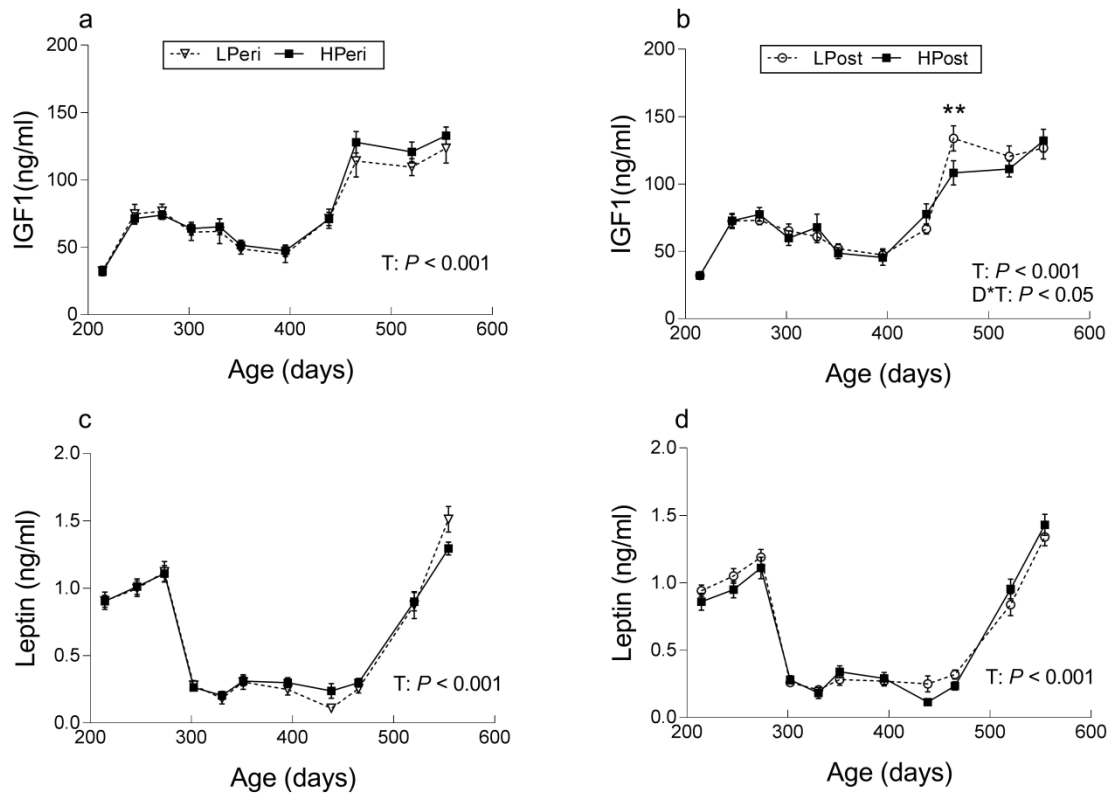
Figure 5. Circulating concentration of IGF1 (ng/mL) and leptin (ng/mL) in male progeny (n = 40) between 214 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (a,c) PERI-conception period (-60 to 23 dpc) or (b.d) POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, T, D x T: Maternal diet, time and maternal diet-by-time interaction effects respectively. Means with superscripts indicate differences between groups within age.   ** $P < 0.01$

vessel parameters were altered by dietary treatment (Tables 4-5). A higher proportion of seminiferous tubules within the testis (Table 4; $P = 0.04$) due to a greater number of tubules within the tissue (Table 4; $P = 0.04$) were observed in the LPeri diet fetal gonad compared to the HPeri gonad. There were no observed effects of maternal diet in the adult progeny in seminiferous tubule parameters (Table 4; $P > 0.05$).

The LPost fetal gonad displayed decreased numbers of blood vessels within the capsule of the testis (Table 5; $P = 0.02$) whilst the tissue area of blood vessels within the parenchyma of the testis (Table 5; $P = 0.03$) was decreased in the LPeri fetal gonad compared to the HPeri. In the adult testis, the number of blood vessels was increased by the LPost maternal diet (Table 4; $P = 0.03$).

Table 4. Area, number and percentage coverage of seminiferous tubules and blood vessels (BV) in 98 dpc fetus and adult (20 month old) bulls following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	*P*-value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
Fetal							
N	9	12	11	10			
Sem tubule area (μ²)	2115.9 ± 214.0	2691.3 ± 653.7	2560.0 ± 723.0	2317.8 ± 200.1	0.691	0.286	0.421
Sem tubules no.	27.1 ± 2.7^a	20.3 ± 2.0^b	24.6 ± 2.8	21.8 ± 2.2	0.035	0.166	0.088
Sem tubules %	21.8 ± 1.0^a	17.9 ± 1.4^b	18.5 ± 1.7	20.7 ± 0.9	0.041	0.844	0.320
BV area (μ²)	1042.8 ± 87.4^a	1457.0 ± 154.1^b	1221.7 ± 124.1	1343.0 ± 176.3	0.032	0.466	0.139
BV no.	83.6 ± 9.0	65.2 ± 7.6	74.9 ± 8.8	71.07 ± 8.51	0.134	0.531	0.435
BV %	1.9 ± 0.3	2.0 ± 0.3	2.2 ± 0.4	1.7 ± 0.3	0.913	0.318	0.490
Adult							
N	16	24	22	18			
Sem tubule area (μ²)	3842.3 ± 198.5	3901.1 ± 142.7	3877.2 ± 175.7	3878.0 ± 145.5	0.713	0.890	0.199
Sem tubules no.	15.1 ± 0.5	15.5 ± 0.4	15.6 ± 0.5	15 ± 0.3	0.585	0.611	0.408
Sem tubules %	54.4 ± 1.2	57.0 ± 1.1	56.7 ± 1.2	55.1 ± 1.1	0.146	0.325	0.319
BV area (μ²)	2451.2 ± 973.8	962.8 ± 229.2	1237.1 ± 486.0	1950.5 ± 731.4	0.704	0.548	0.653
BV no.	2.3 ± 0.2	2.4 ± 0.1	2.6 ± 0.2^c	2.1 ± 0.1^d	0.377	0.025	0.621
BV %	2.1 ± 0.7	1.1 ± 0.2	1.4 ± 0.3	1.6 ± 0.5	0.928	0.796	0.828

Mean ± SEM. Values with different superscripts differ significantly ($P < 0.05$).

Sem tubules = seminiferous tubules, BV = blood vessels, Area = average of total area of tissue occupied by blood vessel or tubule.

Table 5. Area, number and proportion of blood vessels (BV) within the parenchyma and capsule in 98 dpc fetus following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	P-value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	9	12	11	10			
Fetal BV Capsule							
BV area	1620.4 ± 163.0	2125.0 ± 302.2	1858.3 ± 287.4	1964.1 ± 261.0	0.273	0.549	0.833
BV no.	73.4 ± 8.0	54.7 ± 7.1	50.5 ± 5.7[c]	76.3 ± 8.2[d]	0.130	0.021	0.495
BV %	9.9 ± 1.1	9.5 ± 1.0	9.0 ± 1.1	10.6 ± 0.9	0.901	0.300	0.719
Fetal BV Parenchyma							
BV area	480.6 ± 39.4[a]	715.6 ± 84.6[b]	573.0 ± 68.9	660.9 ± 92.9	0.027	0.309	0.367
BV no.	11.3 ± 2.4	9.6 ± 1.5	10.2 ± 1.8	10.5 ± 2.0	0.540	0.882	0.200
BV %	0.11 ± 0.02	0.16 ± 0.03	0.13 ± 0.02	0.14 ± 0.04	0.328	0.986	0.328

Data represent mean ± SEM. Values with different superscripts differ significantly ($P < 0.05$).

BV = Blood vessel

Discussion

This study is the first to our knowledge to investigate the effects of maternal dietary protein during the peri-conception period and early gestation upon bovine male reproductive development. This was examined during fetal development and postnatally through to adulthood. The key findings were that the LPeri dietary treatment in nulliparous heifers altered reproductive development of their male progeny in the early post pubertal period as reflected by differences in reproductive hormones, testicular cytology and sperm production with a subsequent delay in reaching puberty. Increasing protein intake in the peri-conception period may therefore be economically viable for bull producers as the ability to use yearling bulls reduces production cost and shortens the genetic interval (Barth and Ominski, 2000).

Decreased protein intake during early gestation reduced early fetal growth, as indicated by the difference in biparietal diameter in the LPeri fetus at 60 dpc (Copping et al., 2014) and the reduced fetus weight in the subset of LPost male fetuses assessed at 98 dpc (Copping et al., 2014). This *in utero* effect was not discernible in later gross measures such as birth weight or postnatal growth as previously reported in lambs (Kotsampasi et al., 2009) or calves (Micke et al., 2015) but effects upon postnatal reproductive development were evident: The LPeri diet decreased BV area in the fetal testis. Moreover, seminiferous tubule number and percentage was increased by the LPeri maternal diet although this effect was not evident in the adult. In the developing bull, the LPeri maternal diet lowered sperm quality with this effect being subsequent to significantly lower FSH concentrations in this group at both 320 and 428 days of age compared to the HPeri group.

Testis Histology

The lack of effect upon sertoli, germ and interstitial cells is in contrast to studies which report a reduction in the number of Sertoli cells in newborn lambs undernourished *in utero* during the second trimester of gestation (Bielli et al., 2002; Kotsampasi et al., 2009) but concurs

with studies who excised the testis at the completion of the trial period during fetal life (Da Silva et al., 2003; Andrade et al., 2013).

The observed decrease in vasculature in the LPeri and LPost fetal testis with subsequent reversal of this effect in the adult testis suggests a compensatory ability of either the fetal or pubertal testis. Concomitantly, in the 98 dpc fetus, the LPeri diet caused an increment in the number of seminiferous tubules and proportion of seminiferous tubules per testis but did not affect tubule area. In combination, these results may indicate that the differentiation and proliferation of testicular cells and the development of the seminiferous tubules is not linked to the development of the blood vessels during the first trimester.

In the adult bulls the number and proportion of seminiferous tubules were unaffected by the dietary regimes, further suggesting that compensation may occur during developmental stages after our dietary intervention either in late gestation or postnatally. A prior study observed reduced diameter in the seminiferous tubules of bull calves at five months of age supplemented with protein *in utero,* from day 0 post AI to day 180 post-conception (Sullivan et al., 2010b) which may suggest compensatory mechanisms occur during the pubertal period.

Post-natal development

In this study we have shown that *in utero* LPeri diet increased the age at which bulls reach puberty predicated by the motility, morphology and concentration of sperm produced in the ejaculate (Barth and Oko, 1989; Perry et al., 1990; Holroyd et al., 2002). The higher levels of sperm with non-progressive motility, the overall increased numbers of morphologically-abnormal sperm and the tendency for lower concentration suggest that both epididymal function and spermatogenesis were delayed or disrupted by the LPeri maternal *in utero* diet. As expected in pubertal bulls, the initial high level of proximal droplets observed in ejaculates decreased over time (Lunstra and Echternkamp, 1982; Barth and Oko, 1989; Perry et al., 1991; Evans et al., 1995) but was not altered by *in utero* diet. Midpiece defects and abnormal head and tails were increased in the LPeri diet bulls; both defects reported to be associated

with disturbance of epididymal function (Barth and Bowman, 1994). Knobbed acrosomes were similarly increased in the LPeri bulls at 438, 465 and 520 days of age indicating disturbed spermiogenesis during this peri-pubertal period (Barth and Bowman, 1994; Beggs et al., 2013) compared to the HPeri group. In the current experiment, the bulls reached puberty at a similar age to that previously reported for *Bos indicus* x *Bos taurus* crossbred bulls (Chase et al., 2001; Brito et al., 2004) of this breed type and intermediate to that previously reported for *Bos taurus* (Lunstra et al., 1978; Evans et al., 1995) and *Bos indicus* breeds (Fields et al., 1982; Aponte et al., 2005). The earlier age of puberty observed in the HPeri bull cohort is a desirable production outcome (Barth and Ominski, 2000).

