Evidence for metabolic programming in dairy cattle based on field data

Dissertation submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy (PhD) in Veterinary Sciences
Faculty of Veterinary Medicine, Ghent University, Belgium

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May 2017

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"If you have two options either to have a Government job in developing countries or to study for a PhD in developed world, I would suggest you to choose the latter only because a PhD insists to uphold an internal eye"

NASREEN PARVEEN

(My wife, the most influential person for my PhD)

To my respected PARENTS

and

My beloved daughter RIFA and son RAFID

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AIR	Acute insulin response
BB	Belgian Blue
BCI	Body condition index
BCS	Body condition score
BW	Birth weight
CI	Calving interval
CL	Corpus Luteum
DI	Disposition Index
DNA	Deoxyribonucleic acid
dpi	Days post insemination
DL	Diagonal length
DMI	Dry matter intake
DO	Days open
DP	Dry period
FAO	Food and Agricultural Organization
FCS	Fetal calf serum
FF	Follicular fluid
GH	Growth hormone
GL	Gestation length
Glu_b	Basal glucose level
HG	Hearth girth
HF	Holstein-Friesian
hpi	Hours post insemination
IGF	Insulin-like growth factor

IGFBP	Insulin-like growth factor binding protein
Ins_b	Basal insulin level
IUGR	Intra uterine growth restriction
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
LH	Luteinizing Hormone
LL	Lactation length
LH	Luteinizing hormone
LOS	Large offspring syndrome
M305	Cumulative 305 d milk production
mo	Month
MGEST	Cumulative milk production during gestation
MPCONC	Cumulative milk production from 15 d before to 15 d after conception
MPEAK	Milk production at lactation peak
NEB	Negative energy balance
NEFA	Non esterified fatty acids
NRR	Non-return rate
PBS	Phosphate buffered saline
PGF2α	Prostaglandin F2alpha
QUICKI	Quantitative insulin sensitivity check index
SAF	Surface amplification factor
Small size	Small for gestational age
TCM 199	Tissue culture medium 199
TPEAK	Time to half of the peak milk
WH	Wither height

CHAPTER 1

GENERAL INTRODUCTION

General Introduction

In the last century, our globe has experienced many changes. Since the 1970s, the human population size has doubled to over 7.3 billion, and is growing at a rate of approximately 200,000 per day. Population growth will add an estimated 2.4 billion people to the planet by the year 2050 (Ehrlich and Harte 2015). Most of the growth will occur in developing countries that are already struggling to feed their populations and where the added population pressure will push the poor even further onto marginalized land and into urban areas (Alam et al. 2016; Wahlqvist 2016). Today close to one billion people in developing countries suffer from chronic undernutrition, mainly trying to survive on inadequate starch-based diets. A further two billion in emerging countries aspire to eat more diverse and nutritious diets- with more meat, milk and eggs, at the top of their wish-lists. As well as being rich sources of high quality protein, meat and milk also supply a range of essential micro-nutrients (Reynolds et al. 2015), many of which are scarce or absent in plant-based diets. Consumption of adequate amounts of these nutrients is essential if children are to be healthy and grow and develop normally, going on to fulfill their potential as healthy and productive members of the society.

People in the developed world can enjoy fresh food at a cost of approximately 12-15% of their income (Chattopadhyay et al. 217; Plumer 2015). Unfortunately, due to poverty and lack of technical innovation, people in less-developed countries pay as much as 80% of their income for food (Chattopadhyay et al. 217; Plumer 2015). It is no wonder that the cycle of poverty continues (Alam 2017; Jalal et al. 2015). Besides the problem to feed its inhabitants, our planet currently faces other challenges like for example pollution of the environment and the concomitant climate change, the emergence of antibiotic resistance, and the daily decrease of the amount of arable land. Each year, approximately 5-10 million hectares of arable land become unusable due to severe soil degradation caused predominantly by deforestation, overgrazing, and bad agricultural practices (Islam el al. 2015; Motesharezadeh et al. 2017). Another 0.3-1.5 million hectares become unproductive due to salinization and water logging: not surprisingly, two-thirds of degraded land is located in South Asia and Africa, where the majority of the world's food insecure live (Smith et al. 2016). Serious attention needs to be addressed to restoring these ecosystems, creating/enforcing appropriate policies, and educating farmers on proper integrated farming techniques (Eisler et al. 2014). The health and safety of our ecosystem (plants, livestock, wildlife, and marine life) are closely associated with human health. The areas of One Health, Food Safety, and Food Security are all imbedded within this generally accepted concept (Reynolds et al. 2015).

The global food system could be defined as the activities involved in producing, processing and distributing food to feed the world (Wahlqvist 2016). It links national and local food systems on a global scale through trade, technology, knowledge sharing, labor and capital exchange (BeVier 2012). For over thousand years, food production has evolved from the primitive utilization of vegetative plants and livestock domestication to the precision farming operations of industrialized agriculture we currently know. Despite increasing yields, the global food system has failed to make the world food secure. According to the FAO, to meet the food demand to feed a global population forecasted to be 9.7 billion by the year 2050, a 60% increase in global agricultural production is necessary relative to 2005 (FAO 2007). And this challenge has to be met in the context of increasing resource scarcities while minimizing food safety risks and adapting to/mitigating climate change. It will be crucial to develop win-win solutions that bring together the primary sector and the food industry, considering nutrition, health, water and energy efficiency, zero waste and environmental sustainability in a holistic way. Besides the increased implementation of innovative tools and technologies, especially in third world countries the principal focus should be to optimally make use of basic strategies that already have proven their added value. Genetics, reproduction and other basic health technologies are highly leveraged interventions. Scientific advancements in these areas should also be open for poor farmers in the less developed world since they will certainly contribute in the decrease of the human suffering we face today.

1.1 Human Population and Nutrition in Bangladesh

Bangladesh is one of the world's most densely populated countries with 161 million people (BBS 2016) in an area of only 147 thousands km^2. It has also the highest rates of population growth in the world. Early marriage and short birth interval are the important causes of this high population growth (Streatfield and Karar 2008). One important indicator of early marriage is the proportion of teenage women (aged 15-19 years) currently married. Bangladesh has maintained one of the highest proportions - close to half - of teenage marriage in the world, matched only with a few West African countries (Streatfield and Karar 2008). Currently, the average age at the first birth 18.1 years in Bangladesh (Khan et al. 2016), where this is 28.5 years in Belgium and 29.2 years in Germany (CIA 2017). Moreover, majority of women practice short birth interval that further aggravate the situation of population growth. The high population growth is creating unemployment, poverty, food insecurity and undernutrition of people in Bangladesh.

Adequate nutrition is a precondition for attaining good health, maintaining quality life and accelerating national productivity. But undernutrition remains a severe problem in Bangladesh (Alam 2017; Fiedler 2014; Yosef et al. 2015). A variety of factors cause this undernutrition, the two most prominent being poverty and food insecurity. These two problems limit one's ability to live on a diet that provides all the nutrients necessary for healthy living. The prevalence of undernutrition is relatively higher among rural, illiterate and early married women and among those with a low standard of living (Hossain et al. 2012). Undernutrition can result in adverse pregnancy outcomes and underweight mothers are more likely to give births to babies with relatively lower weight (Ehrenberg et al. 2003). Such babies achieve poor psychological health and possess higher risks of mortality, and in the case of survival, higher risks of having metabolic diseases (Rayhan and Hayat 2006).

Importantly, although often overlooked, milk and meat including other animal products play an important role in achieving food security for several reasons. First, animal products are an important source of high quality, balanced, and highly bioavailable protein and numerous critical micronutrients, including iron, zinc, and vitamins B-12 and A, many of which are deficient in a large portion of the world's population (Randolph et al. 2007). Thus, moderate consumption of animal-sourced foods plays an important role in achieving a nutritionally balanced diet, especially in the developing world (Na et al. 2016; Randolph et al. 2007). Second, because they are recognized as high-quality foods, demand for animal products is almost certain to continue to increase dramatically (Herrero et al. 2010; Na et al. 2016). The drivers of the increased demand for animal products include not only population growth but also increased affluence, especially in the developing world, where most of the increase in population will occur. Third, farm animals are critical to a sustainable agricultural system and especially for smallholders who comprise most of the world's farmers (Herrero et al. 2010; Randolph et al. 2007). Farm animals contribute not only a source of high-quality food that improves nutritional status but also additional resources such as manure for fertilizer, on-farm power, and other by-products, and, in addition, provide economic diversification and risk distribution (Leroy and Frongillo 2007; Smith et al. 2013). Moreover, increased efficiencies in the past several decades through genetic selection and improved management technologies has saved a large amount of resources, including water and land, and have substantially reduced the carbon footprint of animal production (Herrero et al. 2010; Smith et al. 2013).

Currently, two-thirds of the labor force in the less-developed world is focused on farming and livestock, which is in great contrast to the <1% of the population in the developed world

working in agriculture (Eisler et al. 2014; Tedeschi et al. 2015). In agriculture, milk production is a livestock enterprise in which smallholder farmers in Bangladesh can successfully engage in order to improve their livelihoods (Shamsuddin et al. 2006). Regular milk sales also allow them to move from subsistence to a market based income (Rabby et al. 2013). Therefore, livestock improvement can represent an important pathway out of poverty in Bangadesh, since most of the poorest people rearing animals (Shamsuddin et al. 2006). At the currently low levels of daily income, traditional products and services for livestock are simply not available or are too costly. Indigenous dairy cows in Bangladesh produce 2.5 litre/day of milk (Hall et al. 2012), yet in the developed world, cows can produce more than tenfold this amount. The productivity enhancement of livestock will most certainly advance people out of poverty, as well as to provide a source of nutrition to feed a growing population. Biotechnology will provide the mechanism to effectuate the changes required for the less developed world.

1.2 Introduction to Dairy in Bangladesh

The economy of Bangladesh is based on agriculture: about two-thirds of the labour force engaged in it (Fiedler 2014; Lazar et al. 2015; Yosef et al. 2015). The dairy sector is an integral part of agriculture and in Bangladesh is characterized by small-scale, widely dispersed and unorganized dairy cattle keepers, low productivity, an inadequate production inputs and services, and a lack of professional management (Quddus 2012; Uddin et al. 2010). Lower genetic merit, poor nutrition and high prevalence of infectious diseases are the important caused of the low productivity of the cattle in Bangladesh (Hall et al. 2012; Quddus 2012). In addition, the almost total lack of understanding of the nutritional needs of dairy animals, both among farmers and support staff, means that dairy cattle have highly insufficient sources of nourishment, which has a direct and detrimental impact on animal welfare, milk production, fertility and, notably, profitability. There is a long tradition of dairying, with demand driven by a very strong cultural (for example sacrifice of animals during religious festivals and circumcision of children) background in Bangladesh (Sultana et al. 2016). The other key drivers of demand are increasing nutritional awareness, population growth, increases in income and changes in feeding habits (Fiedler 2014). Appropriate research and dairy development interventions are needed to improve dairy productivity and ultimately to improve rural livelihoods.

The South Asian countries including Bangladesh share common milk production systems, consumption patterns, milk collection and marketing (FAO 2007; Saadullah 2001). The dairy farming systems in these countries are part of a crop-livestock mixed agricultural system. There are four major models for dairy production in Bangladesh, based on farm input and output levels (Table 1). The dairy models are based on the needs of smallholders (1-30 cattle) because they play a key role in both the formal and informal dairy markets in Bangladesh (Haque 2009). About 95% of the dairy farmers are smallholders in Bangladesh (Uddin et al. 2011). They supply 100% of the milk for the informal market and 75% for the formal or organized market (Haque 2009). Farms having more than 30 cattle are considered as commercial producer in Bangladesh, and they mostly house cross-bred with Holstein-Friesian, Jersey and Sahiwal. The major characteristics of the smallholder dairy systems can be summarized as follows.

Table 1. Different dairy production systems based on input-output (Uddin et al. 2011).

Description of farming systems	Traditional subsistence	Extensive	Intensive	Bathan*
System boundary	Rural subsistence	Rural to peri-urban	Peri-urban	Peri-urban (cooperatives)
Breed	Local, non-descript, indigenous	Superior local, few a cross-breed with Jersey, Sahiwal	Superior local, mostly cross-bred with HF, Jersey, Sahiwal	Mostly cross-bred with HF and Jersey, very few Pabna milking cows (local)
Herd size	1–6	1–6	2–10	2–30
Milk yield (litres/cow/ lactation)	600–700	600–800	1,000–1,400	1,200–1,800
Feeding system	Cut and carry; tethering	Periodic grazing on public land	Stall feeding supplemented by concentrate and green fodder	Six-month stall feeding followed by six-month bathan feeding
Market access	Limited	Access to spot market only	Good	Good
Access to service (veterinary and AI)	Limited access	Access with high costs	Good access	Good access with low cost

*Bathan is a common pasture land where the animals are kept for a major period of a year.