There was no effect of maternal dietary treatment upon scrotal circumference or paired testis weight at 600 days. This is in agreement with the findings of Rae et al. (2002a) who reported no effect of maternal under-nutrition on scrotal circumference of ram offspring at both 6 weeks and 20 months of age. The absence of *in utero* dietary effect upon scrotal circumference concurs with the observed lack of effect upon fetal testis weight and Sertoli cell count at 98 dpc. Consequently, the effects of maternal protein restriction on sperm parameters and age of puberty were considered to be not directly the result of altered Sertoli cell numbers in the developing post-natal animal (Sharpe et al., 2003).

The effects on sperm parameters were however, subsequent to lower FSH concentrations in the LPeri cohort; FSH is an integral part of the hormonal cascade regulating essential physiological adaptation in the pre-pubertal and pubertal bull enabling sperm production (Perry et al., 1991). This hormonal milieu is also essential for epididymal function (Grover et al., 2005) as well as the regulation of spermatogenesis in the mature bull (Barth and Bowman, 1994; Matsuzaki et al., 2000; O'Shaughnessy, 2014). In ruminants, no comparative studies have documented the associations between prenatal nutrition and sperm abnormalities in the progeny. The results are however, consistent with Toledo et al. (2011) who reported impairment of sperm counts, sperm motility and higher levels of sperm with morphological abnormalities in adult male rats (90 days of age) following *in utero* protein restriction from 0-

21 dpc.

The relationship between reduced FSH and delayed post-natal activation of the reproductive axis observed in the pubertal and post pubertal LPeri cohort concurs with previous research: FSH levels, along with LH, rise transiently between 1 to 4 months of age in the pre-pubertal bull (Rawlings et al., 1978; Evans et al., 1996; Moura and Erickson, 1997; Kaneko et al., 2001; Bagu et al., 2006), a rise reported to be associated with the initiation of rapid testis growth (Moura and Erickson, 1997). FSH levels then fall, remaining low during peri-puberty and puberty (Moura and Erickson, 1997; Kaneko et al., 2001; Brito et al., 2007c, d). In the adult, FSH levels increase as bulls age in association with improvement in sperm quality and quantity (Matsuzaki et al., 2000). Thus, the observed lower basal FSH in the LPeri bulls during the pubertal and post-pubertal period (330 and 438 days of age) may indicate a hormonal regulation pathway contributing to the delayed elevation of sperm traits discussed above.

Circulating testosterone did not differ among the groups during the peripubertal and postpubertal periods, possibly reflecting a lack of effect of the maternal dietary regimens on Leydig cell number, function or both (Barth et al., 2008). Collectively, the lack of effect of maternal diet on testosterone (Rawlings et al., 2008), pre-pubertal inhibin (Kaneko et al., 2001) and pre-pubertal activin A, (Mather et al., 1992), all known to be involved in regulation of post-natal FSH secretion in the developing bull, would suggest that the differences in FSH levels associated with PERI diet were modulated via other pathways. Alternatively, the monthly blood sampling regimen may have been inadequate to detect the effects of maternal diet on testosterone, inhibin or activin A, particularly considering the pulsatile nature of testosterone.

The later age of puberty in the LPeri bulls was also associated with higher AMH levels at 10 months of age. This may suggest a delay in the down-regulation of AMH expression that is known to occur at puberty (Rey and Josso, 1996; Rey et al., 2003) coincident with Sertoli cell maturation (Sharpe et al., 2003). As circulating AMH levels decline sharply in the pubertal

bull (Rota et al., 2002), it is possible the differences measured at one time point may reflect differences in maturity as opposed to differences due to dietary perturbation. However, as birthweight and post-natal liveweights were similar, the observed effects on age of puberty are more likely to have been mediated by the effects of altered FSH secretion rather than persisting influences of prenatal nutrition on postnatal growth. This is further supported by the lack of maternal dietary effect upon progeny IGF1 and leptin profiles; the relationship between energy homeostasis and puberty being well recognised (Blache et al., 2003; Barb and Kraeling, 2004; Zieba et al., 2005; Brito et al., 2007a; Brito et al., 2007c, d; Barth et al., 2008). Collectively these observations indicate that post-natal diet and liveweight were not involved in the observed changes in postnatal reproductive development in the current study in contrast to findings reported in pre-natally growth restricted rams (Da Silva et al., 2001).

Early maternal undernutrition has been reported to disrupt a range of endocrine pathways with long-term effects on progeny health (McMillen and Robinson, 2005; Gardner et al., 2006; McMillen et al., 2008). Furthermore, previous studies support the concept that early maternal undernutrition impacts hypothalamic and/ or pituitary function at later post-natal stages causing alterations to the endocrine system, including the HPG axis. These include, changes in gonadotrophin profiles (Rae et al., 2002a), reduced testosterone concentrations and delayed seasonal increase in testosterone (Da Silva et al., 2001) as well as altered hypothalamic-pituitary responsiveness to post-natal GnRH challenge in sheep (Kotsampasi et al., 2009) and pre-pubertal bulls (Sullivan et al., 2010b). In the current study, a GnRH challenge was not undertaken and the animals were allowed to progress through puberty without any exogenous hormone influence; hence it is not possible to report on pituitary responsiveness in this study. Further studies are required to explore the role of maternal nutrition on the development and function of the hypothalamic-pituitary-gonadal axis in both the fetal and adult male bovine.

Conclusion

In summary, we have uniquely shown that in the developing bull, the LPeri maternal diet delayed the onset of puberty and sexual maturity with negative effects on semen parameters in the early post-pubertal period. These effects were subsequent to lower FSH concentrations in the LPeri maternal diet group. The histology of the fetal and adult testis suggests that the observed early perturbation of the cytology of the testis has been compensated for during later development as no corresponding cytological effects were observed in the adult testis. Whether the effects of this perturbation influenced testicular function through puberty prior to excision of the testis at 20 months, however, is unknown due to the single time point of testis excision in the adult. The circulating hormone data suggest that the peri-conception diet may have altered the development of the hypothalamic-pituitary-gonadal axis and/or the receptivity to circulating hormones during the peri-pubertal period.

Whilst this study provides evidence that low maternal dietary protein has a negative impact on reproductive development in the pubertal and post-pubertal offspring, some of the mechanisms that mediate this effect remain to be elucidated. Further research in bovines is economically warranted to enable exploration of causal relationships between gestational nutrition and consequent post-natal male reproductive development of progeny.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Supplementary Table 1. Fetal gonad weight and relative fetal gonad weight at 98 dpc in male progeny following exposure to maternal diets[1] low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception period (24 to 98 dpc).

Diet	LPeri	HPeri	LPost	HPost	P-value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	9	16	14	11			
Gonad weight (g)	0.103 ± 0.004	0.096 ± 0.005	0.093 ± 0.005	0.104 ± 0.005	0.526	0.334	0.273
Relative Gonad weight (g/g)	0.00031 ± 0.00001	0.00029 ± 0.0001	0.00029 ± 0.00001	0.00030 ± 0.0001	0.240	0.831	0.106

Mean ± SEM. No significant differences between any groups ($P > 0.05$).

[1]LL = Low protein maternal diets peri- and post-conception, LH = Low protein maternal diet in the peri-conception period and high protein post-conception, HL = High protein maternal diet in the peri-conception period and low protein post-conception, HH = High protein maternal diets peri- and post-conception.

Supplementary Table 2. Gestation length, birthweight, final scrotal circumference measure prior to slaughter, testes weight and relative testis weight at slaughter in male progeny following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	*P* - value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	16	24	22	18			
GL (days)	282.3 ± 1.4	282.3 ± 0.9	281.3 ± 1.1	283.6 ± 1.1	0.849	0.242	0.162
Birthweight (kg)	33.6 ± 1.1	32.8 ± 0.8	32.5 ± 0.9	33.9 ± 0.9	0.467	0.630	0.064
Scrotal Circ. (cm)	34.1 ± 0.5	34.2 ± 0.4	34.3 ± 0.4	34.0 ± 0.4	0.671	0.242	0.162
Age at slaughter (days)	597.7 ± 1.4	598.7 ± 0.9	599.4 ± 1.1	597.0 ± 1.0	0.995	0.924	0.741
Paired testes weight (g)	599.1 ± 24.4	605.5 ± 22.8	603.5 ± 22.2	602.4 ± 25.7	0.998	0.635	0.346
Relative testes weight (g/kg)	0.90 ± 0.03	0.91 ± 0.03	0.91 ± 0.03	0.91 ± 0.04	0.964	0.628	0.970

Mean ± SEM. No significant differences between any groups (*P* > 0. 05).

GL = Gestation length

Relative testes weight – paired testes weight: bodyweight (g/kg)

Supplementary Table 3. Testicular cell percentages in 98dpc fetus following exposure to maternal diets low (L) or high (H) in protein during the PERI (-60 to 23 dpc) and POST-conception (24 to 98 dpc) period.

Diet	LPeri	HPeri	LPost	HPost	P-value		
	(LL + LH)	(HH + HL)	(LL + HL)	(HH + LH)	Peri	Post	Peri*Post
N	9	12	11	10			
Sertoli cells %	34.0 ± 1.3	33.0 ± 1.0	33.0 ± 1.1	34.0 ± 1.2	0.400	0.913	0.396
Germ cells %	6.0 ± 0.9	5.0 ± 0.9	5.0 ± 1.1	6.0 ± 1.0	0.293	0.924	0.729
Interstitial cells %	59.0 ± 1.0	62.0±1.0	61.3 ± 1.2	60.0 ± 1.3	0.158	0.910	0.176

Mean ± SEM. No significant differences between any groups ($P > 0.05$).

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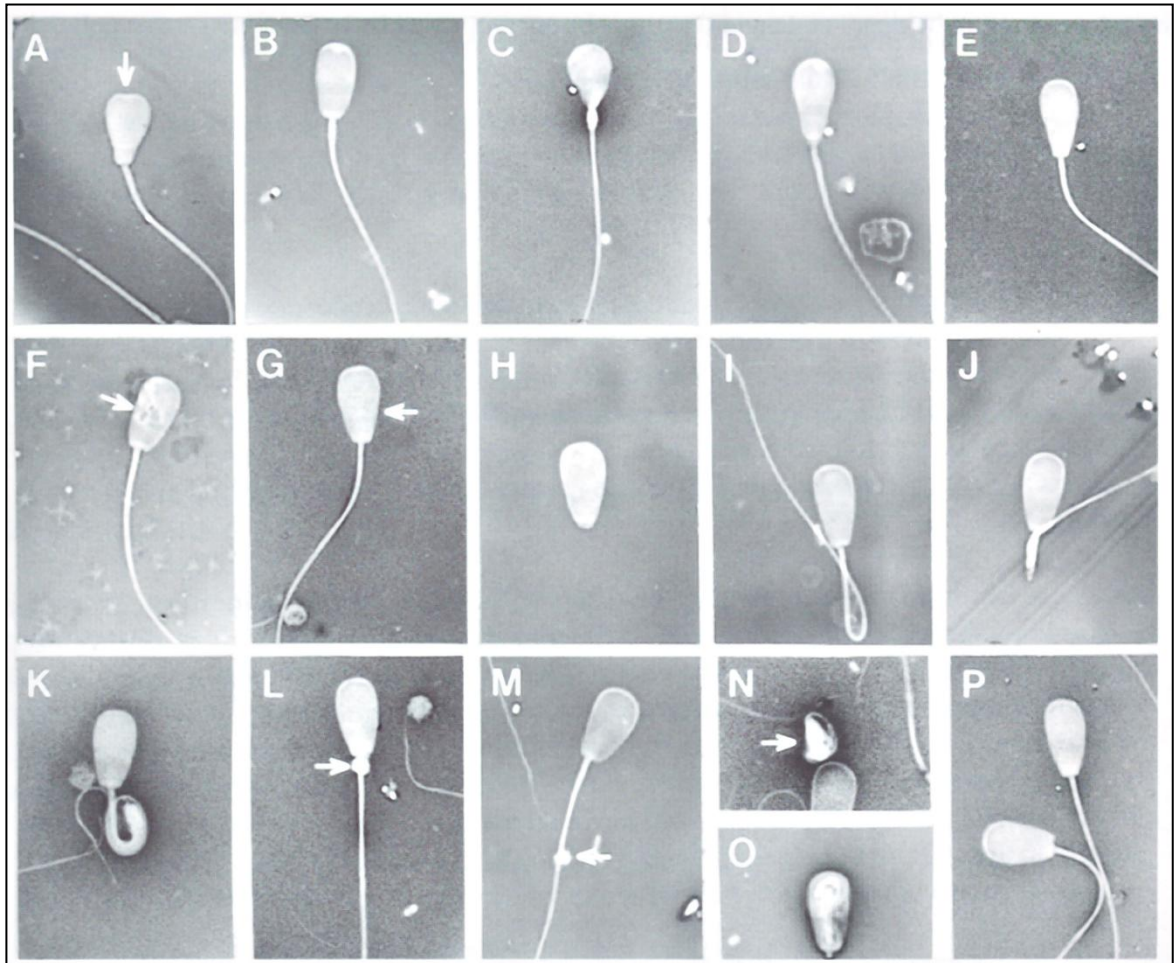
Supplementary Figure 1. Common sperm abnormalities [adapted from (Barth and Oko, 1989)].

A. Knobbed acrosome (common form); B. Knobbed acrosome (beaded form); C. Pyriform head (severe); D. Pyriform head (moderate); E. Pyriform head (slight); F. Nuclear vacuoles; G. Diadem defects; H. Detached head; I. Distal reflex; J. Dag-like defect (broken midpiece); K. Dag-like defect (severely bent midpiece); L. Proximal droplet; M. Distal droplet; N. Teratoid (severe); O. Teratoid (moderate); P. Normal spermatozoa.
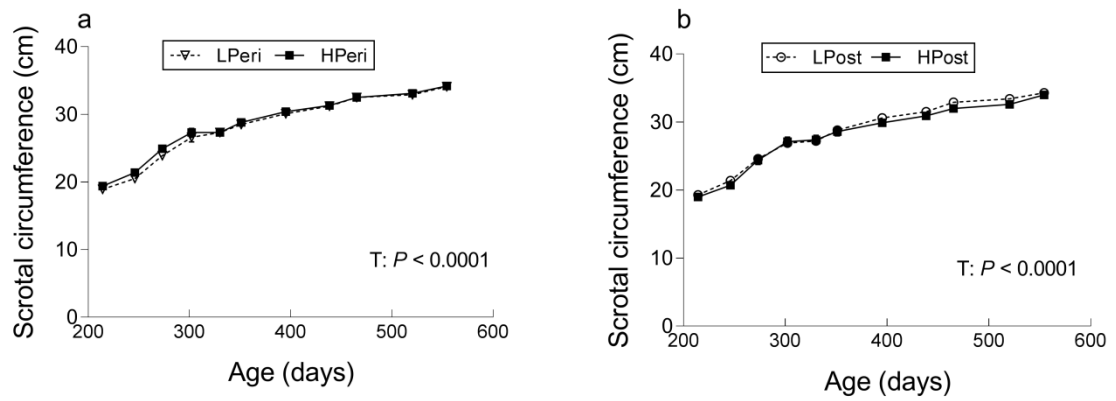
Supplementary Figure 2. Scrotal circumference measurements in male progeny (n = 40) between 214 and 554 days of age following exposure to maternal diets low (L) or high (H) in protein during the (a) PERI-conception period (-60 to 23 dpc) and (b) POST-conception period (24 to 98 dpc). Data are mean ± SEM. D, A, D x A: Maternal diet, time and maternal diet x time interaction effects respectively. No significant differences among groups within age ($P > 0.05$).
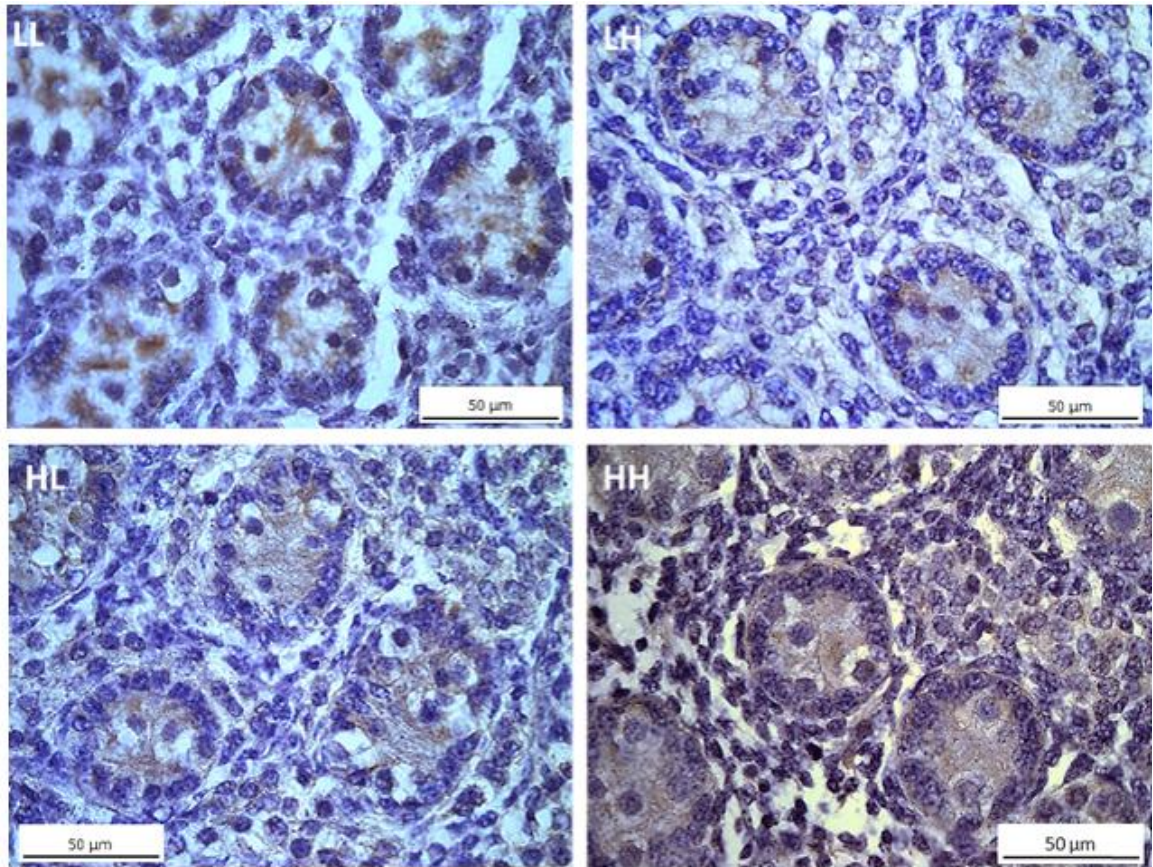
Supplementary Figure 3. Photomicrographs of 98 dpc testis from fetuses exposed to maternal diets[1] low (L) or high (H) in protein during the peri-conception period (-60 to 23 dpc) and post-conception period (24 to 98 dpc). Immunohistochemical staining (Müllerian inhibitory substance MIS-C20 antibody) differentiating germ cells (stained blue) and Sertoli cells (DAB positive brown) contained within the seminiferous tubules and leydig cells in the surrounding interstitial tissue. Scale bars represent 50µm [photomicrograph reproduced from Copping et al. (2017) with acknowledgement to co-author M.D. Ruiz-Diaz].

[1]LL = Low protein maternal diets peri- and post-conception, LH = Low protein maternal diet in the peri-conception period and high protein post-conception, HL = High protein maternal diet in the peri-conception period and low protein post-conception, HH = High protein maternal diets peri- and post-conception.

Chapter 6

The influence of peri-conception and first trimester dietary protein levels in beef cattle on progeny feedlot performance, feed intake and carcass characteristics.

K.J. Copping, M.J. Callaghan, G. Geesink, I.C. McMillen, R.J. Rodgers and V.E.A. Perry

Statement of Authorship

Title of Paper	The influence of peri-conception and first trimester dietary protein levels in beef cattle on progeny growth, feedlot performance, feed intake and carcass characteristics.
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Principal Author

Name of Principal Author (Candidate)	Katrina Copping		
Contribution to the Paper	Experimental design; collection of birth and post-natal growth data, collection of abattoir data, performed statistical analysis and interpreted data, wrote the manuscript.		
Overall percentage 75 (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/11/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mathew Callaghan		
Contribution to the Paper	Formulated maternal diet and constructed diet table for manuscript. Assisted with collection of abattoir data		
Signature		Date	20/06/16

Name of Co-Author	Geert Geesink		
Contribution to the Paper	Assisted with collection of abattoir data.		
Signature		Date	28/06/16

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Caroline McMillen		
Contribution to the Paper	Contributed to initial funding application and concept.		
Signature		Date	14/10/16

Name of Co-Author	Raymond Rodgers		
Contribution to the Paper	Co-supervisor. Contributed to initial funding application. Contributions to sample collection and manuscript evaluation.		
Signature		Date	12/9/2016

Name of Co-Author	Viv Perry		
Contribution to the Paper	Principal supervisor, advisory role, overall project design and co-ordination, contributions to experimental design, procedures, animal monitoring, sample collection, evaluation and editing of the manuscript.		
Signature		Date	15/11/16

Name of Co-Author	Caroline McMillen
Contribution to the Paper	Contributed to initial funding application and concept.

Signature		Date	14/10/16

Name of Co-Author	Raymond Rodgers
Contribution to the Paper	Co-supervisor. Contributed to initial funding application. Contributions to sample collection and manuscript evaluation.

Signature		Date	12/9/2016

Name of Co-Author	Viv Perry
Contribution to the Paper	Principal supervisor, advisory role, overall project design and co-ordination, contributions to experimental design, procedures, animal monitoring, sample collection, evaluation and editing of the manuscript.

Signature		Date	15/11/16

The influence of peri-conception and first trimester dietary protein levels in beef cattle on progeny feedlot performance, feed intake and carcass characteristics.

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ABSTRACT

Nulliparous yearling beef heifers (n = 360) were used to evaluate the effects of maternal dietary protein during the peri-conception and first trimester periods on postnatal growth, feedlot performance, feed intake and carcass characteristics of their male progeny. Heifers were individually fed 1.18 g CP/d (**HPeri**) or 0.62 g CP/d (**LPeri**) beginning 60 d before conception. From 24 to 98 d post-conception, half of each treatment group changed to the alternative post-conception diet and fed 1.49 g CP/d (**HPost**) or 0.88 g CP/d (**LPost**) yielding four treatment groups in a 2 x 2 factorial design. From d 98 of gestation, heifers received a common diet until parturition. Calves were weaned at 183 d and back grounded before feedlot entry. Among the singleton non-castrated male progeny there were no differences between treatment groups in milk intake, pre- and post-weaning BW, ADG and height ($P > 0.05$). Bulls underwent a 70 d RFI feedlot test commencing at 528 d of age prior to slaughter. Progeny of dams that had a change in diet [LPeri/HPost (**LH**) and HPeri/LPost (**HL**)] had 9% higher daily DMI during the RFI test ($P < 0.05$) than progeny of dams that received low diet throughout both the peri-conception period and first trimester [LPeri/LPost (**LL**)]. Progeny feedlot entry and final BWT, ADG, RFI and HCW were not different among bulls ($P > 0.05$), however dressing percentage, estimated retail beef yield and longissimus muscle area were all higher for bulls from HPost dams at slaughter ($P < 0.05$). This study highlights opportunities for the livestock industry whereby maternal dietary supplementation may be used to alter feed intake, enhance progeny muscling and alter fat deposition leading to improvement in efficiency of meat production in beef cattle.