There are two types of marketing models for milk in Bangladesh (Figure 1). First, the traditional model is characterized by high variability in milk price, low milk quality, poor market access, and poor access to veterinary services and extension as well as to artificial

insemination services. In the traditional model, smallholder milk producers sell their milk directly to spot markets, Ghosh (middlemen), sweetmeat shops, tea stalls, consumers or neighbours without written contracts. Usually, in this kind of market, a very small volume of milk (less than 100 litres per day) is traded, and the farmers don't have the burgaining power to sell their milk. Middlemen may provide loans to smallholders in some areas, at interest rates of up to 20% per month (Haque 2009), and in some cases middlemen will pay the smallholders in advance, though in return the farmers are obliged to sell their milk at a discounted price. Therefore, the milk price varies more than the national and global market situation.
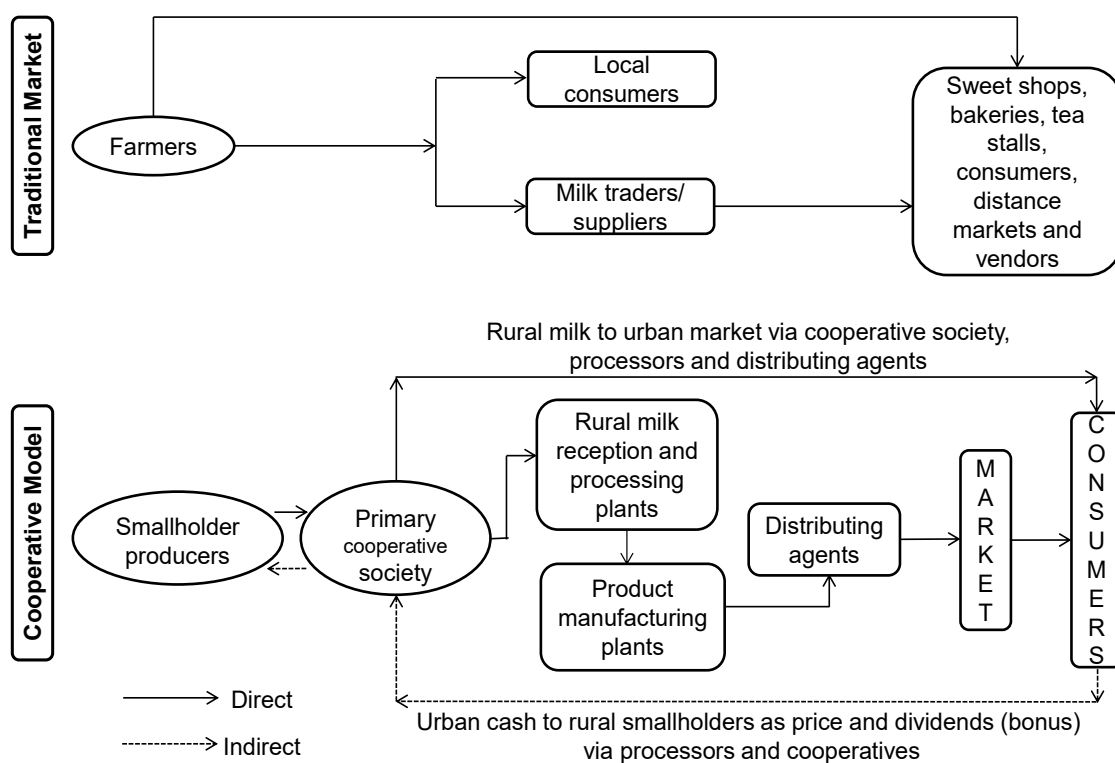

Figure 1. Traditional and Cooperative milk marketing model (Adapted from Haque, 2009).

Second milk marketing process is the Cooperative model. In this model, milk is collected by members of a cooperative society (organized in a village), then pasteurized, processed and distributed to all major cities in the country (Haque 2009; MilkVita 2008). This market model differs from traditional models in that it has only a very small or no price gap throughout the country and it provides milk quality and market assurance. It also provides services such as animal health care, artificial insemination and extension free of charge or at minimal cost. The cooperative dairy model has played a significant role in the country's overall dairy development (Haque 2009; MilkVita 2008). The scenario shows that the dairy sector is

dominated by smallholder dairy farmers and traditional marketing, where the import of milk powder plays a significant role in meeting domestic demand. Cooperative dairying is dominant in India, whereas contract farming is dominant in Pakistan.

1.3 Dairy Production and Consumption in Bangladesh

Bangladesh has achieved a vigorous growth in dairy with a tripling of milk production during the last decade (DLS 2016). This high growth of the milk production is due to the increasing number of crossbred dairy cows and the implementation of different dairy development programmes. The productivity of local cows, however, has not increased. Currently, there are 10 million dairy cattle, including 4 million cross-breeds, produce 7.27 million tons of milk (DLS 2016). In terms of the regional share, the northern part of Bangladesh (one third of the total area) produces nearly half of the country's milk, as a result of the availability of fodder and the establishment of several dairy development programmes (Hemme et al. 2004). Although milk production is increasing in Bangladesh, the gap between milk production and consumption is still high. This is only due to very high population growth. The per capita availability of milk in Bangladesh is 125ml/day (DLS 2016), against the requirement of 250ml/day. Table 2 summarizes the current milk and meat production and consumption status in Bangladesh.

Table 2. Milk and meat production and consumption status in Bangladesh.

Products	In million metric tons			Remarks
	Demand	Production	Deficiency	
Milk	14.69	7.27	7.42	Demand = population × 250ml/day/head
Meat	7.05	6.15	0.90	Demand = population × 120gm/day/head

The consumption of milk in many South Asian countries, including Bangladesh, is substantially increasing because of the growing economies of most of the Asian countries and changing dietary patterns in favour of more dairy products (Owais et al. 2017; Randolph et al. 2007). The rise in purchasing power of certain classes of consumers, leads to increasing demands for high-quality milk and milk products, which offer enormous scope for dairy farmers to provide processed products (Rao and Odermatt 2006). Based on the consideration that 161 million people in Bangladesh should consume at least 250ml milk per day, the annual milk demand is about 14.69 million tons. Therefore, meeting potential milk demand is a huge national task and the question arises how well-positioned Bangladesh is to meet this demand.

School milk programmes have proven to be an effective means to reduce undernutrition (Owais et al. 2017). Combining the strengths of dairying as a powerful means of enhancing rural livelihoods and of rural school milk programmes in reducing undernutrition, this programme aims at developing and demonstrating innovative models to improve poor peoples' livelihoods and child nutrition, especially in rural areas (Owais el al. 2017).

1.4 Why Metabolic Programming?

In humans, there has been an explosion of data showing that *in utero* undernutrition is associated with increased risk for insulin resistance, obesity, and cardiovascular disease during adult life. The process by which early undernutrition at critical stages of development leads to permanent changes in tissue structure and function is known as intrauterine programming (Fowden et al. 2006). The global prevalence of metabolic diseases in adult life is increasing rapidly and their associated complications bring high morbidity and mortality (Lozano et al. 2013). Bangladesh is also undergoing demographic transition with a rapidly rising type 2 diabetes prevalence, increasing from 4% in 1990 to 10% in 2010 and projected to have over 10 million people with type 2 diabetes by 2030 (Finer et al. 2016), the concept of metabolic programming is considered a potential contributor to this rising prevalence (Thurner et al. 2013). Moreover, the tradition of early (teenage) marriage and short birth interval in human population of Bangladesh is being associated with metabolic diseases in later life (Khan et al. 2016; Streatfield and Karar 2008). However, the phenomenon of the adverse pregnancy oucomes in terms of later life is not clear. Therefore, there is immense importance how the intrauterine nutritional situation related to dam growth (early marriage) and lactation (short birth interval) during pregnancy causing metabolic alterations in neonatal period. Furthermore, intrauterine programming is also a potential phenomenon in dairy cattle, which evolved from low to high milk production per year over a period of last decades. The condition of cattle in Bangladesh is further aggravated by the poor quality and lower availability of animal nutrition.

1.5 Implications for Human Health/Dairy Production

Metabolic programming is an emerging area of science dealing with the origins of non-communicable diseases. Impaired development due to suboptimal intrauterine conditions may cause short term as well as long term effects on the function of the organisms subsequently determining the health and disease in later life (Kiani and Nielsen 2011). Epigenetics - a change in phenotype without a change in genotype - set as relatively innovative sciences in

the field is thought to bring significant extra value in animal production. So far, the knowledge about developmental programming and its application in the dairy industry are to the best of our knowledge rather scarce. Therefore, the present thesis describes the effect of metabolic programming in terms of gestation during dam growth and lactation in dairy cattle. Reading this thesis is recommended to all physicians, epidemiologists, nutritionists, animal scientist, endocrinologists and health policy makers.

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GENERAL REVIEW

Metabolic Programming in Dairy Cattle: A Review

The area of metabolic programming is of great interest to animal production. Metabolic programming in the context of livestock production refers to events specific to the embryo or fetus, independent of postnatal maternal or other confounding influences, which result in permanent alterations to efficiency and outputs within the animal's productive life (Wu et al. 2006). Therefore, studies are increasingly focusing on implications of maternal nutrition during early- to mid-pregnancy when organogenesis of tissues occurs, including those of commercial importance to livestock producers (Gonzalez-Recio et al. 2012; Long et al. 2009; Zhu et al. 2007). Clearly, during development, there are critical periods during which a system or organ has to mature. These periods are brief, they occur at different times for different systems and they occur *in utero* for most systems. Developmental plasticity is the term used to describe the process by which the foetus experiences its environment *in utero* and makes developmental adjustments to generate a phenotype that improves immediate survival. For example, in a situation of suboptimal nutrient availability, the foetus may prioritize nutrient partitioning for the most critical organs at that moment such as the brain, at the expense of other organs like the kidney and the lungs which are not functionally active in the womb. The low-priority for development of some organs may, however, have serious consequences for their functionality in later life and hence for lifelong health of the individual (Barker 1995).

In animals, it has been shown to be surprisingly easy to produce lifelong changes in the physiology and metabolism of the offspring by minor modifications to the diet of the mother before and during pregnancy (Banos et al. 2007; Funston et al. 2010). Experiments have shown that alterations in maternal diet around the time of conception and during gestation can change the fetal growth trajectory. The sensitivity of the embryo to its environment is being increasingly recognized with the development of assisted reproductive techniques. Malnutrition (both under- as well as overnutrition) and other adverse influences like reduced maternal oxygen availability (Gulick et al. 2016; Higgins et al. 2016), reduced uterine blood flow (Vonnahme et al. 2012), heat stress (Guo et al. 2016) or metabolic and infectious diseases (Wathes 2012) affecting the dam during development, permanently alter gene expression in the fetus leading to a specifically designed phenotype (Berry et al. 2008; Gutierrez et al. 2012). Efficiency of the production of meat, wool and milk and the susceptibility to disease of domestic livestock continues to vary widely among and within similarly managed herds and flocks of relatively uniform genetic background. At least some

of this hitherto unexplained variation may be ascribed to carryover effects of metabolic perturbations during different phases of embryonic and fetal development. The best described effects are those on early muscle and adipose tissue development, with putative consequences for the capacity for lean tissue growth, propensity for fattening and therefore feed efficiency in meat animals (Du et al. 2010).

Animal models have identified common mechanisms by which events very early in life could have programmed effects on health in later life. Mechanisms conserved between different models and between species are likely to represent fundamental biological processes (Tarry-Adkins and Ozanne 2011). One mechanism by which effects of the early environment could have a permanent effect on tissue function is through alterations in organ structure. For example, it is known that the endocrine pancreas is particularly susceptible to changes in nutrition during fetal life that permanently alters the structure of this tissue. A variety of animal models, including the maternal protein-restriction, maternal-caloric-restriction, and intrauterine placental-ligation models, have shown a reduction in β-cell mass and islet vascularization (Garofano et al. 1999; Simmons et al. 2001). Secondly, conclusive evidence is now emerging that demonstrates that epigenetic alterations can form integral underlying mechanisms in early life programming. Epigenetics can be defined as any change in phenotype or gene expression caused by modifications independent of changes in genotype. These modifications include methylation of DNA and modifications of histones, including acetylation. Epigenetic regulation of transcription factors is now emerging as a common mechanism of early life programming, including the peroxisome proliferator-activated receptor-α (Lillycrop et al. 2005) and Pdx1 (Park et al. 2008), including effects on both DNA methylation and histone marks. Transcription factors represent attractive targets of nutritional programming as alterations in transcription factor expression can affect a wide range of other downstream target genes. Third underlying mechanism could be accelerated aging at the cellular level through accelerated telomere shortening. This is accompanied by increased pancreatic islet gene expression of p21 and p16^{INK4a} (Tarry-Adkins et al. 2009), indicative of accelerated cellular aging. Increased reactive oxidative species and oxidative stress in pancreatic islets have been reported in intrauterine artery ligation IUGR, which has been suggested to be related to mitochondrial dysfunction (Simmons et al. 2005).

2.1 Unique Features of Dairy Cattle Management

Dairy cows exhibit a mammalian tendency to nurture the neonate from tissue stores by lipolysis and muscle catabolism (Roche et al. 2008), losing body condition for approximately 40 to 100 days after calving before replenishing lost tissue reserves (Pryce and Harris 2006; Roche et al. 2007; Sumner and McNamara 2007). In dairy cattle, the body condition is most commonly quantified by body condition scores (BCS) that describe the relative fatness of a cow (Edmonson et al. 1989). However, what makes dairy cows unique among all other mammalian species has been the intense trans-generational genetic selection for early lactation and total milk production during the last 50 years (Compton et al. 2017). Such selection pressures have resulted in many physiological changes that facilitate greater mobilization of energetically important tissues in dairy cows than other mammals (Chagas et al. 2009; Lucy et al. 2009). Genetic selection programs based solely on increased milk production have resulted in cows that are genetically predisposed to a greater degree of negative energy balance (NEB) in early lactation (Patton et al. 2007). The increased genetic merit for milk yield is thus associated with a greater degree of BCS loss in early lactation and less BCS throughout lactation, reflecting a greater degree of NEB (Pryce et al. 2001). The calving and nadir (i.e., the lowest point) BCS are nonlinearly associated with the height of the lactation curve and the slope of the post-peak decline i.e., lactation persistency (Berry et al. 2007; Roche et al. 2007b), with lactation milk yield increasing with BCS loss postcalving and decreasing with further BCS loss (Figure 2).