Key Words: Appetite, beef cattle, carcass, protein supplementation, fetal programming.

INTRODUCTION

Under Australian extensive pasture production systems, protein is the most common limiting factor to production (Bortolussi et al., 2005). Nutritional perturbations may result in the fetus being unable to express its full genetic potential. In cattle, altered maternal dietary protein intake during gestation has been shown to lead to both short and long-term impacts upon the developing fetus, as well as the growth, physiology and metabolism of the offspring in adult life (Cafe et al., 2006; Larson et al., 2009; Sullivan et al., 2009d; Long et al., 2010b; Micke et al., 2010a; Micke et al., 2010b; Sullivan et al., 2010b).

Early gestation is an important period of development for the programming of appetite and body composition. The persistence of such changes may contribute to the programming of an altered appetite in adulthood with this hypothesis forming the basis for investigations into the fetal origins of human obesity (Ong and Muhlhausler, 2014). Compared to the brain, both muscle and adipose tissue have a lower priority for nutrient partitioning, making development of these fetal tissues vulnerable to undernutrition (Zhu et al., 2006). Early and mid-gestation are also critical developmental stages for skeletal muscle and adipose tissue in the ruminant (Stickland, 1978; Du et al., 2010). Alterations to cell differentiation in both muscle and adipose tissue during this period of development may modify postnatal body composition and carcass characteristics (Du et al., 2011).

Our aim, therefore, was to evaluate the effect of dietary protein at levels experienced under Australian range conditions in unsupplemented and supplemented heifers calving at 2 years of age upon progeny performance. Previously we showed in 3 year old heifers that nutrient intake during the first and second trimesters caused persistent programming of postnatal liveweight and carcass weight in the progeny in a sex-dependent manner (Micke et al., 2010a; Micke et al., 2011a). To interrogate further these windows of impact upon fetal development, we tested the hypothesis that low maternal dietary protein intake during the peri-conception

period and the first trimester in heifers calving at 2 years of age would decrease post-natal growth, feedlot performance and carcass traits whilst increasing feed intake in the male progeny.

MATERIALS AND METHODS

Experimental animals

Use of animals and the procedures performed in this project were approved by the University of South Australia IMVS, The University of Adelaide and The University of New England Animal Ethics Committees. (Approval numbers: 18/11, S2012-249 and AEC14-037, respectively).

Experimental design

The purpose of this experiment was to evaluate the impact of maternal dietary protein during the peri-conception (PERI; -60 to 23 dpc (conception being the day of AI and implantation being 18 to 22 dpc (Wathes and Wooding, 1980; Spencer and Hansen, 2015b) and first trimester (POST; 24 to 98 dpc) periods in nulliparous heifers upon postnatal growth, appetite (as measured by DMI) and carcass traits in the male progeny. The dietary protein levels reflected pasture conditions in Australian rangelands without (**Low**), and with (**High**), protein supplement.

The study was a two-by-two factorial design. The study animals were the singleton male progeny of 2-yr old heifers described previously (Copping et al., 2014). Briefly, three hundred and sixty nulliparous Santa Gertrudis (*Bos taurus* x *Bos indicus*) heifers were selected from S Kidman and Co herds located at 'Glengyle' and 'Morney Plains', south western Queensland. Heifers were transported to 'Tungali', Sedan, South Australia (34º29´S, 139 º18´E) where they underwent a 60 d acclimatization prior to commencement of the study. At 12-mo of age, 60 d prior to AI, heifers were stratified by BW and randomly assigned to two equal PERI treatment groups, High and Low protein (**HPeri** and **LPeri**). Each heifer was

fed a High protein diet (71 MJ ME/d and 1.18 kg CP/d) or Low (63 MJ ME/d and 0.62kg CP/d) (as fed basis; Table 1) consisting of a pelleted diet supplemented with a commercial vitamin and mineral preparation (Minmix, Ridley Agriproducts, Toowong, Qld). The ration was individually fed in stalls with straw (5% CP) available *ad libitum*. Heifers underwent a progesterone based estrous synchronization program commencing 10 days prior to AI (Hernandez-Medrano et al., 2015) when all heifers were inseminated with frozen semen from one Santa Gertrudis bull. At 23 dpc, half of each nutritional treatment group changed to an alternative post-conception (POST) treatment in the first trimester, High (**HPost**: 102 MJ ME/d and 1.49 kg CP/d) or Low (**LPost**: 98 MJ ME/d and 0.88kg CP/d) (as fed basis; Table 1), giving rise to four treatment groups: [LPeri/LPost (**LL**), LPeri/HPost (**LH**), HPeri/LPost (**HL**), HPeri/HPost (**HH**)]. The ration continued to be individually fed with straw (5% CP) available *ad libitum*. The diets were as isocaloric as possible for ruminants fed the forage component of the diet under group housing. There was a 1.9 to 2.1-fold difference in CP content and 1.1-fold difference in energy content between the High and Low diets. Thus, protein intake was restricted during both the peri-conception period and first trimester in the Low group whilst both groups received similar energy intake..

Pregnancy was confirmed at 36 dpc and fetal sex was determined at 60 dpc by transrectal ultrasound (Copping et al., 2014). Non-pregnant heifers were subsequently removed from the trial. A sub-set of heifers (singleton fetus; $n = 46$, 21 females and 25 males) was necropsied at 98 dpc (Copping et al., 2014). From 98 dpc (the end of the first trimester of gestation), the remaining heifers were individually fed a common diet, which was formulated to provide additional growth of 0.5 kg/d until parturition (79 MJ ME/d and 0.92 kg CP/d: as fed basis, Appendix 1). Heifers continued to receive the measured pellet portion of their diet individually on a daily basis with straw (5% CP) *ad libitum* until parturition. Sixty-four heifers completed the study and gave birth to 18 live singleton female (n: LL= 3; LH = 4; HL = 4; HH = 7), 43 live singleton bull progeny (n: LL= 9; LH = 10; HL = 14; HH = 10) and 1 set live twin bull progeny (n: LL = 2; excluded from the analysis). Two bull progeny were

removed from the study after birth, due to poor mothering. An additional calf was subsequently found to be a cryptorchid (LL group) and was excluded.

At parturition, individual feeding ceased. Progeny remained with their dams grazing native and improved pastures as one group until weaning at 183.3 ± 0.8 d (age $\pm$ SEM). Progeny were weighed at birth (Copping et al., 2014), then approximately monthly along with measurement of height at the cranial dorsal iliac spine (Micke et al., 2010a). During the post-weaning period, male and female progeny were segregated. The male progeny remained at Sedan, South Australia in one group grazingnative and improved pasture until the commencement of the RFI Test. The male progeny were left un-castrated to enable the assessment of their reproductive development
 (Copping, unpublished data; Chapter 5).

Milk intake

The milk intake of the male progeny was measured using the weigh-suckle-weigh (WSW) protocol, previously described by Beal et al. (1990) and Sullivan et al. (2009e), on 34, 65, 92, 125, 154 and 183 days of post-natal life. Measurements were obtained at by the summations of the changes in calf BW during three supervised sucklings undertaken at two 6-hour intervals and a 12-hour interval over a 24-hour period. The difference in pre- and post-suckling calf BW was considered to be the amount of milk consumed (Beal et al., 1990).

Residual feed intake test

Non-castrated singleton male progeny were transported from Sedan, South Australia to the 'Tulimba' Research Feedlot, Kingstown, NSW (30º28´S,151 º11´E) several weeks prior to the commencement of an RFI test. Prior to the test, the animals underwent an adjustment period (Arthur et al., 2004). One bull was removed from the feedlot at the end of the pre-test introduction period after failing to adjust to the feeding environment. The bull remained on

feed in an open bunk pen but was excluded from the study reported herein from 528 d

onwards. The remaining bulls (n: LL= 8; LH = 8; HL = 13; HH = 10) commenced a standard

70-day RFI test (also referred to in Australia as net feed intake, or NFI, test) (Exton, 2001).

Briefly, during the RFI test animals had *ad libitum* access to a finisher diet (13.5 MJ and 90%

DM). Individual feed intake was measured using computerised automatic feeders (GrowSafe,

Airdrie, Alberta, Canada) with each animal fitted with an electronic ear tag and individual

feeding events recorded over the duration of the test period. Bulls were randomly allocated to

two, adjacent feedlot pens at the beginning of the trial. The average age and liveweight (±

sem) at the commencement was 528.3 ± 0.8 days and 537.5 ± 4.9 kg, respectively. Individual

animal liveweight was measured throughout the test period as per the standard RFI protocol

(Exton, 2001). Metabolic midtest BW (MMW) and average daily gain (ADG) was calculated

from the regression of the animal's fortnightly BW against day of test. Residual Feed Intake

was calculated from the linear regression of average daily feed intake during the test (kg

DM/day) against MMW (kg) and ADG (kg/day) with the residual being its RFI, as detailed by

Arthur et al. (2001).

Carcass traits

Following the completion of the RFI test, the bulls were transported to a commercial abattoir

at Warwick, Queensland where they were euthanised by captive bolt stunning and

exsanguination and then assessed for carcass traits.

After slaughter, carcasses were weighed to give a hot standard carcass weight (HCW).

Carcasses were prepared following standard AUS-MEAT procedure (Anon, 2007). Rump

(P8) fat was recorded, before the sides of each carcass were chilled overnight. Carcasses were

quartered the next morning between the 12th and 13th ribs. Carcass grading was undertaken

by an accredited assessor. Traits measured were: eye muscle area (EMA), AUS-MEAT

marble score [AusMS: 0 (nil) to 6 (abundant)], fat colour [0 (near white) to 4 (dark cream) by

units of 1] and meat colour [1A (pale pink) to 1C (dark pink); 2 (pale red); 3 (red)]. Estimated

retail beef yield (RBY%) was calculated using the prediction equation Yield = 64.8 - (0.2 *

P8) - (0.14 * EMA) (John Wilkins, NSW Department of Primary Industries, NSW, Australia,

personal communication). Dressing percentage (dressing%) was calculated as HCW divided

by the final non-fasted weight at the feedlot (Cafe et al., 2006).

Statistical analyses

Two-way ANOVA (STATA/IC 13.0, StataCorp, College Station, Texas), was used to

interpret the effects of maternal nutrition treatment group during PERI and POST conception

treatment periods and their interaction term on liveweight, FI, ADG, DMI, RFI, HCW, EMA,

P8 Fat depth, fat colour, AUS-MEAT meat colour, AusMS, dressing % and estimated retail

beef yield (RBY%). One-way ANOVA with Tukey-Kramer post hoc test was used to explore

significant interactions between nutritional treatment groups during PERI and POST as

required. All traits were analysed as if continuous with meat colour coded: 1A = 1, 1B = 1.3,

1C = 1.7, 2 = 2, 3 = 3 (Herd et al., 2014). Feedlot pen influenced daily weight gain and thus

was included as a co-variate along with animal age. Additionally, to investigate the

interactions between maternal diet and time on liveweight, height and milk intake, linear

mixed-effects models were performed, adjusting for repeated measures over time for each of

the bulls. An autoregressive 1 covariance structure was used. Post-hoc comparisons were

made of the differences of least squares means. The statistical software used was SAS 9.3

(SAS Institute Inc., Cary, NC, USA). Statistical significance was accepted at $P < 0.05$, and a

tendency at $P < 0.10$.

RESULTS

Pre-weaning and post-weaning progeny performance

Progeny liveweight, ADG and height at weaning and post-weaning prior to feedlot test are

summarised in Table 1. At birth, there was no difference in progeny birthweight due to

maternal nutrition treatment as previously reported (Copping et al., 2014). Post-natal progeny

liveweight, ADG and height did not differ due to maternal dietary treatment (P > 0.10). There was no effect of gestation length on progeny liveweight, ADG and height (P > 0.10). When data from birth until 600 d were considered in a single analysis, liveweight and height changed with time (P < 0.05; Fig. 1) but did not alter due to maternal diet during the peri- or post-conception period, nor were there any interaction effects (P > 0.10).

Milk intake

Milk intake did not differ between 34 d and 198 d (weaning) due to maternal diet (P > 0.10; Fig. 2).

Feedlot growth and efficiency

Mean age, feedlot start liveweight and final liveweight, ADG, FI, FCR and RFI are presented in Table 2. There were no significant differences (P > 0.05) in age, liveweight at the start or end of the feedlot period between bulls from the different maternal nutrition treatment groups. Feed Intake varied with a significant interaction (P < 0.05) between maternal PERI and POST diet. Bulls whose dams had a change in diet at the end of the PERI diet period from High protein to Low protein and *vice versa* (HL and LH progeny) had 9% higher daily feed intake on test (P < 0.05) than those whose dams received a constant low protein diet throughout both the PERI- and POST-conception diet period (LL progeny). A similar pattern was apparent in feedlot growth rate (ADG) and RFI during the feedlot period but the differences were not statistically significant (P > 0.10).

Table 1. Liveweight (BW), height and ADG of bulls at weaning and post-weaning prior to commencement of residual feed intake test, following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[1].

	Treatment				SEM			
PERI	Low		High				P-value	
POST	Low	High	Low	High		PERI	POST	PERI*POST[2]
Item								
Weaning, 183 d of age								
n	8	8	14	10				
ADG, kg/d	0.84	0.93	0.90	0.84	0.02	0.657	0.555	0.069
Height, cm	116.5	119.5	118.1	117.0	0.5	0.645	0.316	0.075
BW, kg	185.1	204.7	200.1	184.7	3.4	0.619	0.524	0.100
Post-weaning, 183-520 d of age								
n	8	8	14	10				
ADG, kg/d	1.00	0.94	0.95	0.95	0.01	0.592	0.138	0.415
Height at 520 d, cm	137.1	136.9	139.0	136.3	0.7	0.662	0.334	0.432
BW at 520 d, kg	520.8	520.0	519.3	505.2	4.5	0.418	0.388	0.423

Values are unadjusted mean ± SEM.

[1]Dams were individually fed diets Low or High in protein during the periconception (PERI;-60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[2]PERI*POST = Interaction term (2 x 2 factorial design). Significant interactions explored by post hoc test as required.
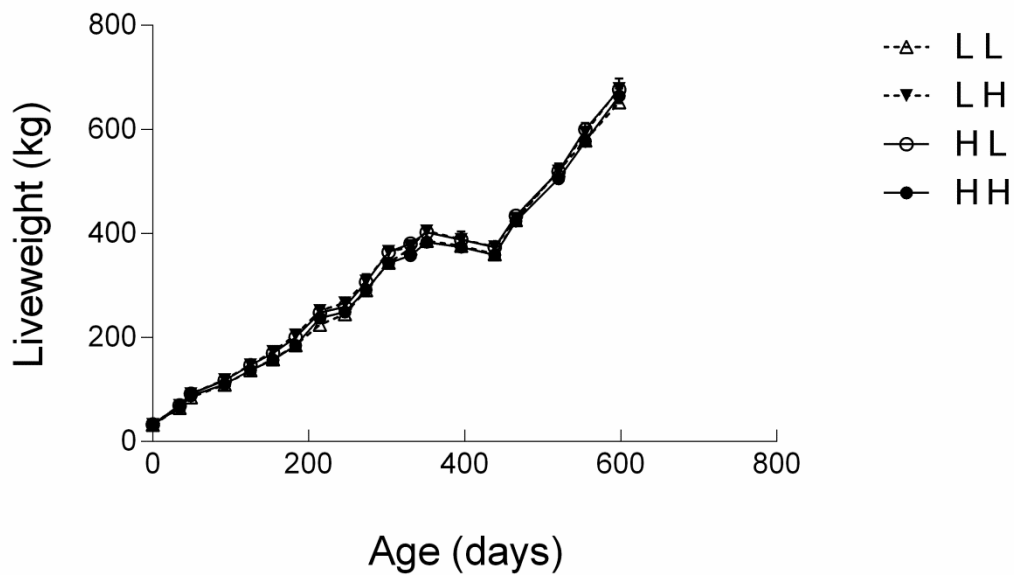
Figure 1. Progeny liveweight and height from birth by low (L) and high (H) protein maternal diet during the PERI- (-60 to 23 dpc) and POST- conception (24 to 98 dpc) periods of gestation. Values are unadjusted mean ± SEM. (n = 40).

LL = low protein maternal diet during both PERI and POST conception period.

LH = low protein maternal diet during PERI and high protein during POST conception period.

HL = high protein maternal diet during PERI and low protein during POST conception period.

HH = high protein maternal during both PERI and POST conception period.

Carcass traits

Liveweight prior to slaughter, HCW, AusMS, AUS-MEAT meat colour or fat colour (Table 3) did not differ (P > 0.10) due to the PERI- and POST-conception maternal diet, nor their interaction term.

Dressing percentage was lower (P < 0.05) in those animals whose dams received a low protein diet during the first trimester (24 to 98 dpc), with LPost (LL + HL) bulls dressing 1.5 percentage points less than HPost (HH + LH) bulls. P8 fat depth did not differ as the result of maternal nutritional treatment although those animals born to dams receiving LPost (LL + HL) tended to have increased P8 fat depth (P = 0.06). When adjusted for HCW, there was no difference in fatness although animals born to dams receiving LPost (LL + HL) diet during first trimester still tended (P = 0.07) to be fatter (Table 3).

EMA was smaller (P < 0.05; Table 3) in those animals whose dams received a low protein diet during first trimester (24 to 98 dpc), with LPost (LL + HL) bulls being 6.9 cm^2 smaller than HPost (HH + LH) bulls. This difference was significant both without, and with, adjustment for HCW (P < 0.05).

RBY% was lower (P < 0.01;Table 3) in those animals whose dams received a low protein diet during first trimester (24 dpc to 98 dpc) with LPost (LL + HL) bulls being 1.5% point lower for RBY% compared to HPost (HH + LH) bulls. This was despite there being no significant difference in liveweight at slaughter, or in HCW.

The consideration of progeny birthweight or maternal liveweight at birth as a covariate in the statistical model (Robinson et al., 2013) did not alter the significance of the effects of maternal diet on the carcass traits described above.
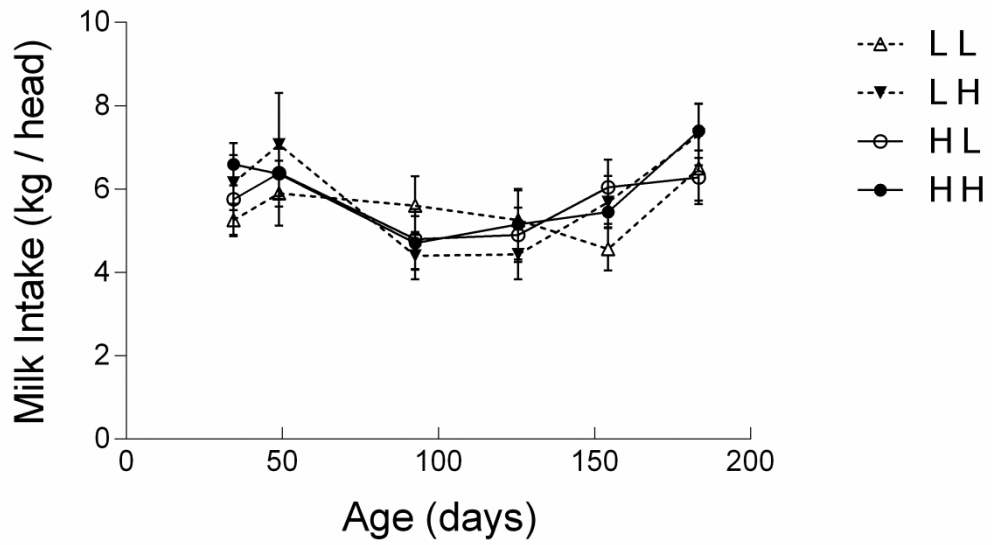
Figure 2. Progeny (n = 40) daily milk intake (kg/head) from 34 to 198 d of age (weaning) by low (L) and high (H) protein maternal diet during the PERI- (-60 to 23 dpc) and POST-conception (24 to 98 dpc) periods of gestation. Values are unadjusted mean ± SEM.

Table 2. Performance of bulls during 70 d residual feed intake test following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation [1].

	Treatment				SEM	P-value		
PERI	Low		High			PERI	POST	PERI*POST[2]
POST	Low	High	Low	High				
Item								
N	8	8	13	10				
Initial BW at 528 d of age, kg	532.8	545.1	541.5	530.2	4.9	0.774	0.917	0.296
Final BW at 598 d of age, kg	647.4	678.6	676.6	652.3	6.9	0.961	0.870	0.125
ADG on RFI[3] test, kg/d	1.66	1.94	1.96	1.77	0.05	0.594	0.873	0.128
DMI on RFI test, kg /d	12.4[a,d]	13.6[b,c]	13.6[b,c]	12.7[c,d]	0.2	0.836	0.809	0.032
RFI, kg/d	-0.12	0.18	0.15	-0.10	0.10	0.966	0.911	0.297

[a,b]Within a row, means without a common subscript differ at $P < 0.05$ for treatment. Values are unadjusted mean ± SEM.

[1]Dams were individually fed diets Low or High in protein during the periconception (PERI;-60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[2]PERI*POST = Interaction term (2 x 2 factorial design). Significant interactions further explored by post hoc test.

[3]RFI = Residual Feed Intake

Table 3. Carcass characteristics of bulls at slaughter at 598 d of age following exposure to maternal diets low or high in protein during the peri-conception and first trimester periods of gestation[1]

	Treatment				SEM	P value		
PERI	Low		High					
POST	Low	High	Low	High		PERI	POST	PERI*POST[2]
Item								
N	8	8	13	10				
P8 Fat[3],mm	17.0	15.0	19.7	16.2	0.75	0.233	0.065	0.921
LMA[4], cm^2	80.3	89.5	81.7	86.8	1.59	0.910	0.027	0.318
Dressing[5],%	53.2	54.9	53.4	54.8	0.33	0.918	0.030	0.620
RBY[6], %	72.6	74.33	72.30	73.7	0.29	0.466	0.007	0.387
HCW , kg	344.3	373.1	361.3	357.3	4.5	0.960	0.320	0.130
Meat colour	1.76	1.78	1.88	1.81	0.03	0.210	0.978	0.470
Fat Colour	1.50	1.00	1.20	1.15	0.09	0.502	0.214	0.256
marble score	0.13	0.13	0.15	0.50	0.07	0.131	0.231	0.416

Values are unadjusted mean ± SEM.

[1]Dams were individually fed diets Low or High in protein during the periconception (PERI;-60 to 23 d post-conception) and first trimester (POST; 24 to 98 d post-conception) periods of gestation.

[2]PERI*POST = Interaction term (2 x 2 factorial design). Significant interactions explored by post hoc test.

DISCUSSION

This study has demonstrated for the first time that low dietary protein during the first trimester (24 to 98 dpc) in beef heifers calving at 2 years of age altered carcass traits in their 20-mo old non-castrated male progeny. A decrease in yield was consequent to decreased EMA in this cohort. This period of nutritional intervention during the first trimester corresponds to the period of primary myogenesis and the start of secondary myogenesis (Stickland, 1978; Bonnet et al., 2010). Protein supplementation during this period therefore may be essential to maintain expression of carcass traits.

Maternal nutrition and offspring carcass characteristics

In utero development of fetal skeletal muscle is crucial to final muscle definition as the number of muscle fibres does not alter after birth, only fibre size (Stickland, 1978; Zhu et al., 2004). During partitioning of nutrients to the fetus, skeletal muscle is thought to be of a lower priority compared to more metabolically active tissues such as the heart or brain (Reynolds and Caton, 2012). Thus skeletal muscle is potentially more vulnerable to deficiencies in maternal nutrition (Close and Pettigrew, 1990; Zhu et al., 2006). .