To maximize milk production during herd life, currently farmers are stimulated to breed their young stock at young age in order to have their first calf at 24 months (Wathes et al. 2014), and subsequently have their cows calved with intervals no longer than 385 to 400 days (Inchaisri et al. 2010). The latter implies dairy cows to be rather atypical since they have to manage the compatibility of optimal reproductive performance and (early) gestation with continued growth or the production of large quantities of milk. Rather than being an absolute shortage of energy substrates *per se*, this metabolic priority for growth and lactation (after calving) is known to generate adverse conditions hampering optimal ovarian functioning, follicular growth, oocyte maturation and early embryonic development (Leroy et al. 2008). On the other hand, suckling beef cows are typical in the sence that they produce only a small amount of milk during their gestation. A pregnant cow's capacity to care for her embryo is **however** largely determined by the way she partitions nutrients to support embryonic, placental and fetal development together with her own growth, maintenance and milk production.
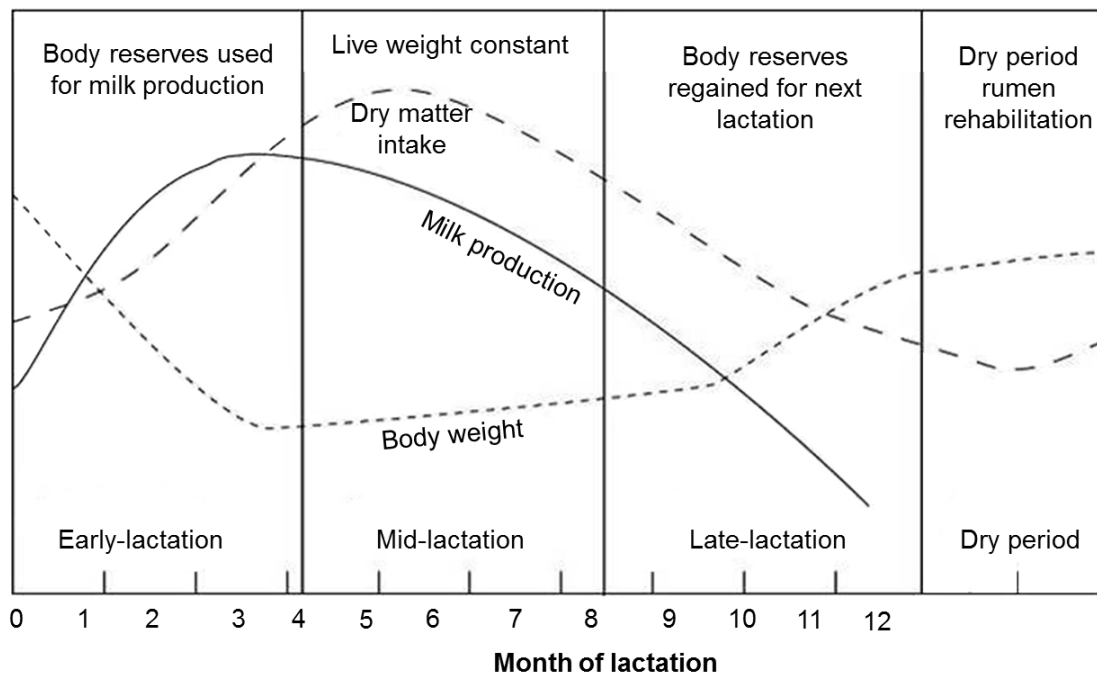
Figure 2. Dry matter intake, lactation cycle and live weight changes in a cow during her lactation cycle (Adapted from Hoffman et al. 2000).

2.2 Characteristics of Bovine Reproduction

Cattle are a year round breeder. Among cattle, a heifer is a young cow that has not yet given birth to a calf. To assure a high level of milk production Holstein heifers should be raised to weigh 350-375 kg at 15 months of age, the age they should be inseminated to allow calving at 24 months (Wathes et al. 2014). However, generally the accepted target weight for yearling replacement heifers at breeding was 65% of their mature weight. Heifer calves are born with oocytes in the diplotene stage of meiosis and ovarian activity is characterized by a follicular wave pattern (usually 2 or 3 follicular waves per cycle) leading to estrus and ovulation of a mature oocyte every three weeks. Typically, the growth of follicles destined to ovulate in the cow, takes place over 3-4 months (Webb et al. 2004) and involves several critical stages that can be disrupted during environmental or physiological insults leading to development of defective follicles, oocytes, and subsequent poor embryo quality. For example, the negative energy status during the early postpartum period might impair oocyte and embryo quality results in subfertility in lactating dairy cows (Leroy et al. 2008). Fertilization of the oocyte occurs in the oviduct and is dependent on multiple factors like AI technique, quality of spermatozoa, timing of AI, the presence or absence of pro-inflammatory substances in the uterine lumen, to be successful. The oviduct is named to contribute to the optimal early embryo development (Wiltbank et al. 2016). The bovine embryo enters the uterus 4 days after

ovulation. Prior to embryo attachment to the uterine caruncles, the embryo is free floating and is dependent upon uterine secretions into the uterine lumen, termed histotroph, both for energy and proteins. Thus deficiencies or excess of energy or specific amino acids may have an impact on further embryo development. This is to be mention here that the gestation length in cattle averages 280±10 days, and modern dairy cattle have an average lifespan between 5 to 6 years.

The term 'nutrient partitioning' refers to the processes by which available nutrients are channelled, in varying proportions, to different metabolic functions. A narrower definition commonly applied in dairy nutrition refers specifically to the partition of nutrients between milk outputs and body reserves (Friggens and Newbold 2007). The nutrient partition changes with stage of lactation (Kirkland and Gordon 2001) and at different stages of the reproductive cycle (Chilliard et al. 2000; Theilgaard et al. 2002) due to a change in the endocrine profiles (Bauman 2000). The net result of such changes is that nutrients are channelled to differing extents to different organs, life functions and end-products. This occurs not as a function of changing nutritional environment but rather as a function of (physiological) time. The onset of lactation provides the classic example of this with the uncoupling of GH and IGF and the resulting channelling of nutrients to the mammary gland (Bauman 2000). 'Negative' energy balance, insulin 'resistance' and reproductive 'failure' are all expressions that imply that the machine, i.e. the cow is being on the very thin line between top production and health. There are clear differences between breeds in their partition of energy between milk and body reserves that change with stage of lactation and also with parity (Dillon et al. 2003; Yan et al. 2006). For example, Holstein-Friesian cows can produce more milk energy than Norwegian cows, mainly as a result of higher metabolizable energy intake and because of a greater ability to partition more energy into milk and less into body tissue (Yan et al. 2006).Combining the genetically derived teleophoretic aspects and the environmentally affected homeostatic aspects is the crucial step for the next significant advance in models of nutrient partitioning, and this requires consideration of genotype environment interactions (Friggens and Newbold 2007). The key underlying process is called plasticity or environmental sensitivity. An animal with low plasticity for milk production is an animal that maintains milk production at the expense of other life functions. Although dairy cows currently show a substantial ability to cope by reducing body fat levels or by reducing disease resistance (Collier et al. 2005), they do so at the expense of other life functions such as reproduction (Royal et al. 2002) and health (Windig et al. 2005).

2.3 Embryonic Growth and Development in Ruminants

In cattle, the critical period of maternal recognition of pregnancy occurs between days 15-18 after ovulation, followed by the initial stages of early placentation (Haeger et al. 2016). The early ruminant embryo secretes copious quantities of a protein called interferon tau. Exposure of the endometrium to this hormone dampens the secretion of PGF2α, thereby blocking the signal for luteolysis (Haeger et al. 2016). As a result, the corpus luteum survives and progesterone levels are maintained for the recognition of pregnancy. The placenta attaches at 5 weeks to discrete sites of the uterine wall called caruncles. These caruncles are arranged in two dorsal and two ventral rows throughout the length of the uterine horns. The placental membranes attach at these sites via chorionic villi in specific areas called cotyledons. The caruncular-cotlydonary unit is called a placentome and is the functional area of physiological exchanges between cow and calf. Cattle have been shown to have the synepitheliochorial type of placentation (Wooding and Burton 2008). In association with the formation of the placentome, the caruncular area is progressively vascularized to meet the increasing demands of the conceptus. Fetal survival is dependent upon proper placental growth and vascularity early in pregnancy, while also further intrauterine growth is mainly dependent on the placental supply of maternal nutrients and oxygen to the fetus (Reynolds and Redmer 2001). Therefore, establishment of a functional fetal/placental vascular system is one of the earliest requirements during conceptus development (Vonnahme 2008). Transport efficiency is related to uteroplacental blood flow (Reynolds and Redmer 1995). Although placental growth slows during the last half of gestation, blood flow to the placenta increases three to fourfold from mid to late gestation to support the exponential rate of fetal growth (Reynolds and Redmer 1995). However, during pregnancy, the placenta is exposed to a variety of environmental insults which can alter fetal organogenesis and growth, leading to improper pre- and postnatal growth and eventually lower life performance (Schlafer et al. 2000). In placental mammals, post-embryonic growth becomes quantitatively significant only after mid-gestation. However, this is preceded by rapid hyperplastic growth of the placenta which attains all or most of its mass of dry tissue, protein, and DNA by mid-gestation (Ehrhardt and Bell 1995). Foetal growth then follows its familiar, flattened sigmoid pattern during the latter half of gestation as it proceeds from an early exponential phase through a rapid, linear phase, and then, as term approaches, begins to diminish in rate. In most species, there is little or no increase in placental weight during this period; the ovine placenta actually diminishes in weight, mostly due to loss of extracellular water (Ehrhardt and Bell 1995). However, the

placenta undergoes extensive tissue remodelling after mid-estation, including major proliferative growth of the umbilical vasculature, which is associated with a progressive increase in its functional capacity (Haeger et al. 2016). Patterns of foetal and placental growth in the normal and growth-retarded sheep conceptus are illustrated in Figure 3.
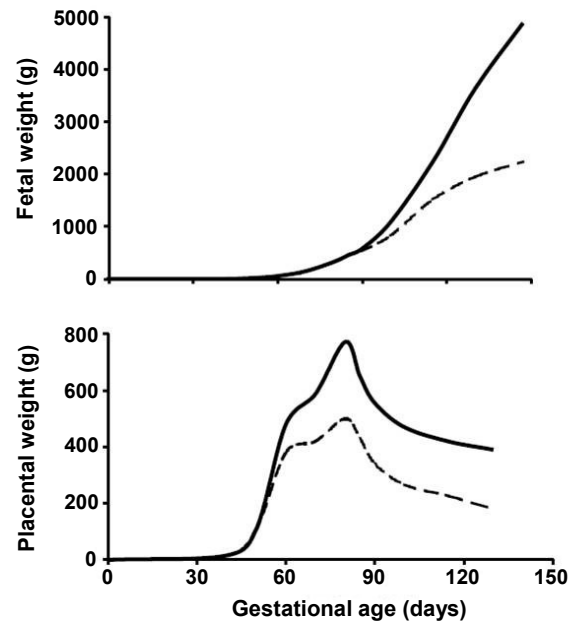

Figure 3. Patterns of foetal and placental growth in the normal (—) and growth-retarded (---) sheep conceptus. Adapted from Ehrhardt and Bell (1995) and Greenwood et al. (2000). The growth-retardation can be imposed by maternal nutrient restriction, carunculectomy, uterine artery ligation, hypoxia, heat stress, overfeeding etc.

The placenta is pivotal in the competition between mother and fetus for resource allocation. Maternal nutrition influences growth of the foetus and size of the newborn either directly as a result of the adequacy of nutrient intake and circulating substrate concentrations, or indirectly due to effects on the capacity of the placenta to transport nutrients to the foetus. Because placental growth precedes foetal growth on a weight specific basis (Ehrhardt and Bell 1995), residual effects of nutrition during early and mid pregnancy on subsequent foetal growth may be mediated, at least in part, by effects on placental size. This has stimulated interest in understanding how nutrition may be used during early to mid pregnancy to increase placental capacity for nutrient transport in sheep (Heasman et al. 1998; Wallace et al. 1999) and cattle (Cooper et al. 1998; Perry et al. 1999) prior to the period of maximal foetal growth potential during late pregnancy. However, effects of nutrition on placental growth during early to mid pregnancy are highly variable, and may be influenced by a range of factors that uncouple the

normally tight association between placental and foetal weights, including nutritional status of the dam prior to mating (Kelly 1992).

2.4 Distinctive Factors Affecting Embryonic Development

2.4.1 Growth in Adolescent Animals

Generally, reproductive capacity of nulliparous heifers is higher when compared to multiparous animals. This finding can be attributed to the fact that oocytes and embryos of the nulliparous heifers have not been challenged by the metabolic stress of milk production (Gonzalez-Recio et al. 2012), but one should also not forget the decisive role of the uterus in terms of pregnancy success. Uteri of nulliparous heifers have not been confronted yet with a parturition event which is in the vast majority of the cases associated with bacterial infection. Besides the better reproductive performance of nulliparous heifers, significant differences in terms of production, reproductive capacity, longevity, and resilience against metabolic challenges, between offspring of first versus higher parity animals are reported (Banos et al. 2007; Gonzalez-Recio et al. 2012). In most cases, the offspring of first parity animals is in a favorable condition (Swali and Wathes 2007). The latter results are a clear indication of the deleterious effect of lactation during gestation (Swali and Wathes 2006; Gonzalez-Recio et al. 2012).

All too often however, researchers have used first parity heifers as non-lactating and hence 'negative' controls when examining the effect of lactation and its concomitant metabolic consequences on the animal's reproductive capacity and more particularly embryonic growth. However, when reproduction has to coincide with continued growth of the first parity dam, both the gametes as well as the embryo/fetus may face intense competition for nutrients from its mother's own metabolic needs while she is still growing. Hence, the normal hierarchy of nutrient partitioning between maternal body growth and fetal growth may be altered (Wallace et al. 2006). In sheep for example, there is a general consensus nowadays that overnutrition during gestation in adolescent ewes gives rise to a lighter progeny, while the dam generally experiences a significant increase in BCS. In this paradigm, rapid maternal growth results in placental growth restriction and often premature delivery of low birth weight lambs when compared with moderately nourished ewes of equivalent age (Wallace et al. 2006).

Since farmers are currently stimulated to maximize daily growth in their growing young stock in order to maximize milk production in the first and subsequent lactations, they accentuate

the mismatch between the milieu the offspring is prepared for and the milieu the neonates actually arrive in, which may lead to even more deleterious effects. Examples hereof are well known in human medicine, where it has been shown that babies that had experienced intrauterine growth retardation and thereafter experience a catch-up growth, are more prone to reproductive disorders such as polycystic ovarian syndrome (Ibanez et al. 2007). Epidemiological studies both in beef (Funston et al. 2010) as well as in dairy cattle (Brickell et al. 2009; Swali et al. 2008) have indeed shown that heifers growing fast in the first months of life, have a significantly earlier pubarche but need more inseminations to become pregnant, ending up with a similar age at first calving in comparison with their slower growing peers.

2.4.2 High Milk Yield and the Concomitant Metabolic Stress in Lactating Animals

The genetic drive to produce large quantities of milk makes modern dairy cows more vulnerable for factors generally known to impair overall health and fertility. Hence, since dairy cows are challenged by such a variety of environmental factors during the period they should also reproduce, they represent a 'natural' model to describe the effects of periconceptional environmental challenges on their reproductive capacity. Typically furthermore, modern dairy cows' reproductive capacity is under serious pressure especially because of very high rates of (early) embryonic mortality (Wiltbank et al. 2016). The latter might be a reflection of the high number of insults the gametes and early embryos are confronted with in the periconceptional period (Leroy et al. 2008; Ribeiro et al. 2013).

Modern dairy cows have been predominantly selected for high milk yield in early lactation which is associated with a very high capacity to mobilize body reserves during this period. Calculations showed that cows can produce as much as between 120 and 550 kg of milk from body reserves on the basis of energy (average 324 kg). Maximum mobilization in 8 weeks amounted up to 41.6 kg empty body weight, 30.9 kg fat and 4.6 kg protein (Tamminga et al. 1997). Most cows can cope with this metabolic load which is defined as: 'the total energy burden imposed by the synthesis and secretion of milk, which is met by mobilization of body reserves'. Metabolic load has however been opposed to metabolic stress which is defined as 'the amount of metabolic load that cannot be sustained by body mobilization, leading to the down-regulation of some energetic processes, including those that maintain general health' (Knight et al. 1999). Hence, the 'over' mobilization of body reserves during the period of negative energy balance (NEB) is a key factor for disease susceptibility in modern dairy cattle. The genetically and hormonally driven body mobilization is further significantly

aggravated by the mismatch between the energy need and the cow's capacity to take in energy. Maximal feed intake in dairy cows occurs commonly at 6 to 8 weeks in lactation, which is much later than peak production, causing cows typically to be in negative energy balance for 5-7 weeks postpartum (Tamminga et al. 1997).

Typically, the negative energy balance and concomitant body fat mobilization is characterised by specific alterations in peripheral plasma metabolite concentrations such as high non-esterified fatty acids (NEFAs), low glucose and insulin and high levels of ketobodies. These alterations are not only occurring in the peripheral circulation but are also reflected in the follicular fluid at the ovaries (Leroy et al. 2004). The effect of the elevated/lowered concentrations of metabolites being associated with high milk yield on follicular cells (Vanholder et al. 2005b; Vanholder et al. 2006) and oocytes (Leroy et al. 2005; Leroy et al. 2006) are associated with lower fertilization and blastocyst rates *in vivo* (Leroy et al. 2005) and *in vitro* (Leroy et al. 2008).

Whether these changes can also induce pertinent changes in the offspring's metabolism and body functions and hence has an effect on its health later in life, is still a matter of debate. It is however demonstrated that the altered microenvironment give rise to altered patterns of gene expression (Lillycrop and Burdge 2012).

2.5 Birth Size in Metabolic Programming

Size at birth is important for calving ease of the dams and neonatal survival of the calves. Offspring born at an above average weight have an increased chance of survival compared with those born at a below average weight in all domestic livestock species, including the cow, ewe, and sow. Complications of low birth weight reported in livestock include increased neonatal morbidities and mortalities (Hammer et al. 2011), intestinal and respiratory dysfunctions, slow postnatal growth, increased fat deposition, differing muscle fiber diameters and reduced meat quality (Wu et al. 2006). Epidemiological evidence suggests that small size (small for gestational age) at birth both in humans and animals is associated with increased predisposition to metabolic diseases during adult life (Symonds et al. 2010; Vuguin 2007). It should be noted that birth size is a complex trait influenced by the interaction between genetic and non-genetic intrauterine factors (Kocak et al. 2007). However, the intrauterine environment has been stated to be a more important regulator of birth size than the parental genome (Sharma et al. 2012; Swali and Wathes 2006). The use and interpretation of birth size in relation to fetal programming assumes that birth size indeed reflects specific fetal responses

to particular variations in the nutritional status of the dam. Since epigenetic adaptations in the embryo may influence future health and fertility (Van Soom et al. 2013; Wu et al. 2006), the intrauterine environment is currently being assessed with regard to health and welfare of the offspring (Rutherford et al. 2012). The association of young age in heifers and high milk production during gestation in cows with reduced longevity and productivity of their offspring (Banos et al. 2007; Berry et al. 2008; Gonzalez-Recio et al. 2012) furthermore emphasizes their potential impact on birth size of the calves.

Livestock producers are interested in utilizing nutrients in the most efficient way to optimize growth of their animals. While growth is often thought to take place after birth, the majority of mammalian livestock (i.e. swine, sheep, and cattle) spend 35-40% of their life within the uterus, being nourished solely by the placenta. Therefore it is especially relevant to understand the impacts of the maternal environment on placental growth and development as this directly impacts fetal growth. The trajectory of prenatal growth is sensitive to direct and indirect effects of maternal environment, particularly during early stages of embryonic life (Robinson et al. 1995), the time when placental growth is exponential. Moreover, it is recognized that the maternal system can be influenced by many different extrinsic factors, including nutritional status and level of activity, which ultimately can program nutrient partitioning and ultimately growth ad development of the fetus (Godfrey and Barker 2000; Wu et al. 2006). Therefore, the continual desire to enhance management methods to produce healthy livestock has led to increased research in the area of developmental programming of our livestock species.

2.6 Placental Mechanisms in Metabolic Programming

The placenta characteristics in mammals are presented in Table 3. Despite some differences in placental structure and function, the placenta in all mammals has a large surface area for nutrient and gas exchange with complex associations of maternal blood vessels on the uterine side that develop through both angiogenesis and vasodilation to increase blood flow to the placenta and a dense network of capillaries on the fetal side. The details of comparative placental structure have been well described earlier (Wooding and Flint 1994). Trophoblast cells of the placenta regulate fetal growth by affecting the development and function of maternal arteries that bring blood into the placenta (Cross 2015). In sheep and cattle, in which there is no trophoblast invasion directly into maternal arteries, the effect on maternal arterial blood flow may be developmental and physiological because trophoblast cells express

vascular endothelial growth factor (VEGF) and vasodilators (Cross 2015). The surface area and density of nutrient transporters at the exchange surface of the placenta also regulate fetal growth. Several types of nutrient transporters are expressed by trophoblast cells on the surface of the villi and their levels are altered in some cases of IUGR (Burton and Fowden 2012). After development of the branched trophoblast villous tree, a dense network of fetal blood vessels develops. Similar to other organs, this is regulated by local VEGF that can be produced by the trophoblast cells (Pfarrer et al. 2006).

Table 3. Pregnancy and placenta characteristics in mammals (Cross 2015)

Species	Cow	Sheep	Human	Mouse
Gestation length (days)	285	145	270	19
Number of young	1	1-3	1	5-12
Placenta type				
Gross	Cotyledon	Cotyledon	Discoid	Discoid
Cell layers	Synepithelio-chorial	Synepithelio-chorial	Haemochorial	Haemochorial

A drawing of bovine placentome and fetal membrane attached to the endometrium is presented in Figure 4. Different types of experimental manipulation of the placenta or the intrauterine environment have shown that different types of early responses can be initiated that would be interpreted as being adaptive attempts to mitigate the effects on fetal growth (Sferruzzi-Perri and Camm 2016). Trophoblast proliferation and activation of angiogenic responses are reported in the sheep placenta in response to altered maternal nutrition (Cross and Mickelson 2006; Redmer et al. 2009). There is evidence of adaptation in fetal vascular density in the placenta as an attempt to compensate for a less developed trophoblast villous tree (Bainbridge et al. 2012). We know, the placenta transports the substrates (oxygen, glucose, amino acids and fatty acids) to the fetus by passive diffusion, transporter mediated processes and endocytosis-exocytosis (Duttaroy 2009; Sibley et al. 1997). In addition to morphological characteristics of the placenta, transporter-mediated processes are influenced by the expression, localization, affinity and activity of specific transporters in the placental plasma membranes as well as the materno-fetal concentration gradient across the placenta (Jansson and Powell 2006). Changes in any of these placental parameters can, therefore, affect fetal fuel acquisition and growth with consequences for adult health and disease (Fowden et al. 2008).
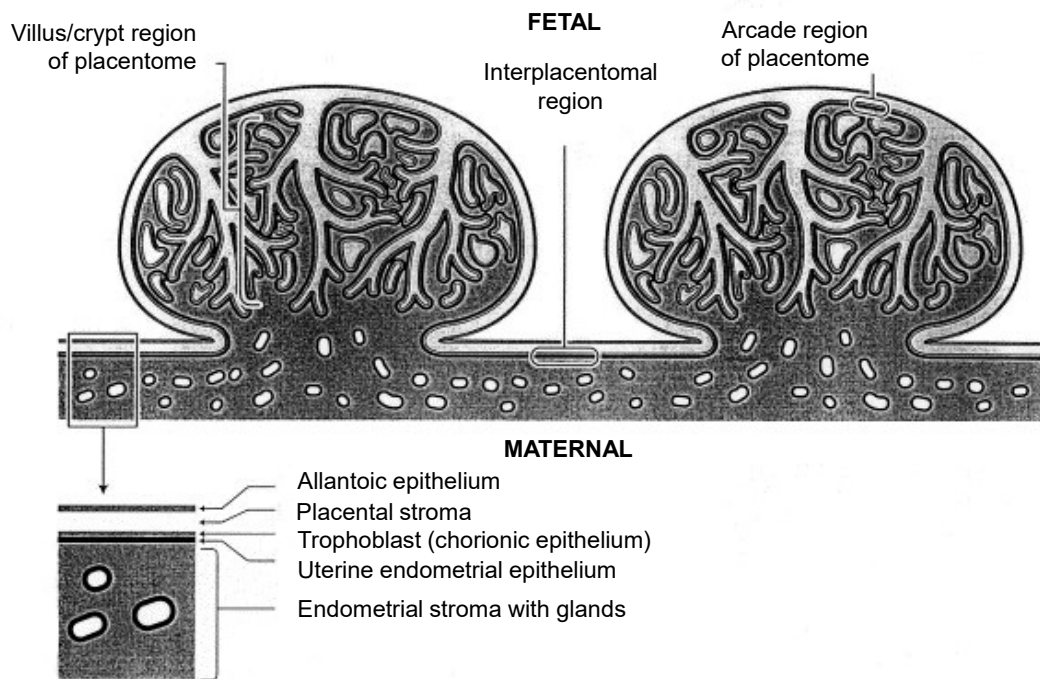
Figure 4. Drawing of two bovine placentome and fetal membranes attached to the endometrium (Schlafer et al. 2000).

Due to the importance of placental development on fetal nutrient transfer, studies have been conducted in cattle to determine how maternal nutrition can influence placental development, or placental programming. It is reported that nutrient restriction of beef cows from day 30 to 125 of gestation resulted in reduced caruncular and cotyledonary weights from nutrient restricted cows compared to control cows, and fetal weights from nutrient restricted cows tended to be reduced compared to control cows (Zhu et al. 2007). Following realimentation during day 125 to 250 of gestation, caruncular and cotyledonary weights were still reduced for nutrient restricted cows; however, fetal weight was not different. Using the same cows, it is evidenced that there is increased placental angiogenesis as well as angiogenic factor mRNA abundance in the caruncular and cotyledonary tissues at the end of the nutrient restriction period (Vonnahme et al. 2007). Therefore, the lack of significant fetal weight differences in regard to maternal nutrient restriction may have resulted from the increase in cotyledonary arteriolar density allowing for adequate nutrient transfer (Vonnahme et al. 2007; Zhu et al. 2007).

Placental development responds to both fetal signals of nutrient demand and maternal signals of nutrient availability and, by adapting its phenotype, regulates the distribution of available resources (Fowden and Moore 2012; Fowden et al. 2006). Placental adaptation may occur by

changing its surface area for nutrient transfer, the thickness of its interhaemal barrier separating the maternal and fetal circulations, its abundance of nutrient transporters, metabolic rate and blood flow as well as its synthesis and metabolism of specific hormones (Fowden and Moore 2012; Vaughan et al. 2012). Fetal intrauterine growth retardation due to nutritional constraints is closely linked to placental development and function (Cetin and Alvino 2009; Fowden et al. 2008). Basically, under conditions of impaired nutrient supply that may limit normal fetal growth, the placenta has shown to adapt so as to increase its transport capacity (Burton and Fowden 2012). Earlier studies in beef cattle and sheep have demonstrated that placentas may adapt their phenotype to be able to accommodate fetal nutrient demand and that this process may include either morphological or functional mechanisms acting during different stages of feto-placental development (Fowden et al. 2010; Long et al. 2009; Sullivan et al. 2009).

2.7 Critical Windows for Embryonic Development

In the uterus, the embryo may experience challenges by a variety of environmental insults and that at different time points. In the early stages, most investments by the dam should go to the development of the placenta (Redmer et al. 2009). Hence, environmental challenges like undernutrition or hypoxia when occurring during early pregnancy may affect placental development and therefore indirectly embryonic growth and development. Due to the minimal nutrient requirement during early gestation, inadequate nutrition during this time was thought to have little significance. However, during the early phase (from day 25) of fetal development critical events for normal conceptus development occur in a sequential manner for different organs including the pancreas, liver, testicle, ovaries, adrenal glands, lungs, thyroid, spleen, brain, thymus, and kidneys (Funston et al. 2010; Hubbert et al. 1972; Nilsson and Skinner 2009). Review have made earlier that lists examples of fetal programming in livestock models in individual organs including heart (Han et al. 2004), lung (Gnanalingham et al. 2005), pancreas (Limesand et al. 2005; Limesand et al. 2006), kidney (Gilbert et al. 2007), placenta (Reynolds et al. 2006), and small intestine (Greenwood and Bell 2003). Hence, deleterious insults taking place at the specific time during pregnancy might be associated with impaired function and a lowered ability to ensure homeostasis in later life.