In the present study, high protein maternal nutrition from 24 to 98 dpc (first trimester) resulted in a larger EMA at slaughter, with EMA being a measure of muscle development in the progeny. This period of nutritional intervention corresponded to the period of primary myogenesis and early secondary myogenesis (Russell and Oteruelo, 1981; Du et al., 2010) - when it is suggested that maximum fetal muscle fibres numbers are developing in the bovine (Bonnet et al., 2010). Differences in EMA are known to be reflected in retail beef yield (McKiernan et al., 2009) and the observed larger EMA, combined with a tendency to be leaner, likely contributed to the increased RBY% in the HPost bulls compared with LPost animals. Interestingly, the effects of HPost maternal diet on EMA were still apparent when

EMA was adjusted for HCW suggesting that the HPost bulls had increased muscularity independent of overall bodyweight.

The observed tendency for the progeny of the LPost dams to be fatter is in agreement with previous reports of progeny of nutrient restricted dams being predisposed to adiposity (Micke et al., 2011a). As proposed by the 'thrifty phenotype' hypothesis (Hales and Barker, 1992), this may be a survival advantage under poor nutritional conditions. Adipogenesis in beef cattle starts to occur before mid-gestation (Bonnet et al., 2010). Adipocytes are reported to be seen first in visceral fat depots as early as 80 dpc, sequentially followed by detection in subcutaneous and intermuscular depots, and finally, in intramuscular depots by 180 dpc (Taga et al., 2011; Du et al., 2013). The 24 to 98 dpc nutritional intervention window in the current study corresponds to the very start of the window for initiation of adipocyte formation in the visceral and subcutaneous depots. The observed tendency for the progeny of HPost dams to be leaner, have improved dressing% and higher RBY%, but not different in either pre-slaughter liveweight or HCW, suggests a shift in fat deposition that may improve carcass value. Of note, there was no difference observed in AUS-MEAT marble score and scores were low overall. Previous literature reports differences in adiposity based on gender and castration status (Berg and Butterfield, 1981) and the low marbling scores observed are likely to be related to the non-castrated status of the animals and their genetic type. Furthermore, the AUS-MEAT marble score reported is not a continuous scale which may have also decreased our ability to detect differences between treatments. However, the similarity in marble score is supported by the laboratory assessment of intramuscular fat in samples from this cohort (Alvarenga et al., 2016). Intramuscular adipocyte formation is thought to predominately occur during late gestation through to about 250 days post-natal (Taga et al., 2011); this being outside the supplementation window of the current study. Hence, the lack of effect of the peri-conception diet upon any of the carcass trait measures assessed is not unexpected given that evidence suggests that although myocytes, adipocytes, and fibroblasts originate from a common progenitor during early embryogenesis (Maltin et al., 2001; Du et al., 2013), the

majority of myogenesis and adipogenesis growth occurs outside the peri-conception window assessed in the current study (Du et al., 2010).

In other species such as pigs, similar changes in body composition, as indicated by smaller loin area and increased fatness have been reported in the offspring of sows fed a low-protein diet during gestation (Rehfeldt et al., 2011). Zhu et al. (2006) reported that lambs from ewes that experienced nutrient restriction during mid-gestation were fatter and had a lower lean–to–fat ratio compared to progeny from non-restricted dams. However, previous studies in the bovine evaluating maternal nutrition effects on the carcass characteristics of offspring have reported inconsistent effects (Greenwood et al., 2009; Larson et al., 2009; Micke et al., 2010a; Underwood et al., 2010; Long et al., 2012; Mohrhauser et al., 2015). The inconsistent results may be attributed to differences in the timing and length of the diet intervention period, the degree of nutrient restriction, dam age and parity, the specific nutrient evaluated, the sample size or the sex evaluated. Our study reported here being unique in that the individual level of controlled feed intake of the dams occurred from the peri-conception period through to parturition. Further, our time-window of evaluation commenced earlier than in other studies (Larson et al., 2009; Long et al., 2010b; Mohrhauser et al., 2015) and lasted longer than all studies except our previous studies (Micke et al., 2010a; Micke et al., 2011a).

Maternal nutrition and offspring feed intake and efficiency

Altered levels of nutrition during fetal and early postnatal development have been shown to influence offspring appetite and body composition (Ong and Muhlhausler, 2014). Exposure to both excessive over and under-nutrition before birth has been reported to permanently change the regulation of appetite in humans, rodents and sheep (Muhlhausler et al., 2006; Muhlhausler and Smith, 2009). The hypothalamic region of the brain plays a crucial role in the control of voluntary food intake and appetite drive in ruminants (Muhlhausler et al., 2006). The activity of neural pathways is modulated by factors such as leptin that circulate in the bloodstream providing information on the body's nutritional status (Muhlhausler et al.,

2007a). In ruminants, these neural pathways develop early in gestation and are susceptible to maternal nutrition (Muhlhausler et al., 2006). Total milk intake assessed during the pre-weaning period was unaffected by maternal nutrition. As the dietary energy provided to the heifers met requirements for nulliparous heifers (Gunn et al., 2014), the genetic background of the heifers was similar (Gregory et al., 1992; Freetly and Cundiff, 1998) and the calf sire was the same (Saner and Gaillard, 1988), the similar milk production was not unexpected. Reports of altered milk intake in lambs following maternal diet perturbations in several studies have been restricted to measures in very early post-natal life with effects not persisting, or not measured, beyond the first few weeks of life (Muhlhausler et al., 2006; De Blasio et al., 2007). It is possible, that differences in food intake only emerged, or became more pronounced, in the post-weaning phase, and when factors such as dam milk production, maternal behavior and maternal-calf interaction would no longer have an influence. A study in goats showed that maternal feed restriction during late pregnancy modified feeding behavior in a small number of artificially reared female offspring for up to 2-yr of age (Laporte-Broux et al., 2012) but the naturally reared male cohort exhibited no differences in milk intake or feeding behavior in early neonatal life prior to slaughter (Laporte-Broux et al., 2011).

Feed is a major cost in livestock production, particularly during a feedlot finishing phase. The observed increase in DMI during the feedlot period in progeny from dams that had a change in diet between peri-conception and first trimester may have the potential to influence production efficiency. Residual feed intake (RFI), which is considered the most appropriate method to assess feed efficiency in the bovine (Cafe et al., 2014), showed a similar pattern to DMI in this study but did not differ between the groups . It is possible that the relatively small number of animals studied meant that there insufficient power to detect anything but large differences in RFI (Herd et al., 2014). Few studies have reported in the bovine on the differences in appetite and RFI in progeny whose dams have experienced either restricted or excessive nutrition during pregnancy. Summers et al. (2015a) reported that RFI,

as measured via a GrowSafe system, was improved in progeny born to dams that received isonitrogenous and isocaloric supplements with varying levels of RUP in late gestation (~ 142 to 242 dpc) (Summers et al., 2015b) compared to those born to unsupplemented dams. Feed intake, however, was greater in the calves from unsupplemented dams. This window of maternal supplementation being later in gestation than the period evaluated in our study. Currently, the mechanisms regulating the differences in appetite are poorly understood in the bovine. Preliminary results exploring the effects of maternal nutrition upon the hypothalamic appetite regulatory system in the bulls reported herein indicates that the hypothalamic gene expression for the appetite stimulating neuropeptide, agouti-related protein (AGRP), is significantly up-regulated in the HL progeny (Copping, unpublished data). These changes may represent a potential mechanism contributing to the observed higher FI in this group as AGRP is known to stimulate food intake in ruminants (Wagner et al., 2004). Further investigations are continuing into the relationships between the expression of orexigenic and anorexigenic hypothalamic neuropeptides, food intake and adiposity (Muhlhausler et al., 2006) in the fetal and adult progeny and will be reported in a forthcoming paper.

Maternal nutrition and offspring growth

In the present study, protein nutrition during either the PERI (-60 to 23 dpc) or POST (24 to 98 dpc) periods had no effect on either birthweight (Copping et al., 2014) or post-natal growth (pre and post-weaning liveweight, ADG and height), nor upon feedlot performance or HCW at slaughter. The results for birthweight, ADG and HCW are consistent with those of Long et al. (2010b) who reported that nutritional restriction for energy and CP (55% of NRC vs 100% of NRC) during early pregnancy (32 to 83 dpc) in 2-year old heifers did not affect birthweight or postnatal growth (ADG) in steer progeny. However, the results for progeny liveweight differed from those of Long et al. (2010b) and from our previous findings in steers from 3 year old heifers (Micke et al., 2010a). Long et al. (2010b) reported that steers from the nutrient restricted dams were heavier at the beginning of the finishing period and tended to be

heavier at slaughter, although empty bodyweight was not different. Micke et al. (2010a) reported that a low CP diet during the first trimester (0 to 93 dpc) also resulted in heavier post-natal liveweight at feedlot entry but did not influence HCW in the steer progeny. In contrast, heifers exposed to the low CP diet during the first trimester had a lower HCW at slaughter (Micke et al., 2010a). Such inconsistencies between studies may result from differences in the age of the dams, and the castrate status of the progeny. In addition, they may be the result of the timing of maternal nutrition intervention compared to the periods of supplementation (-60 to 98 dpc) explored in the current study; altered progeny weaning weight having been reported in a number of studies investigating maternal protein supplementation in cows during mid to late gestation (Stalker et al., 2006; Larson et al., 2009; Underwood et al., 2010). Interestingly, the post-natal circulating levels of IGF1 and leptin in the male progeny (Copping, unpublished data; Chapter 5) did not differ due to maternal nutrition in our study which is consistent with the lack of difference in liveweight and ADG; associations between postnatal IGF concentrations with ADG and growth rate having previously reported in the bovine (Micke et al., 2010a).

Conclusion

This study highlights opportunities for the livestock industries whereby maternal dietary supplementation can be used to alter feed intake, enhance muscling and alter fat deposition, which in turn, leads to modest improvements in efficiency of meat production in beef cattle. This study provides evidence that protein supplementation during the periconception period and first trimester may alter offspring appetite and carcass traits. Improved understanding of the mechanisms in the bovine that regulate appetite and fetal cell lineage commitment into myocytes, adipocytes or fibroblast cells is needed and additional studies utilizing larger experimental cohorts are warranted. Studies are currently underway in our laboratory that may enable maximisation of the benefits of dietary supplementation by elucidating these mechanisms targeting specific developmental stages in the bovine.

Chapter 7

General Discussion and Conclusion

General Discussion and Conclusion

In extensive grass-fed beef production systems, there is substantial seasonal variation in both the nutritional value and the quantity of pasture available to pregnant cows. In the northern pastoral regions of Australia, protein, rather than energy, is often the major limiting nutrient (Norman, 1963) in beef herds. Therefore, the use of protein supplement for developing replacement heifers is a common management practice (Bertolini et al., 2006; Burns et al., 2010).

In a recent study, researchers leading the current project showed that dietary protein restriction in the first and second trimester of gestation in the older beef heifer (mated at 24 months of age) influenced fetal and postnatal reproductive development and production traits in the offspring (Sullivan et al., 2009a; Micke et al., 2010a; Sullivan et al., 2010b; Micke et al., 2011b). The aim of the work contained within this current thesis was to extend previous research by; a) exploring an earlier period of gestational dietary intervention; the peri-conception and first trimester, and b) examining these effects in the adolescent heifer (mated at 14 mo of age) as mating of younger heifers is increasingly becoming common practice within the beef industry. The gestational dietary protein levels used in this study reflect pasture conditions in the Australian rangelands, with, and without protein supplementation. Specifically, our studies focused on the effects of dietary protein intake upon feto-placental and neonatal calf development and further, whether these effects translate into functional consequences in postnatal growth, reproductive development and carcass traits of the offspring. The reported long-term effects of maternal diet are relevant to both the veterinary and human medical community as well as the commercial beef industry which largely funded this ARC Linkage research program.