During the second trimester of pregnancy, the fetus continues to develop and grow and reach to about 25% of its birth size at the end of this stage. Muscle and adipose tissue formation occurs primarily during mid to late gestation (Du et al. 2010). Because the dam in most cases

won back positive energy balance at that time, the risk of major metabolic challenges is lower during that stage. On the other hand, the development of major organ systems is still going on, so that major insults might still have pronounced effects on later health and productivity. The largest increase in fetal tissue size of about 75% of fetal growth generally takes place during the final trimester of pregnancy, insults taking place at that time being mostly reflected in a significantly lowered birth weight (Sharma et al. 2012; Zhu et al. 2004). For that reason, many people believed that cow nutrition could only affect calf growth during the last trimester of gestation. However, depending on when the nutrient restriction happens during gestation, the outcome of this insult might have different consequences to calf performance.

An impaired nutrient supply during intrauterine development leads to metabolic adaptations to enhance survival in the short term but may become detrimental in postnatal life (Fleming et al. 2012). The unique metabolic adaptations to reduced nutrient supply *in utero* are well coordinated in an attempt to maximize the uptake and use of nutrients by the most vital organs like the brain (Fleming et al. 2012). As a consequence, these adaptations often occur at the expense of those organs that are less critical for survival (e.g. the pancreas) (Gutierrez et al. 2012; Limesand et al. 2013; Long et al. 2010a). Hence, suboptimal intrauterine nutrition may end up in birth of growth restricted newborns with an impaired β-cell mass, visualized by a lowered insulin secretion when challenged with a standard glucose bolus (Limesand et al. 2013; Owens et al. 2007; Rozance et al. 2006). The environmental sensitivity is viewed as an adaptive mechanism by which the developing organism adjusts its metabolic and homeostatic systems to suit the anticipated postnatal environment. The long-term effects of these adaptations predispose the offspring to adiposity and metabolic diseases in later life if nutrition in postnatal life does not match that foreseen by the fetus on the basis of its intrauterine environment (De Rooij et al. 2006; Micke et al. 2011).

2.8 Vulnerability of Conceptus in Different Trimesters

A summary of feto-placental development during different trimesters of gestation including long-term effects in the offspring after maternal nutrient restriction is presented in Figure 5.

Early Gestation (0 to 3 months of gestation): Cows must conceive within 80 days postpartum if a yearly calving interval is desired. Cows' milk production and nutrient requirements peak at 60 days postpartum; however, intake lags behind. This results in negative energy balance during early lactation, especially if cows are managed to calf during the dry or winter seasons when poor quality and less forage mass is available. Thus, nutrition inadequacy often occurs

in cattle production systems (Caton and Hess 2010). Unfortunately, a limited amount of published results exists regarding the effects of cow nutrient restriction during early gestation on calf performance. Growth performance and organ development of calves born to cows experiencing nutrient restriction during early gestation has been studied in depth (Long et al. 2010b). Cows were separated into two groups that were fed at 55 or 100% of their nutrient requirements for the first 83 days of gestation. Following 83 days, both groups were provided 100% of their nutrient requirements until calving. No differences were observed on calf birth weight, weaning weights, and average daily gain from birth to weaning or during the feedlot finishing phase. However, lung and trachea weights of steers born to heifers provided 55% of their nutrient requirements were significantly less than steers born to heifers fed 100% of their nutrient requirements. Although growth performance was not affected, it would be misleading to interpret these results as if nutrient restriction during early gestation does not impact calf performance.
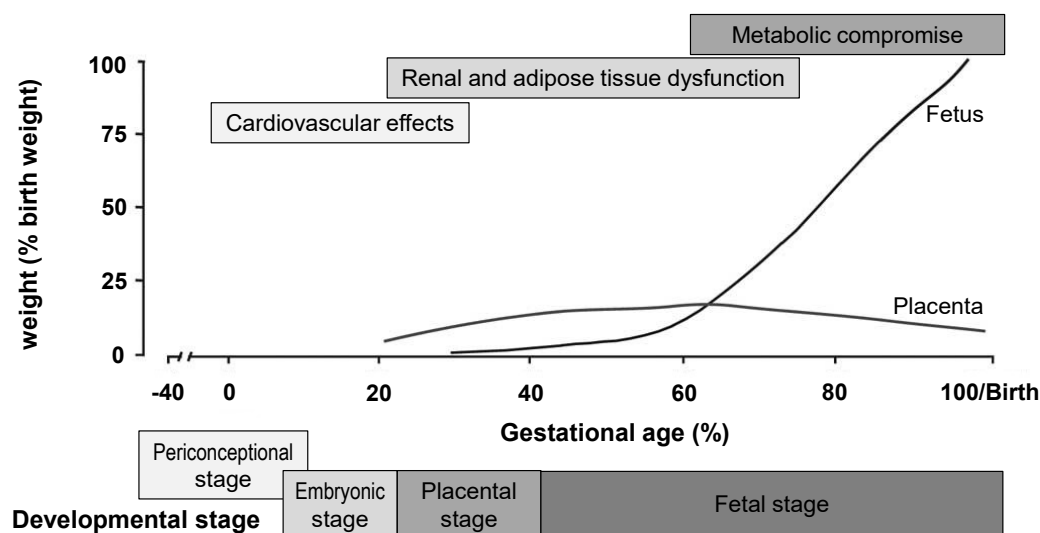

Figure 5. Summary of feto-placental development during gestation including long-term effects in the offspring after maternal nutrient restriction in sheep (Symonds et al. 2010).

Mid Gestation (3 to 6 months of gestation): Production-oriented tissues, such as muscle, appear to be responsive to fetal programming effects *in utero* (Caton and Hess 2010). Muscle formation is divided into two waves of muscle fiber synthesis. The first wave begins at mid gestation, whereas the second wave occurs from six to nine months of gestation (Du et al. 2010). Thus, nutrient restriction during mid gestation is expected to decrease muscle fiber formation, leading to lower birth and weaning weights. At the University of Wyoming, researchers evaluated the growth performance of steers born to cows grazed on low-quality,

native pastures (6% crude protein) or high-quality, fertilized and irrigated pastures (11% crude protein) for 60 days from 120 to 150 days through 180 to 210 days of gestation (Underwood et al. 2010). In that study, cows that grazed on improved pastures during mid gestation produced calves that were heavier at weaning and harvesting, and that had greater meat tenderness at slaughter. Nutrient restriction during mid gestation also may have consequences on organ development. Angus × Gelbvieh cows were randomly allotted into groups and fed at 70 or 100% of their nutrient requirements from day 45 to 185 of gestation. They were then commingled and fed at 100% of their nutrient requirements from day 185 of gestation until calving (Long et al. 2012). Although body weight at birth and at weaning did not differ between treatments, heifers born to cows fed at 70% of their nutrient requirements had smaller ovaries and luteal tissue. Luteal tissue is crucial for progesterone synthesis and pregnancy maintenance. Therefore, smaller ovary and luteal tissue could affect cows' reproductive performance during their first breeding season.

Late Gestation (6 to 9 months of gestation): Late gestation is probably the most important gestation period in terms of potential impact on production-oriented tissues such as muscle and adipose tissue. As mentioned before, major portions of cattle muscle and adipose tissue form during late gestation (Du et al. 2010). Muscle fiber number is set at birth, meaning that after the calf is born, there is no net increase in the number of existing muscle fibers. Thus, if nutrient restriction during late gestation reduces muscle fiber number (Zhu et al. 2004), calf growth performance following birth might be compromised. In addition, maternal nutrient restriction may also compromise adipocyte populations, resulting in carcasses with lower quality and marbling scores. In addition, heifers born to cows that were supplemented achieved puberty at younger ages (Funston et al. 2010) and had greater pregnancy rates (Martin et al. 2007) than heifers born to cows that did not receive protein supplementation.

2.9 Pancreatic Islets and Insulin in Metabolic Programming

The functional role of the pancreatic islets has been well demonstrated as one of the key targets of metabolic programming (de Oliveira et al. 2011; Rodriguez-Trejo et al. 2012), which might predispose the endocrine pancreas to exhaustion, resulting in a diabetic condition. Both overnutrition and undernutrition have been linked to pancreatic β-cell dysfunction induced by epigenetic changes, which appears to induce the onset of type 2 diabetes (Berends and Ozanne 2012; Pinney and Simmons 2010). During the embryonic stage of pancreatic organogenesis and β-cell development, several transcription factors are

indispensable to appropriate pancreatic cell formation, maturation, and future function (Rodriguez-Trejo et al. 2012). Among other alterations in the transcription factors that promote pancreatic β-cell development and function, it is primarily associated with lower expression of the Pdx-1 gene (Park et al. 2008). Dietary management schemes, such as low-protein or food-restriction diets, which are used to induce IUGR, can alter the epigenetic markers that regulate gene expression through DNA methylation and/or histone modifications which showed decreased Pdx-1 mRNA expression associated with the development of type 2 diabetes mellitus in adulthood (Stoffers et al. 2003). Pancreatic β-cell development and maintenance of function are pivotally dependent on the control of these factors (Bernardo et al. 2008), which make these observations importantly suggestive of the malprogramming of pancreatic β cells early in development and the possibility of inducing metabolic disease onset in adulthood. Moreover, early environmental influences also appear to induce morphologic, rather than epigenetic, development, resulting in permanent changes in organ structure and adult metabolism (i.e., not all developmental plasticity can be explained by epigenetics) (Waterland and Michels 2007). Nevertheless, epigenetic mechanisms might underlie several examples of metabolic imprinting, as shown in the review (Barella et al. 2014).

Another factor affecting fetal growth, and one that is much less studied than the others, is a change in maternal metabolism that affects nutrient partitioning between the mother and conceptus. Although the fetal IGF system is well described, human genetic studies indicate that maternal genes account for threefold more variation in birth weight than fetal genes (Cross 2015). The mother undergoes major physiological changes during pregnancy and utrient delivery to the fetus is increased because of development of insulin resistance in the mother during pregnancy, in which her tissues require more insulin to take up glucose, leading to elevated post-meal blood levels, enhancing glucose nutrient delivery to the fetus (Torgersen and Curran 2006). The pregnant female adapts to insulin resistance by increasing insulin synthesis, and reducing the threshold for glucose-stimulated insulin secretion (Brelje et al. 1994; Weinhaus et al. 1996). Failure of these adaptations leads to gestational diabetes (Devlieger et al. 2008; Zhang et al. 2010), a higher risk for Type 2 diabetes later in life (Shaat and Groop 2007) and babies that are more likely to develop heart disease and diabetes as adults (Frías et al. 2007). Therefore, insufficient maternal β-cell adaptation affects both maternal and fetal health. Summary of effects of ewe nutrition on the body fatness and glucose tolerance of progeny during postnatal life is presented in Table 4.

Table 4. Summary of effects of ewe nutrition before and during pregnancy, and of placental insufficiency on the body fatness and glucose tolerance of progeny during postnatal life (Bell and Greenwood 2016)

Treatment	Birthweight	Relative fatness	Glucose tolerance
		Maternal undernutrition	
Periconceptional	↔	↑[A]	n.m.
	↔	n.m	↓
Early-mid pregnancy	↔	↑	↓
Late pregnancy	↔	↑	↓
	↓	↑	↓
Throughout pregnancy	↔	↔	↓
		Maternal overnutrition	
Periconceptual	↔	↔	n.m.
Late pregnancy	↔	↑	n.m.
Throughout pregnancy	↔	↑	↓
		Placental insufficiency	
Overfed adolescent ewe	↓	↔	↓
Carunculectomy	↓	n.m.	↓[A]
Large litter size	↓	↑	n.m

n.m., not measured; ↑, increase; ↓, decrease; ↔, no change; [A]Male offspring only

During periods of metabolic challenges such as NEB or overfeeding, insulin concentrations deviate from normal concentrations (around 0.2-0.6 ng/mL) and circulating levels are either decreased or elevated (Butler 2003; Graugnard et al. 2012). Insulin acts as a crucial metabolic signal in coupling the growth hormone, insulin-like growth factor (IGF) axis (Butler et al. 2003), which stimulates cell growth and proliferation. As the existence of insulin receptors has been confirmed in oocytes, cumulus cells (Purcell et al. 2012), and in embryos from the zygote to the blastocyst stage (Schultz et al. 1992), an influence of insulin on all these cell types and embryos can be expected. Extreme insulin levels could be detrimental for the developmental potential of the oocyte and the cause of poor pregnancy outcome (Leroy et al. 2008). This leads not only to economic losses for the farmer but may also impair the health status of live offspring later in life due to an unfavorable environment during early embryonic development. Interestingly, children born during a period of food deprivation (Roseboom et al. 2006) suffer later in life from the same diseases and metabolic disorders as children from obese mothers (Catalano and Ehrenberg 2006). Part of the numerous health defects reported may be due to the dysregulation of energy metabolism during oocyte maturation and early development. The metabolic programming induced by epigenetic changes due to an

environment with energy excess or deprivation during the periconception period can affect the offspring's health and body condition during their whole life (Patel and Srinivasan 2002). An interesting fact to mention is the so-called 'large offspring syndrome' (LOS), epigenetic effects causing deregulated overexpression of genes in the *H19/IGF2* region has been implicated in LOS (Gong et al. 2002). IGF2 is among the most important known embryonic growth factors, and its overexpression leads to overgrowth phenotypes, whereas lower than normal expression leads to suppressed growth and in the extreme case (Davies et al. 2002).

2.10 Nutrition in Reproduction

The effect of nutrition as a potential insult for the periconceptional environment of the gamete and young embryo, should be evaluated at three different levels: undernutrition, overnutrition and on the level of diet composition, meaning that the diet could be inadequate (excess or deficit) in terms of specific compounds like for example amino acids, sugars or even vitamins and/or minerals. Nutrient restriction is broadly defined as any series of events that reduce fetal and/or perinatal nutrient supply during critical windows of development. Basically, nutrient restriction can result from altered maternal nutrient supply, placental insufficiency, deranged metabolism and regulation, physiological extremes and environmental conditions. From a practical standpoint, maternal nutrient supply and environmental conditions leading to stress responses are the most likely observed causes of nutrient restriction in ruminant livestock.

2.10.1 Undernutrition

Schematic outline of some important factors linking maternal undernutrition and placental insufficiency to intrauterine growth retardation is presented in Figure 6. In modern dairy farming, undernutrition should be considered as a very rare phenomenon in developed countries, since animals that are to be inseminated (both nulliparous heifers as well as lactating multiparous cows), are generally fed according to their requirements. However, the cattle in developing countries suffer from undernutrition due to supply of less amount and lower quality feed. In extensive beef farms, undernutrition may still occur especially in specific seasons when animals are outdoors and the development of crops and grass is far below what should be expected. Therefore, in lactating dairy cows, undernutrition is mainly regarded as the incompetence to cope with the NEB during the immediate postpartum period. As mentioned earlier, the main challenge for the cows at that time is to optimize their dry matter intake in order to let the NEB not to become too deep nor to last exceptionally long. As outlined in earlier studies (LeBlanc 2010; Mulligan et al. 2006), mostly an inadequate

management is the main underlying reason why cows fail to handle the NEB and finally experience severe metabolic stress. All too often, the latter leads to subclinical metabolic disease like subclinical ketosis, or eventually even clinical ketosis and fatty liver.
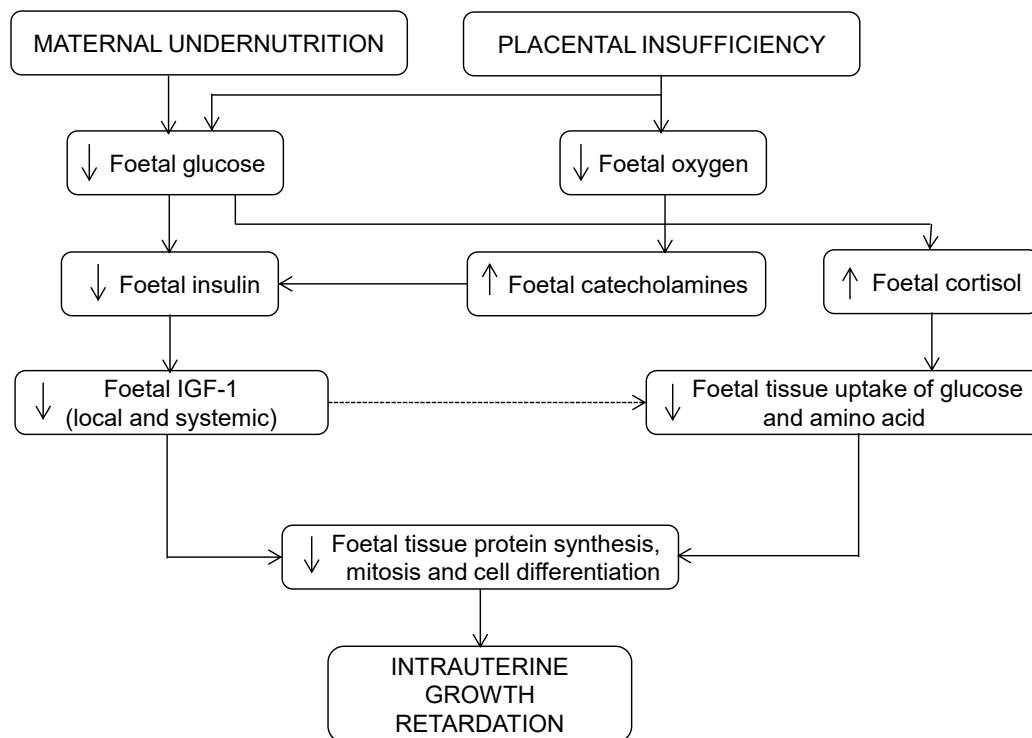

Figure 6. Schematic outline of some important factors linking maternal undernutrition and placental insufficiency to intrauterine growth retardation (Bell et al. 2003).

Undernutrition in the postpartum dairy cow should therefore be seen as insufficient dry matter intake leading to inabilities for the cows to cope with NEB. In terms of reproduction, the latter will first of all be accompanied by a retardation of resumption of normal ovarian cyclicity. Modern high yielding dairy cows that do experience NEB have been shown to resume ovarian activity significantly later in comparison to earlier studies in which the NEB challenge for the cows was lower. Furthermore, significantly more ovarian disturbances have been demonstrated in modern high yielding dairy cows (Opsomer et al. 1998; Opsomer et al. 2000). Besides, also the expression of heat symptoms has been shown to be significantly lower in those cows which necessitates farmers often to inseminate cows based on secondary heat symptoms which is known to be associated with lower pregnancy results.

Most dairy cows develop the first dominant follicle postpartum within approximately 2 weeks after calving, but only about 40% of these follicles produce sufficient estradiol to stimulate ovulation despite having normal ultrasound appearance and growth (Cheong et al. 2015). The

mechanism leading to a correctly timed ovulation of a fertile oocyte is based on a well-orchestrated crosstalk within the hypothalamic-pituitary-ovarian axis (Cheong et al. 2015). Nutritional and metabolic effects on the central nervous system are well researched and reasonably clarified. In dairy cows selected for a high level of milk production, peripheral levels of glucose, insulin and IGF-1 are known to be substantially reduced. Lower peripheral insulin levels have been associated with non-ovulation of the dominant follicle, finally giving rise to cystic ovarian disease (Vanholder et al. 2005a). Underlying reason has shown to be a compromised theca cell function, finally leading to estradiol levels inadequate to provoke an ovulatory LH-peak and hence ovulation. The reason for the compromised theca cell function has been suggested to be related to the elevated levels of non-esterified fatty acids concomitant with overall fat mobilization (Vanholder et al. 2005b; Vanholder et al. 2006), although not all authors sustain this hypothesis. Overall, it is recently concluded that cows that fail to ovulate the first postpartum dominant follicle are characterized by lower periparturient energy balance, increased insulin resistance, lower LH pulsatility and lower intrafollicular concentrations of androstenedione and estradiol (Cheong et al. 2015).

Undernutrition or more particularly for the dairy cow, insufficient dry matter intake and thus a more extensive NEB may cause adverse changes of metabolites in the ovarian follicular fluid giving rise to concomitantly lower oocyte quality and hence lower fertilization and blastocyst rates as outlined higher. The latter are generally recognized as being major causes for the high range of (early) embryonic mortality in dairy cattle. Undernutrition of the recently inseminated cow may appear to be unimportant because of the limited nutrient requirements of the early embryo and fetus for growth and development and this during the complete first half of gestation. However, it is during the early phase of fetal development that maximal placental development and growth, differentiation and vascularization occurs, as well as fetal organogenesis, all of which being critical events for normal conceptus development.

2.10.2 Overnutrition

Excessive energy intake particularly from high carbohydrate diets can reduce fertilization and embryo quality in some but not all circumstances. The latter has been shown to be related with increased circulating insulin levels during the final week of follicle growth, although the underlying mechanisms are still not clear (Wiltbank et al. 2014). High protein diets have been found to reduce embryo quality by day 7 after breeding, possibly due to elevated levels of circulating urea although the latter has not been sustained by all authors. Adamiak et al.

(2005) demonstrated that high feeding levels were beneficial to nulliparous heifers in low body condition, but detrimental to oocytes from animals of moderately high body condition. Also here, elevated levels of insulin were probably the underlying cause for this negative influence. Later, these findings were confirmed by feeding heifers diets high in starch, although they were able to avoid the adverse effects on oocyte quality when leucine intake was increased (Rooke et al. 2009). Increasing dietary leucine increases plasma glucagon rather than insulin concentration and thus the positive effect of dietary leucine on embryo development observed when high starch diets were fed appears to be related to reductions in the insulin to glucagon ratio (Rooke et al. 2009).

2.10.3 Composition of the Diet

A number of reviews have highlighted the importance of nutrition in regulating bovine reproductive efficiency (Cardoso et al. 2013; Grummer et al. 2010; Santos et al. 2010) with focus on the effects of energy and protein nutrition in the dam on bovine preimplantation embryo development (Wiltbank et al. 2014). First, effects during the early postpartum period have been postulated to alter the oocyte and subsequent embryo development after fertilization of a perturbed oocyte (Britt 1992). Second, changes in circulating factors such as insulin, glucose, urea, or amino acids during the final stages of oocyte development, prior to ovulation, can profoundly impact fertilization or embryo development (Adamiak et al. 2005; Adamiak et al. 2006; Bender et al. 2014). A third obvious target of nutrition on the embryo is, during the first week of embryo development when changes in oviductal and uterine environment could alter development of the embryo to the blastocyst stage (Steeves and Gardner 1999, 1999b; Steeves et al. 1999). Finally, changes in circulating energy sources, such as glucose and propionate, and building blocks for cells, such as amino acids, could alter the uterine lumen and subsequently alter hatching and embryo elongation. The elongating embryo secretes the protein interferon-tau that is essential for recognition of pregnancy and rescue of the corpus luteum and can alter expression of specific proteins, such as amino acid transporters in endometrial epithelial cells, and thus alter the concentrations of many substances in the uterine lumen (Gao et al. 2009; Groebner et al. 2011; Hugentobler et al. 2010). Complete characterization of all of these nutritional effects on reproduction is likely to be impossible; however some of these aspects have been recently reviewed (Leroy et al. 2014; Santos et al. 2008; Velazquez 2011). Furthermore, application of diets specifically designed to improve fertility by counteracting mechanisms related to the NEB or by supporting a

specific pathway that is necessary for successful fertility, has always been a very attractive way to circumvent the impairment of reproduction during early lactation.

Glucogenic Diets. Although the reproductive system is known to be influenced by multiple hormones that are also involved in the adaptation towards high milk production (like GH, IGF-1 and leptin), only insulin is known to be relatively sensitive to the composition of the ration. Ovarian follicles have been shown to bear insulin receptors (Bossaert et al. 2010) and cows with lower peripheral insulin levels in the immediate postpartum period have been demonstrated to suffer from retarded postpartal ovarian resumption and normal cyclicity among others by a higher risk to suffer from cystic ovarian disease (Vanholder et al. 2005a). Therefore, glucogenic diets have been advocated in the immediate postpartum period aiming to enhance the peripheral insulin concentrations and advance normal ovarian resumption (Gong et al. 2002). However, insulin has been shown to have detrimental effects on oocyte and embryo competence (Fouladi-Nashta et al. 2005) and has been shown to stimulate enzymatic catabolism of progesterone in the liver (Lemley et al. 2008). The latter suggests glucogenic diets only being of advantage when offered in the immediate postpartum period, while to be avoided when cows are inseminated.

Proteins. Rations leading to high peripheral urea levels are generally mentioned to be associated with lower pregnancy rates due to its detrimental effects on the embryo. However, the mechanistic pathways by which this detrimental effect may be caused and the threshold peripheral urea level, are both still matters of debate. Special attention in this respect should furthermore been given to the supplementation of soybean meal as the main protein source in the ration. In a recent study (Cools et al. 2014), it is demonstrated that commercially available soybean meal contains isoflavones in concentrations that are able to induce increases in the blood concentration of estrogenically active isoflavone metabolites (equol, O-desmethylangolensin, dihydrodaidzein) in high yielding dairy cows postpartum, even when supplemented in relatively low amounts (1.72 kg per day on average). When compared with rapeseed meal, soy supplementation was furthermore associated with a decreased angio- and steroidogenesis at the level of the corpus luteum based on biopsy sampling at day 9 of the estrous cycle (Cools et al. 2014). Therefore, although the results of that study suggest negative effects of soy feeding on CL function in recently calved dairy cows, the contribution of this effect on the peripheral progesterone concentration and consequently on overall fertility of supplemented cows warrants further research.

Fats. Addition of fats in feeds has been another strategy that has been extensively tested to reduce the impaired reproductive capacity of dairy cows. However, limiting energy output via milk by supplementing the ration with exogenous fats are not successful since cows simply produced more milk when reducing the NEB (Hostens et al. 2011). Omega-6 fatty acids are believed to have pro-inflammatory and thus PGF2α-stimulating properties rendering them of extra value early postpartum, while omega-3 fatty acids can weaken this inflammatory potency, leading to a higher chance of survival of the embryo when supplemented during the periconceptional period. Unfortunately, research results rarely provide a consensus in this perspective (Jahanian et al. 2013; Otto et al. 2014). Fat feeding may alter the micro-environment of the growing and maturing oocyte of the early and older embryo and thus may affect reproductive outcome. Research has shown that dietary-induced hyperlipidaemic conditions can be harmful for embryo development and metabolism. However, to date, research results remain somewhat conflicting most probably due to differences in fat sources used, in diet and duration of supplementation and in experimental set-up in general. Furthermore, peripheral blood in lactating dairy cows would contain a mixture of fatty acids of dietary origin and from body-tissue breakdown, the latter being largely abundant in the immediate postpartum period and containing a high proportion of saturated fatty acids. Especially the latter have been shown to have a significantly detrimental effect on both the oocyte as well as embryo quality (Leroy et al. 2005).

A relatively new approach is to implement relatively short-term changes in the quantity or composition of the diet at key stages in the reproductive process. Therefore, the term focus-feeding, which refers to implementing short periods of nutritional supplements that are precisely timed and specifically designed to ameliorate the reproductive process including embryonic and fetal growth and development, has been introduced (Martin and Kadokawa 2006). In this context, possibilities to supplement rumen protected fats (sometimes called C16 fatty acids or rumen-bypass fats) in the ration of dairy cows were recently discussed (Wiltbank et al. 2014). These fats passes through the rumen without affecting fermentation, but can still be digested in the cow's intestine. Of special interest herein is the supplementation of methionine since this is a rate limiting amino acid for milk production and is known to be a methyl donor which may potentially affect the DNA-methylation and hence the expression level of genes. Apparently methionine supplementation did not affect embryo development and quality, but it did affect gene expression levels. More research is however warranted to further decipher the effect of supplementation of amino acids both during oocyte

maturation as well as during early embryo development on gene expression level and hence potential effects on health in later life.