In the first three experimental chapters of this thesis (**Chapters 2, 3 and 4**), we identified the effects of maternal diet during the peri-conception period and first trimester on fetal growth and morphology and associated maternal performance. The influences of dietary

protein on maternal metabolic hormones: insulin-like growth factor (IGF) 1 and IGF2 and leptin; metabolites: NEFA and urea and circulating markers of placental function: progesterone, bovine pregnancy associated glycoprotein (bPAG) and bovine placental lactogen (bPL) were determined. The metabolic effects of dietary protein intake in nulliparous heifers were generally as expected whereby increased protein intake in association with an isocaloric diet moderately increased bodyweight and average daily gain during the periods of supplementation and increased plasma levels of IGF1 and urea. The effects on IGF2, leptin and NEFA were less clearly defined, occurring at different stages in gestation and importantly varying dependent upon fetal sex. This may, to some extent, reflect the design of the experimental diets which were as isocaloric as possible and formulated to allow for positive liveweight gain throughout pregnancy. This contrasts with the more severe restriction models utilised in many fetal programming studies resulting in maternal weight loss during pregnancy (Stalker et al., 2006; Mossa et al., 2013) and thereby necessarily display a larger effect upon circulating maternal NEFA, IGF1 and leptin levels. The placentally produced bPAG and bPL, and the ovarian and placentally produced progesterone were evaluated as circulating indicators of placental function. Placental function as indicated by bPAG and bPL was not influenced by dietary protein intake. However, low dietary protein during the peri-conception period was associated with lower circulating progesterone at several time points. Inadequate circulating progesterone has been associated with increased levels of early embryonic loss in a number of studies (Lonergan, 2011; Lonergan et al., 2016).

As expected from a previous study on bovine maternal dietary protein intake in the first trimester (Micke et al., 2010b), protein restriction negatively affected fetal growth from 36 dpc. Furthermore, in a published paper from these experiments (where the candidate is the second author) the dietary protein regimen altered maternal blood flow to the fetus (Hernandez-Medrano et al., 2015). This was concurrent with preferential distribution of nutrients to fetal organs, confirmed in the necropsied fetus at 98 dpc. Intrauterine growth restriction (IUGR) was evidenced by; a) reduced fetal weight in both sexes, b) indices of

disproportionate growth, and c) reduced organ weights, with these effects being predominately in the male fetus, confirming the asynchronous development of fetal organs and morphology in a sex-specific manner. First trimester protein restriction was also associated with decreased placentome number and volume in the male placenta at 98 dpc. The IUGR evident at 98 dpc was no longer apparent in the gross measures of placental and calf morphometry and weight that were obtained at birth. The lack of dietary effect upon measures in the neonate may indicate compensatory growth of the feto-placental unit occurred during the second and third trimesters when the dams were returned to NRC recommended rations following the experimental intervention. Importantly, the sex-related differences in weight between the male and female calves, which were present in the 98 dpc fetus, were no longer apparent at birth. This suggests that the fetal growth trajectory in the second and third trimester following the experimental nutritional perturbation differed between the sexes such that growth of the female fetus was enhanced compared to the male. We propose that a possible mechanism for this occurrence may lie in the observed correlation at 128 and 190 days post-conception between maternal circulating levels of placental bPL and the growth factor IGF1 in the female feto-placental unit. This did not occur in the male. This correlation may indicate a signalling mechanism between the fetus and mother whereby enhanced bPL from the fetus increased hepatic IGF1 production. The placenta is permeable to maternal IGF1 suggesting a mechanism of enhancing growth in the female fetus. Due to the low pregnancy rate there were insufficient numbers of heifers to allow the necropsy of a second group of heifers later in gestation. This would have enabled further determination of the feto-placental growth trajectory and compensatory mechanisms following the cessation of dietary perturbations.

The absence of dietary effects upon birth weight does not necessarily indicate that the neonate has not experienced IUGR prior to birth, as observed in this study in the necropsied fetus and placenta at 98 dpc. Prior studies have shown that nutrient restriction may have lasting effects on the post-natal health, growth and development of the offspring without

incurring a birthweight effect (McMillen and Robinson, 2005; Martin et al., 2007; Mossa et al., 2013). This scenario represents a challenge for beef producers as offspring birth weight (a frequently observed production measure) may not be a satisfactory indicator of IUGR, thereby restricting recognition of the concomitant effects upon postnatal development of the progeny (Long et al., 2009).

The observed effects upon postnatal growth and reproductive development of the progeny in this study are explored in **Chapter 5 and 6** of this thesis. Due to the overall low conception rate and skewed sex ratio identified in **Chapter 3**, along with the subsequent skewed numbers of males versus female calves born (**Chapter 4**), in **Chapter 5 and 6** I investigated the postnatal development of the un-castrated male progeny only. The initial aim of this thesis was to have examined the postnatal development of the female offspring through to calving (F2 stage). However, the comparatively small numbers of female calves required a re-evaluation of this experimental aim. There are a small number of studies in the bovine that have reported on the effects of maternal diet on the postnatal reproductive development of the heifer progeny (Martin et al., 2007; Sullivan et al., 2009a; Mossa et al., 2013). In contrast, there is only one reported study on the effects of maternal diet on post-natal reproductive development in bull progeny (Sullivan et al., 2010b), which in fact examined development in pre-pubertal bulls to 5 months of age only.

In **Chapter 5**, therefore, the aim was to examine the effects of maternal diet during the peri-conception period and first trimester of gestation upon the reproductive development of the bull calves through to maturity. Using standard industry parameters to assess reproductive development and fertility, we showed that bull progeny of heifers fed a high protein diet in the peri-conception period had increased sperm quality at a younger age and consequently reached puberty earlier. These effects upon sperm production were associated with lower levels of circulating anti-Müllerian hormone (AMH) at 290 days of age and higher FSH concentrations at both 320 and 420 days of age in the bull progeny exposed to the high protein diet compared to the progeny of mothers fed a low protein diet in the peri-conception

period. AMH and FSH are integral to the initiation and maintenance of the complex process of reproductive development including spermatogenesis; AMH secretion by the Sertoli cells is down regulated at puberty when Sertoli cells mature (Sharpe et al., 2003) while FSH production by the anterior pituitary is essential to the initiation and continued spermatogenic capacity of the testis and epididymal transport with levels increasing as bulls age in association with improvement in sperm quality and quantity (Matsuzaki et al., 2000). The histology of the fetal and adult testis in this cohort of animals showed that the observed early perturbation of the cytology of the testis in the 98 dpc fetus was compensated for during later development as no corresponding effects were observed in the adult testis. We hypothesise, therefore, that the high peri-conception diet initiated the observed earlier elevation of FSH, in accord with the earlier downregulation of AMH, thereby enhancing the development of the hypothalamic–pituitary-gonadal axis and/or the associated receptivity to circulating hormones.

The **6th Chapter** of this thesis aimed to investigate the long-term effect of heifer dietary protein intake in the peri-conception period and first trimester on the postnatal growth pathway, appetite, feed efficiency and carcass traits of the bull calves. The liveweight of the male progeny was unaffected by maternal diet at any post-natal stage of growth from birth to slaughter. Similarly, in the male, plasma concentrations of IGF1, leptin and milk intake prior to weaning was not affected by gestational diet. This is dissimilar to the effects observed upon intake in the female progeny where maternal dietary protein altered milk intake observed in the early post-natal calf prior to weaning (Copping, unpublished data). The bull progeny were moved from South Australia to the University of New England facilities at 528 days of age where a net feed intake trial was completed to assess feedlot growth performance, appetite and feed efficiency. Maternal dietary protein did not affect liveweight, average daily gain or feed efficiency. However, there was an interaction between maternal peri- and post-conception diet such that bull progeny from heifers that had a change in diet at the end of the peri-conception period from high protein to low protein (HL group), and *vice versa* (LH

group), had 9% daily higher feed intake on test than progeny of mothers that remained on a constant low protein diet throughout the peri- and post-conception period (LL group). Interestingly, Micke et al. (2010a) reported that a change to maternal diet from high to low protein, or *vice versa*, at the end of the first trimester resulted in greater plasma IGF1 concentrations in the male offspring during the postnatal period compared to their counterparts. In the current study this effect upon IGF1 was not observed and may demonstrate additional studies on the effects of maternal nutrition on appetite in cattle may be warranted as in sheep, gestational diet has been shown to affect offspring appetite (Muhlhausler et al., 2006). Dressing percentage, estimated retail beef yield and *longissimus dorsi* (eye muscle) area were all higher in the offspring of heifers that received the high protein diet in the first trimester and there was a tendency for these carcasses to be leaner, despite no observed effect on carcass weight. Furthermore, these differences in eye muscle area and fatness were observed with, and without, adjustment for carcass weight. This confirms that the observed effects upon body composition were not driven by differences in the size of the carcass. These findings are not consistent with the earlier study that explored first and second trimester protein restriction in steers (Micke et al., 2010a; Micke et al., 2011a, b) that reported high protein maternal diets during the first trimester decreased liveweight, increased fatness and decreased cross – sectional area of the *semitendinosu*s and *longissimus dorsi* muscles. However, the effects of protein restriction were examined in steers, as distinct from bulls, and reported significant effects upon fatness and cross-sectional muscle area in the live animal as measured by ultrasonography (Micke et al., 2010a). Subsequent carcass measures of these parameters were not different once adjusted for carcass weight (Micke et al., 2011a, b). Similarly, despite a difference in liveweight prior to slaughter, in the study by Micke et al. (2010a) this was not reflected in changes in carcass weight. As discussed below, the effects of sex -related hormones on body composition may partly explain the differences reported in this thesis.

An important finding of this thesis was the identification of clear fetal sex-specific differences in both maternal hormonal and metabolic responses, and in corresponding feto-placental and neonatal development. In **Chapter 2 and 3** we observed that exposure to low protein diets in the peri-conception period reduced fetal size as early as 36 dpc and development began to be affected in a sex-specific manner. Subsequently this interaction between maternal diet and fetal sex continued to be observed later in development (**Chapter 4**) where in the 98 dpc fetus the maternal dietary effects upon fetal morphology, organ growth, asynchronous growth indices, and placental parameters were predominantly sex-specific. In combination, these results suggest that whilst exposure to a maternal diet low in protein during the peri-conception period and first trimester in nulliparous heifers was associated with altered early fetal development in both sexes, these effects were generally more pronounced in the male at 98 dpc. Sex-specific differences have been reported previously in developmental programming studies. Male embryos have been shown to develop not only more rapidly than female embryos but also display an increased response to exogenous metabolites (Mittwoch, 1993; Bermejo-Alvarez et al., 2011). Thus, it has been suggested that in the bovine, males are more likely to respond and adapt to perturbations in very early pregnancy (peri-conception and first trimester) whereas the female fetus more readily adapts to perturbation from the second trimester. Such an early adaptive response may explain the wide-ranging effects observed in the males at 98 dpc in the present study.

The loss of the normal sex-related differences in birthweight (Bellows and Short, 1978; Holland and Odde, 1992) in the cohort of animals taken to term suggests sex-dependent compensatory growth of the fetus occurred in the 2nd and 3rd trimesters when the animals were fed to meet their nutritional requirement. Perhaps placental adaptations after restriction ceased were sufficient to meet the lower nutritional requirement of the usually smaller female but not adequate to completely mitigate the early suppression of growth in the male (Burton and Fowden, 2012; Rosenfeld, 2015). The mechanisms behind these differences remain unclear; however, the sex-specific differences in both Doppler feto-placental perfusion indices and

response of these indices following the maternal dietary interventions (Hernandez-Medrano et al., 2015) may represent a sexually dimorphic placental adaptation. This may potentially be driven by signalling from the female fetus mediated via the observed association between bPL and IGF1 in the second trimester enabling enhanced growth of the female feto-placental unit at this stage in gestation. There is clearly a requirement for further research on addressing the underlying mechanisms behind the differential fetal adaptations to maternal nutritional perturbations in one sex over the other.