Vitamins and minerals. Supplementation of extra vitamins and minerals to the diet has often been suggested by concentrate companies as a golden bullet solution to reduce the fertility decline. Usually farmers are highly sensitive to this kind of advice since it doesn't involve extra labor which is their paramount constraint nowadays. Evaluating whether the amount of these compounds is sufficient in the ration is often very difficult for the practitioner since it is usually impossible to even estimate the content of these substances present in the basic ration. In herds in which cows are given a high amount of concentrates to sustain peak yield in the immediate postpartum period, the risk of suffering from such deficiencies may be lower due to the fact that concentrates are usually highly supplemented with vitamins and minerals. Special attention should be given to Vitamin E and Selenium in terms of their effect on immune response and embryo quality (Bayril et al. 2015; Pontes et al. 2015).

2.11 Heat Stress in Reproduction and Programming

Heat stress is known to affect many components of the reproductive system including gonadotrophin profiles, follicular growth, granulosa cell function, steroidogenesis and oocyte and embryo quality (Roth 2008). As a result, seasonal infertility is prevalent in cattle herd in tropical and subtropical regions. Embryo development is sensitive to the transient increases in body temperature arising as a consequence of elevated environmental temperature. Especially in dairy cows, increasing milk yields are known to challenge the cows even more to regulate their body temperature during warm weather further exacerbating the deleterious effects on fertility. Internal heat production increases at higher feed intakes and milk production, which is why high-producing cows are more sensitive to heat stress than lower-producing cows. Interestingly, observations of impaired fertility of dairy cattle in the autumn subsequent to a hot summer have been reported (Kadzere et al. 2002; Sonmez et al. 2005; Wakay et al. 2015). It seems that heat stress not only affects antral follicles emerging in the follicular wave, but probably also affects the ovarian pool of small antral follicles resulting in a carry-over effect on follicular function and oocyte developmental competence.

Maternal heat stress during the dry period affects metabolism of the offspring. Calves born to heat-stressed dry cows have lower insulin concentration compared to those from cooled cows (Guo et al. 2016). Such metabolic alteration in calves persists until the preweaning period (Tao et al. 2014; Monteiro et al. 2016) and has been reduced heifer milk production during the

first lactation (Monteiro et al. 2013). Similar effects are also observed in sheep where intrauterine growth restriction caused by maternal heat stress during early to mid-gestation resulted in compromised insulin synthesis and secretion in the lambs compared with those that develop under thermoneutrality (Limesand et al. 2006). In contrast, heifers born to dry period heat-stressed cows have similar basal plasma insulin concentration and pancreatic insulin sensitivity to glucose, but lower peripheral tissue insulin sensitivity during the preweaning period compared with those from cooled cows (Monteiro et al. 2016). Moreover, it is observed that the calves born to heat-stressed dry cows had a lower plasma concentration of cortisol immediately after birth compared with calves born to cooled cows, indicating that maternal heat stress alters the fetal development of hypothalamus-pituitary-adrenal axis and related stress responses during the postnatal period (Tao et al. 2012). The metabolism of an animal is of importance in growth and body composition, which in turn alters her future productive and reproductive performance. Therefore, it is important to examine metabolic adaptation of heat-stressed calves during the transition from intra- to extrauterine life, postnatal metabolic responses to stress, and the effect on the calf's future performance.

2.12 Health Problems and Inflammatory Reactions

Despite the very orchestrated homeostatic controls and homeorhetic adjustments to adapt to the changes in the intermediary metabolism associated with the establishment of milk production, 40 to 70% of dairy cows across different levels of milk production, breeds, and management systems, develop metabolic or infectious diseases in the immediate postpartum period (Dobson et al. 2007; Ribeiro et al. 2013). These health problems not only cause reductions in milk production and animal well-being, but are furthermore seen as an important contributor to the general complaint of the lowered reproductive capacity of modern dairy cows. Indeed, there is evidence that the calving-to-pregnancy interval is extended for at least 7, 8, 26 and 31 days in cows treated for mastitis, retained fetal membranes, hypocalcaemia or endometritis, respectively, compared with healthy herd-mates. Lameness is associated with even worse reproduction performance, as up to 40 days can be lost to get lame cows in-calf again even though the lameness has been treated (Dobson et al. 2007). In part, these poor fertility data may be related to delayed resumption of ovarian cyclicity after calving and on a lowered expression of heat symptoms. On the other hand, some events seem to have more long-lasting effects. Signs of dystocia, or immediate postpartum hypocalcaemia, endometritis or mastitis can be 'cured' within days by clinical treatment but the cows are subfertile many weeks later during the breeding period. Obviously, inflammatory diseases taking place in the

first weeks of lactation are associated with a reduced fertilization of cows inseminated between 50 and 60 days postpartum.

In a recent study (Ribeiro et al. 2013), it is shown that the carryover effects of disease on reproduction of dairy cows cannot be explained simply by the nutritional status and its consequences to body condition score and oestrous cyclicity at the onset of breeding postpartum. The inflammatory mediators produced by the injured or infected tissues can also reach the reproductive tract including ovaries and uterus, but also the brain, which ultimately affects the physiological processes that control normal reproductive cyclicity. For example, cows that suffered from uterine disease postpartum had delayed growth of the first dominant follicle postpartum and reduced concentrations of estradiol (Sheldon et al. 2002). Presence of lipopolysaccharides in the follicular fluid of cows with uterine diseases has been postulated as a potential reason for compromised steroidogenesis, follicle growth and impaired oocyte developmental competence (Bromfield et al. 2015).

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CHAPTER 3

AIMS OF THE STUDY

Aims of the Study

Optimal animal health is the first step towards high milk production and profitability of a dairy herd. Several studies have indicated that the health of an individual is not only determined by the actual environment at a certain moment in life but also a reflection of the environment to which that individual has been exposed in the past (Finer et al. 2016; Sferruzzi-Perri et al. 2017; Smits et al. 2012). Therefore, farmers need to be aware that the basis for a healthy, productive and fertile life of a dairy cow is initiated very early, even before its birth. This process in which prenatal and early postnatal events are affecting later health and longevity, is called "early life programming" (Eriksson JG. 2016; Marciniak et al. 2017) and was proposed for the first time in 1989 by the British professor Dr. Barker and has since then been defined as the "Barker hypothesis" (Barker 2001; Hales and Barker 2001). This hypothesis also postulates that the achievements of an individual (such as the level of milk production) are not only the result of genetic selection and the present environment, but are also determined by the environment to which the individual has been exposed to as a fetus, including the nutritional factors that have been experienced by its mother during pregnancy.

The conflict in nutrient allocation between mother and offspring during pregnancy is especially intriguing in modern dairy cattle. The gestation in heifers takes place when they are still growing. The cows in early lactation are confronted with a substantial negative energy balance in response to the rapidly increasing milk production. During this metabolically challenging period, the farmer wants his cows to become pregnant again, which means that the oocyte and the young fetus are 'victims' of this suboptimal physiological status while being *in utero*. For our research, we hypothesized that young age in heifers and high milk production during gestation in cows affect the intrauterine feto-placental development and subsequent organ function of the calf.

Knowledge about the effect of these environmental factors in the uterus during conception and pregnancy on the health and production on the long term can lead to practical advice for dairy cattle farmers. Therefore, the aims of this PhD thesis were to investigate the effects of young age in pregnant heifers and of high milk production during gestation in cows on the feto-placental development and subsequent organ function of the calf.

In order to obtain these specific aims, this PhD thesis investigated the following research objectives:

1. To evaluate environmental and dam factors, including age at calving in heifers and level of milk production during gestation in cows that might be associated with birth size in Holstein calves.

2. To study the impact of maternal factors like age at conception and level of milk production during gestation on gross placental morphology at parturition in dairy cattle.

3. To evaluate potential associations between environmental factors and dam characteristics and major insulin traits in newborn Holstein calves.

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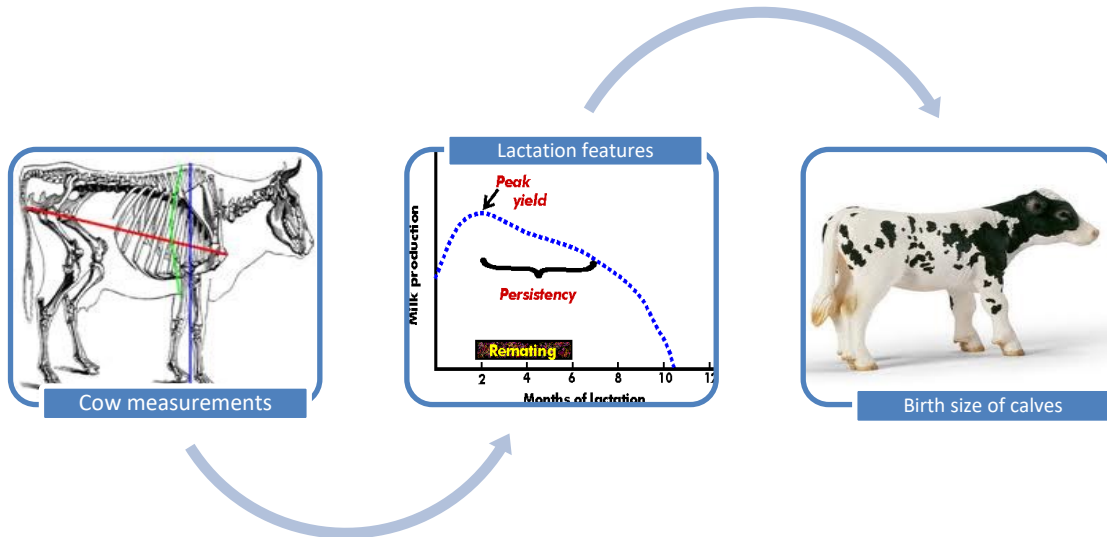
FACTORS INFLUENCING BIRTH SIZE OF HOLSTEIN CALVES

Abstract

The objective of the present study was to evaluate environmental and dam factors associated with birth size of Holstein calves. Data of 1,594 births from dairy herds in Belgium and Germany were analyzed in a retrospective cross-sectional study. Immediately after birth, the birth weight (BW) of the calves was measured. On the next day, the heart girth (HG), wither height and diagonal length of the dams and calves were measured. Parity, body condition score, gestation length (GL) and age at calving were recorded for all dams. For the cows, days open, lactation length, length of the dry period (DP) and calving interval were also calculated. The magnitude and shape of the lactation that took place during gestation was quantified using the MilkBot model based on monthly milk weights. Using the same procedure, cumulative milk production from conception to drying off (MGEST) was calculated. After descriptive analyses, mixed models were used to identify factors that are significantly associated with the BW (most consistent measure of size at birth) of the calves born to both heifers and cows. Of the variables offered to the offspring BW model in heifers (n = 540), calf gender, season of calving, GL, HG, wither height, diagonal length and age at calving were significant. The mean BW of the calves born to heifers was estimated to be 41.3 ± 1.01 kg. In comparison to calves born to old (25.5 to 37.3 mo; n = 99) heifers, the BW was estimated to be respectively 2.75, 3.29 and 2.35 kg heavier when the calves were born to very young (20.3 to < 22 mo; n = 98), young (22 to < 23.5 mo; n = 145) and standard aged (23.5 to < 25.5 mo; n = 198) heifers. Of the variables offered to the offspring BW model in cows (n = 1,054), calf gender, season of calving, GL, parity, DP and MGEST were significant. The mean BW of the calves born to cows was estimated to be 44.1 ± 0.99 kg. For cows having an identical HG, the BW of the calves was estimated to be respectively 0.97 and 1.11 kg higher in cows with low (1,400 to < 5,400 kg) and high (6,500 to < 7,200) MGEST in comparison to cows with very high (7,200 to 11,600 kg) MGEST. The decisive effects of age at calving in heifers and of high milk production levels during gestation in cows, on the BW of their calves, may provide a basis for developing managerial interventions to improve long-term health and productivity of the offspring.

Key words: birth size, risk factors, heifer age, milk production

4.1 Introduction

Size at birth is important for calving ease of the dams and neonatal survival of the calves. Large calves are associated with calving difficulties (Johanson and Berger 2003), whereas small calves are more susceptible to neonatal mortality (McCorquodale et al. 2013). Epidemiological evidence suggests that small size at birth both in humans and animals is associated with increased predisposition to metabolic diseases during adult life (Symonds et al. 2010; Vuguin 2007). It should be noted that birth size is a complex trait influenced by the interaction between genetic and non-genetic intrauterine factors (Kocak et al. 2007). However, the intrauterine environment has been stated to be a more important regulator of birth size than the parental genome (Sharma et al. 2012; Swali and Wathes 2006). In the current dairy industry, most heifers are bred whilst still growing and likely most subsequent gestations are carried during lactation. The heifers' age and cows' level of milk production during gestation have both been suggested to be important contributors to the nutritional environment for the developing embryo and fetus (Berry et al. 2008; Brickell et al. 2009; Funston and Summers 2013), since available nutrients need to be partitioned between the growth (in heifers) and milk production (in cows) of the dam and the intrauterine growth of the offspring. We hypothesize that young age in heifers and high milk production during gestation in cows affect the intrauterine fetal development and subsequent birth size of the calf.

The use and interpretation of birth size in relation to fetal programming assumes that birth size indeed reflects specific fetal responses to particular variations in the nutritional status of the dam. Since epigenetic adaptations in the embryo may influence future health and fertility (Van Soom et al. 2013; Wu et al. 2006), the intrauterine environment is currently being assessed with regard to health and welfare of the offspring (Rutherford et al. 2012). The association of young age in heifers and high milk production during gestation in cows with reduced longevity and productivity of their offspring (Banos et al. 2007; Berry et al. 2008; Gonzalez-Recio et al. 2012) furthermore emphasizes their potential impact on birth size of the calves. Moreover, recent publications indicate that the prevalence of perinatal mortality has increased in some dairy industries whereas an increased proportion of this loss is not associated with the long-established risk factors for perinatal mortality (Mee et al. 2008). Hence, there is some evidence to suggest that an increasing proportion of perinatal mortality occurs at unassisted calvings (idiopathic stillbirth or weak calf syndrome) where placental dysfunction and low birth weight may be causative factors (Berglund et al. 2003;

Kornmatitsuk et al. 2004). Although the underlying causes of this rather new phenomenon are not yet fully elucidated, managerial factors typically associated with modern dairy husbandry like young age at first calving and a high level of milk production during gestation, are often referred to as potential candidates.