Future directions that build upon the findings in this thesis must include the completion of studies on histology and gene expression in the many tissues that have already been collected. The evaluation of key genes and pathways will contribute to our understanding of the causal mechanisms of the phenotypic differences observed to date. Important expansions of the outcomes could include further investigations that explore links between peri-conception diet and the programming of the hypothalamus-pituitary-gonadal (HPG) axis, which may require dietary perturbation to cease prior to male gonad differentiation (approximately 40 – 47 dpc in the bull). This period of intervention has not been well explored in developmental programming studies in the bovine to date with most studies on the effects of maternal nutrition in the peri-conception period focusing on embryo development and survival. The implementation of a more intensive hormone sampling and semen testing regimen throughout the peripubertal and pubertal period in the bull progeny would also contribute significantly to our understanding of the mechanisms involved in the *in utero* dietary effects upon sexual development. In the current study, a GnRH challenge was not undertaken as augmenting LH production prior to the normal early gonadotrophin rise by giving calves exogenous GnRH has been reported to hasten pubertal development (Chandolia et al., 1997; Barth et al., 2008). Animals were allowed to progress through puberty without any exogenous hormone influence and hence it was not possible to report on pituitary responsiveness. Nevertheless, consideration of the use of a GnRH challenge in the pre-pubertal offspring is needed to extend our understanding of any diet-mediated alterations to

the endocrine system. Similarly, investigations into the reproductive development of the female cohort need to be considered to expand on the limited existing knowledge about the effects of maternal dietary restriction on female reproductive development in mammals.

The outcomes of the effects of first trimester diet upon carcass traits presented in this thesis would be equally as important to define in steer progeny; the standard industry endpoint for male progeny in a commercial cow-calf operation and thus economically essential to examine. It is possible that the patterns observed in phenotypic muscling, fatness and subsequently carcass yield in the bull progeny in the current study may differ in steer progeny due to the interplay between sex-related hormones and body composition in the postnatal animal.

Many studies on developmental programming effects to date focus on dietary effects at the cellular, molecular or hormonal level either in the fetus or the neonate. It is important to recognise in any future studies in sheep and cattle that for the outcomes to be of direct relevance to the agricultural industry, the focus needs to be on whole animal effects extending beyond early postnatal life. Whilst we have shown that maternal nutrition influenced the developing bull, further studies on the mature animal are needed to investigate if these effects are detrimental to lifetime fertility in the herd. This represents a considerable challenge as the funding and length of time required to perform longitudinal studies in agriculturally important species such as the bovine is significantly greater than in laboratory animals. Furthermore, when considering the implications of this thesis it should be noted that these studies were conducted in young adolescent *Bos indicus* x *Bos taurus* beef heifers. Thus some degree of caution should be exercised when extrapolating the results of this study to older animals, different genotypes and indeed, as previously alluded to, different species.

The dietary regimens employed in the studies reported in this thesis were developed to represent the range in nutritive value of native and/or improved pastures that heifers raised in extensive grass-fed farming systems may encounter prior to mating and during gestation.

Hence, the results have application to the management of heifers calving at 2 years of age to improve productivity in their male offspring. As compared to low protein intake, maternal protein supplementation in the peri-conception period was associated with increased semen quality at a younger age in the developing bull whilst first trimester supplementation improved offspring eye muscle area, dressing percentage and retail beef yield (reflecting altered carcass composition). Furthermore, these effects were not associated with increases in birth weight and were also independent of any changes in postnatal growth pathways which remained similar throughout calf-hood and post-weaning. Maternal nutrition prior to mating and during early gestation, therefore, may represent a critical management window enabling maximisation of early reproductive development and carcass value of the male offspring in the beef herd. Protein supplementation of replacement heifers at the levels reported in this study is already a common management practice in northern Australian rangelands where protein, rather than energy, is often the limiting nutrient. Nonetheless, the economic cost of protein supplementation to yearling heifers in a northern range environment would potentially be difficult to justify in terms of the change observed in carcase traits alone. However, in combination with other positive effects observed on reduced fetal loss and early reproductive development in the male progeny, the manipulation of maternal nutrition has been calculated to be cost-effective.

The findings in this thesis (summarised in Figure 1) add to the existing body of literature that shows a link between maternal nutrition and the development and function of many biological systems in the offspring. This work has shown for the first time in the bovine that dietary protein restriction in the peri-conception period in nulliparous heifers is associated with impaired reproductive development through puberty in their bull offspring. Furthermore, the studies presented in this thesis have also demonstrated that maternal protein restriction in the first trimester is associated with altered fetal growth and placental development to 98 dpc, and altered muscling and carcass yield at slaughter at 20 months of age. When considering the

implications of this work in an industry context, it is important to acknowledge that some of the phenotypic differences reported herein are relatively modest in the postnatal offspring. Nevertheless, when the incremental differences are accumulated across the lifetime of a calf from conception to consumption, they represent significant areas of potential gain in productivity. A number of different areas for future studies to extend the knowledge of developmental programming effects have been identified and discussed above. The challenge for future research into the long-term effects of nutritional fetal programming in the bovine, and other species, will lie in improved understanding of the causal mechanisms driving these effects and enable targeted interventions.
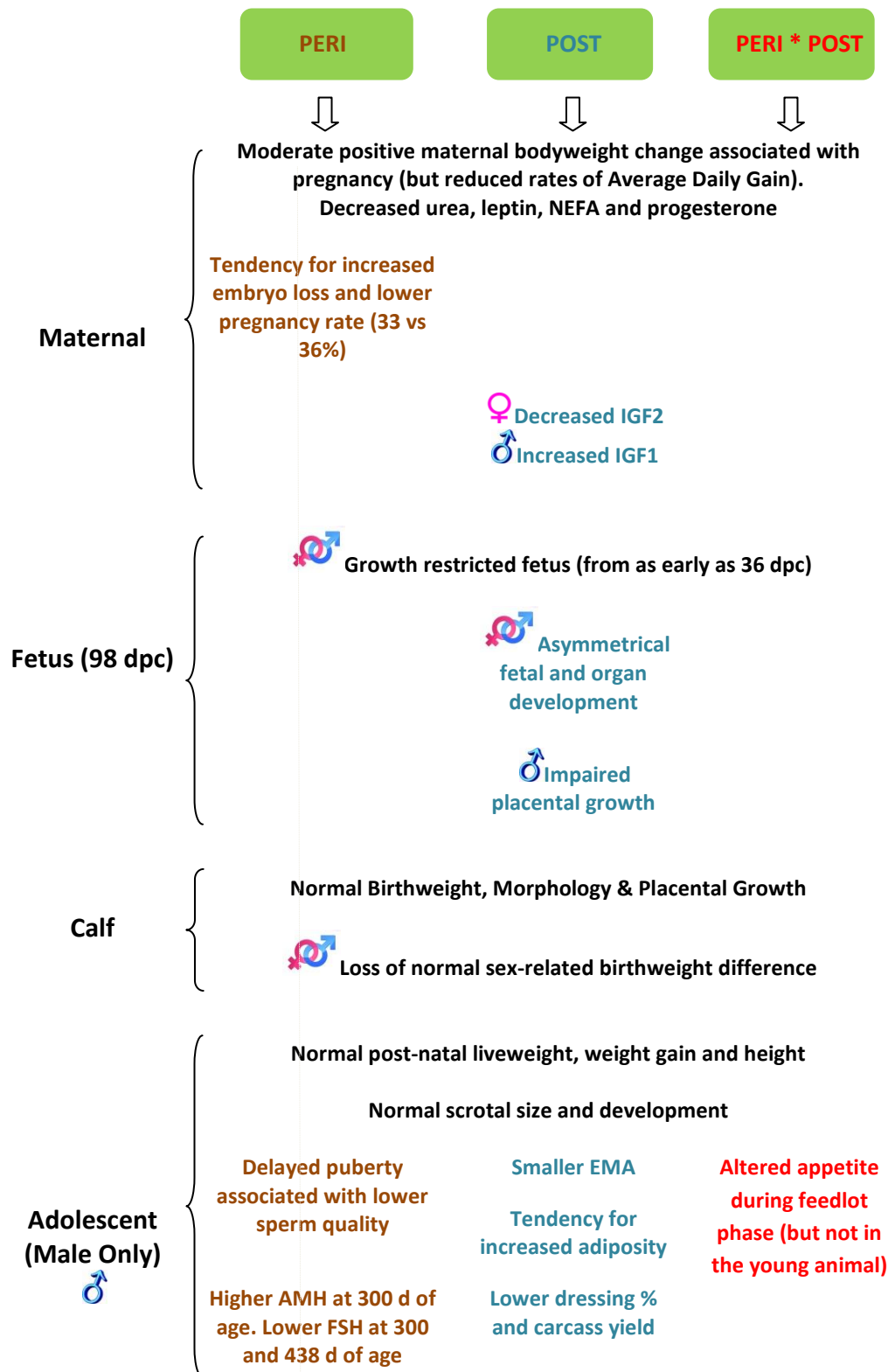
Figure 1. Summary of effects of low maternal dietary protein intake during the peri-conception period (**PERI; -60 to 23 dpc**) and first trimester (**POST; 24 to 98 dpc**) and **their interaction** on pregnancy and offspring outcomes in adolescent *Bos indicus* x *Bos Taurus* cattle. Common effects are shown in **black**. Sexually – dimorphic outcomes denoted by symbols.

APPENDIX 1

Table 1. Ingredients and nutrient content of heifer diets as fed for induction period, peri-conception (PERI; -60 to 23 dpc), post-conception (POST; 24 to 98 dpc) and 2nd and 3rd trimesters of gestation.

Item	Induction	PERI		POST		2nd and 3rd Trimester
		L	H	L	H[4]	
Ingredient (as fed basis)						
Wheat, kg	0.66	1.81	0.48	2.12	0.56	0.60
Canola meal, kg	2.23	-	-	-	-	0.89
Soybean meal, kg		0.48	1.83	0.56	2.14	0.44
Barley straw, kg	7[1]	5.5	6.7	10.2	10.7	8.6
Molasses, g	90	72	72	84	84	60
Biofos MDCP, g	-	19	-	22	-	-
Salt, g	15	12	12	14	14	10
Vitamin / trace mineral						
Premix, g	3	2	2	3	3	2
Dry matter, kg	9.1[2]	7.2	8.3	11.8	12.3	9.6
Nutrient content						
Total energy, MJ ME		63	71	98	102	79
% of energy requirements[3]		85	96	136	142	125
Total CP, kg		0.62	1.18	0.88	1.49	0.92
% of protein requirements[3]		67	127	72	123	88
% CP (total diet)		8.6	14.2	7.4	12.1	9.6
% Fat[2]		1.5	1.4	1.4	1.4	1.5
% Starch[2]		15.1	4.7	10.9	3.8	4.8
Total calcium, g		22	26	37	38	33
% of calcium requirements[3]		110	130	185	190	132

Total phosphorus, g		17	17	21	21	20
% of phosphorus requirements[3]		130	130	160	160	125

[1]assumed value.

[2]predicted value.

[3]Dietary requirements were calculated using Nutrient Requirements of Domesticated Ruminants (Freer, 2007). Input values were based upon nutrient analysis of component ingredients in the total diet, BW and age of heifers at each diet change, mature cow wt of 550 kg and the desired growth target.

[4]L = Low protein H = High protein.

Key assumptions:

Calculations use the formulated values for pellets and actual values for straw.

Peri-conception diet is based upon 340 kg Santa Gertrudis heifer gaining 0.5 kg/d.

Post-conception diet is based upon 400 kg, 60 dpc Santa Gertrudis heifer gaining 0.5 kg/d.

2nd and 3rd trimester diet is based upon 480 kg, 200 dpc Santa Gertrudis heifer gaining 0.5 kg/d.

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