Numerous studies report on the effect of environment and nutrition (Kocak et al. 2007; Symonds et al. 2010; Zhang et al. 2002) and multiple dam morphometrics (Kertz et al. 1997; Lundborg et al. 2003; Swali and Wathes 2006) on the birth size of their calves, but information on factors inherent to the dam, such as age at calving in heifers and milk production during gestation in cows has, to the best of our knowledge yet to be adequately documented. In studies examining the effect of the level of milk production on size and well-being of the neonatal calf, authors used the amount of milk produced during the whole lactation or during the 305 d of lactation (Berry et al. 2008; Swali and Wathes 2006) and not the amount produced during gestation or during specific moments in gestation when the overall growth of the fetal calf is of major importance. Furthermore, despite the multifactorial nature of fetal growth in Holsteins, the current literature is lacking in research where a multifactorial approach has been used to identify the key risk factors associated with birth size. The objective of the present study was to evaluate environmental and dam factors, including age at calving in heifers and level of milk production during gestation in cows that might be associated with birth size in Holstein calves.

4.2 Materials and Methods

4.2.1 Farms, Animals and Management

Data were collected from four small dairy herds (on average 70 lactating cows) in Flanders (Belgium) and one large herd (> 2000 lactating cows) in Rostock (Germany). Herds were selected based on their long history of successful collaboration with the Ghent University Ambulatory Clinic. All herds participated in an official milk recording system and artificial insemination program and in a veterinary herd health program to closely monitor both health and productivity. In all herds, only Holstein cows were milked and average 305 d milk production was > 9,400 kg. The heifers and cows were housed in free-stall barns. They were fed according to their requirements for maintenance and growth (heifers) and production (cows) based on the results of the monthly production tests. Generally, the rations included high quality roughages (maize silage, grass silage, sugar beet pulp and fodder beets) and sometimes supplemented with concentrates. The cows were generally milked twice a day;

whereas in Germany some high producing cows were milked 3 times. Heat detection was performed by the herdsmen and their employees at least three times daily at regular time intervals. In some herds aids were used to optimize heat detection. Observed heats, as well as all other observations concerning health and fertility were carefully noted on a herd health chart or were put in a herd health computer software program. Heifers were generally inseminated at an age of 15 mo whereas the cows were generally inseminated at the first estrus occurring after 50 d postpartum. Heifers and cows approaching parturition were separated in a maternity pen and were closely monitored by the farm employees. After calving, the calves were immediately removed from the maternity pen into an individual calf pen. Calves were fed 4 L of colostrum within the first 10 h after birth by esophageal tube.

4.2.2 Measurements and Data Collection

A retrospective cross-sectional study design was used. The outline of the measurements and data collection is shown in Figure 7. Heifers and cows were enrolled upon calving between August 2011 and April 2013. During the study period, some of the cows calved a second time and were therefore enrolled twice. Calves born following inseminations with semen from non-Holstein bulls (n = 84), born dead or failed to survive during the first 24 h (n = 22), born following an abnormally short (< 265 d, n = 5) or long (> 295 d, n = 2) gestation length as well as twin calves (n = 66), were all excluded from further analyses. Calving ease was scored on a categorical scale: 0 = non-assisted, 1 = easy farmer assistance, 2 = difficult farmer assistance and 3 = veterinary assistance including caesareans. Body condition score (**BCS**) of the dams was determined on a 1 to 5 scale (1 = emaciated, 5 = obese) with 0.25 increments (Edmonson et al. 1989). The birth date of the dam and the date of calving were recorded. The gender of the calves and the identification of their sires were also carefully recorded. Immediately after birth, the birth weight (**BW**) of the calves was measured in kilograms. On the next day, the heart girth (**HG**), wither height (**WH**) and diagonal length (**DL**) of both dams and calves were measured in centimeters. The HG was measured with a plastic-coated fiber tape as the minimal circumference around the body immediately behind the elbows. The WH and DL were measured with specially designed metal calipers on the left side while animals were in a standing position. The WH was defined as the distance from the floor to the top of the withers directly above the center of the shoulder, whereas the DL was defined as the distance from the leading edge of the *tuberculum majus humeri* to the medial border of the *tuber ischiadicum*. The previous calving dates, the dates of conception, the monthly milk weights of the lactation that started at the calving preceding the one that was included in the

study, and the dry off dates were extracted from the herd databases. Since the sire breeding value in terms of offspring BW was not available for all bulls, the breeding value for calving ease was collected and used to estimate the sire effect.
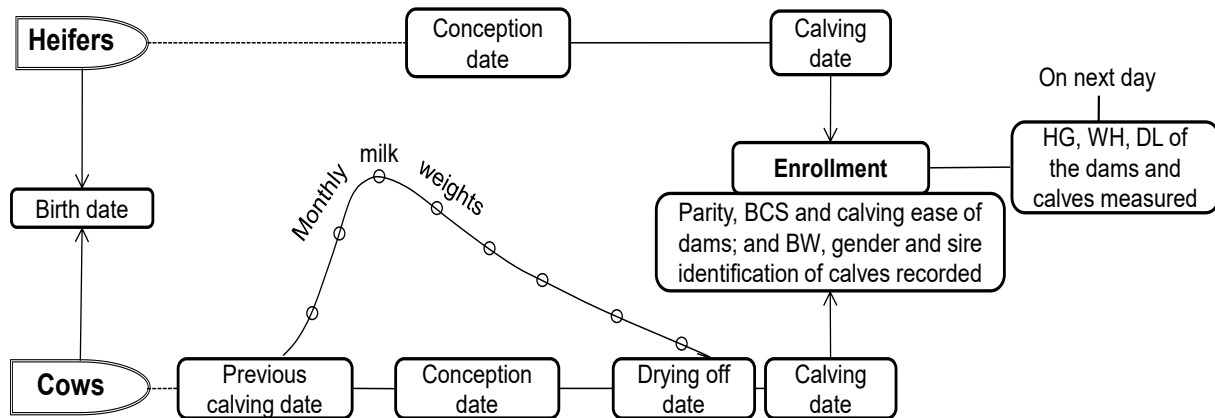

Figure 7. Outline of the measurements and data collection. BCS, body condition score; BW, birth weight; HG, heart girth; WH, wither height; DL, diagonal length.

4.2.3 Calculations and Estimations

The body condition index (**BCI**) of the calves was calculated as BW/(WH*DL). In humans a similar measure (body mass index (BMI), weight/height^2) incorporates standing height, i.e. the length of the spine and long bones, whereas in quadrupeds the length of the spine is not included in height. There were 540 heifer calving records in which the average age was 24.0 ± 2.18 mo. Based on the distribution, age at calving was divided into 4 classes: 20.3 to < 22 mo (very young), 22 to < 23.5 mo (young), 23.5 to < 25.5 mo (standard) and 25.5 to 37.3 mo (old). The gestation length (**GL**) was derived both in heifers and cows from the last recorded insemination and calving date and restricted to 265 to 295 d as mentioned above. Days open, lactation length, dry period (**DP**) and calving interval in the cows were calculated. The season of calving was grouped into two: Summer and Fall (21 June to 20 December) and Winter and Spring (21 December to 20 June). Monthly milk weights were fitted to the MilkBot model (Ehrlich 2011), which can be functionally expressed as

$Y(t) = a(1 - \frac{e^{\frac{c-t}{b}}}{2})e^{-dt}$. This approach was used to summarize the magnitude and shape of each lactation curve, where $Y(t)$ is total daily milk production on day t of the lactation, and parameters a, b, c, and d control the shape of the curve. Specifically, lactation scale is measured by the a parameter in the MilkBot function. It is a simple linear scale with equal

influence at all stages of lactation. The parameter *b*, called the ramp parameter, measures the steepness of the postparturient increase in production, so it is most influenced by changes in early lactation. Higher ramp values correspond to a slower increase in production. The offset parameter, *c*, is the theoretical offset between parturition and the physiological start of lactation. Normal variability in the offset parameter is expected to be small and practically undetectable without daily milk weights in the first days of lactation, thus the offset value was fixed to 0 in the present study. Finally, the decay parameter, *d*, relates to senescence and loss of productive capacity and is influenced by cumulative changes in productive capacity occurring throughout the lactation. As a first-order rate constant, decay can be expressed as half-life, called persistence, corresponding approximately to the time in days for production to decrease by half in late lactation (Ehrlich 2011). This methodology allows scale, ramp, and persistence of individual lactations to be treated as independent variables in statistical models, along with the derived variables cumulative 305 d milk production (**M305**), cumulative milk production during gestation from conception to drying off (**MGEST**), milk production at lactation peak (**MPEAK**), cumulative milk production from 15 d before to 15 d after conception (**MPCONC**), time to peak milk, time to half of the peak milk, which were all easily calculated directly from MilkBot parameter values (Ehrlich, 2011). The MGEST was further divided into 4 classes: 1,400 to < 5,400 kg (low), 5,400 to < 6,500 kg (intermediate), 6,500 to < 7,200 kg (high) and 7,200 to 11,600 kg (very high).

4.2.4 Statistical Analyses

All statistical analyses were performed using the SAS Enterprise Guide (version 5.1; SAS Institute Inc, Cary, NC). In the descriptions given, including Table 5, original data are displayed as means ± standard deviation, whereas model-based estimates are listed as ± standard error. Pearson correlation coefficients (r) were estimated to describe relationships between variables using the PROC CORR procedure. After descriptive analysis, the data were analyzed using a linear mixed model in PROC MIXED. Separate models for heifers and cows were built to identify the significant factors of BW of the calves. The model equation was y = Xβ + Zγ + ε; where y represents univariate data, β is an unknown vector of fixed effects with known model matrix X, γ is an unknown vector of random effects with known model matrix Z, and ε is an unknown random error vector. The 'variance explained' (R^2) by the models was expressed as the ratio between the residual variance of the model of interest and the residual variance of the null model.

The heifer model included fixed effects of calf gender, season of calving, GL, HG, WH, DL, BCS and age at calving. The herds were specified as random effect term. For cows, the model included fixed effects of calf gender, season of calving, GL, HG, WH, DL, BCS, parity/age at calving, days open, lactation length, calving interval, Ramp, Persistence, time to peak milk, time to half of the peak milk, MGEST and DP. In the model for cows, herds were included as random effect, and second-time measurements on the same cows were accounted for by the repeated statement. All fixed effects and their two-way interactions were included in the initial model, but removed if found non-significant (P > 0.05), after which the model was refitted. The Scale, M305, MPEAK and MPCONC were not included in the initial model because of their high correlation (> 60%) with MGEST. Separate similar models were built with these variables by replacing MGEST (data not shown). Both in heifers and cows, the inclusion of sire breeding values for calving ease did not significantly improve the model and therefore were excluded. Data of the final models are reported as model least squares means unless indicated otherwise.

4.3 RESULTS

4.3.1 Descriptions

The characteristics of the dams and calves are presented in Table 5. The overall average BW of the calves was 43.6 ± 5.78 kg, the calves born to heifers (n = 540) being 4.6 kg lighter in comparison to the ones delivered by the cows (n = 1,054) (40.6 ± 4.90 kg versus 45.2 ± 5.58 kg, respectively). The calves born to the heifers were significantly smaller in HG, WH, DL and BCI in comparison to the ones born to the cows (P < 0.001). The BW had the greatest correlation with HG (r = 0.75, P < 0.001) and BCI (r = 0.71, P < 0.001) of the calves. The BW of both male and female calves increased with parity of the dams, and experienced a decrease from the fourth parity (Figure 8). The level of calving assistance increased with increased BW of both male and female calves (P < 0.001). The sire breeding value for calving ease was negatively correlated (r = - 0.22, P < 0.001) with BW of the calves. The heifers had a 1.9 d shorter (278.1 ± 4.36 versus 280.0 ± 4.66) GL than the cows (P < 0.001). The male calves were carried on average 1.3 d longer (280.0 ± 4.69 versus 278.7 ± 4.51) than the female calves (P < 0.001).

Table 5. The characteristics of the dams and their calves (mean ± SD)

Outcomes	Heifers (n = 540)	Cows (n = 1,054)
Dams		
Heart girth (cm)	201.1 ± 6.82[a]	214.0 ± 9.52[b]
Wither height (cm)	140.0 ± 4.39[a]	144.5 ± 5.45[b]
Diagonal length (cm)	159.4 ± 6.33[a]	170.3 ± 7.15[b]
Age at calving (mo)	24.0 ± 2.18[a]	51.6 ± 16.87[b]
Gestation length (d)	278.1 ± 4.36[a]	280.0 ± 4.66[b]
Scale (kg)	- - -	45.7 ± 10.43
Ramp (d)	- - -	24.4 ± 7.85
Persistence (d)	- - -	375.3 ± 241.91
M305 (kg)	- - -	9,409.1 ± 1,639.15
MGEST (kg)	- - -	6,193.1 ± 1,352.79
MPEAK (kg)	- - -	38.6 ± 8.32
MPCONC (kg)	- - -	1,045.1 ± 222.65
Days open (d)	- - -	108.7 ± 55.53
Lactation length (d)	- - -	332.4 ± 51.25
Dry period (d)	- - -	56.2 ± 21.12
Calves		
Birth weight (kg)	40.6 ± 4.90[a]	45.2 ± 5.58[b]
Heart girth (cm)	79.2 ± 3.63[a]	81.9 ± 3.60[b]
Wither height (cm)	74.6 ± 3.70[a]	76.2 ± 3.66[b]
Diagonal length (cm)	68.6 ± 3.99[a]	70.9 ± 3.92[b]
Body condition index	79.3 ± 7.60[a]	83.5 ± 8.63[b]

[a-b]Values in the same row with different superscripts are significantly different (all at $P < 0.001$). M305, cumulative 305 d milk production; MGEST, cumulative milk production during gestation from conception to drying off; MPEAK, milk production at lactation peak; MPCONC, cumulative milk production from 15 d before to 15 d after conception; body condition index: birth weight/(wither height*diagonal length).
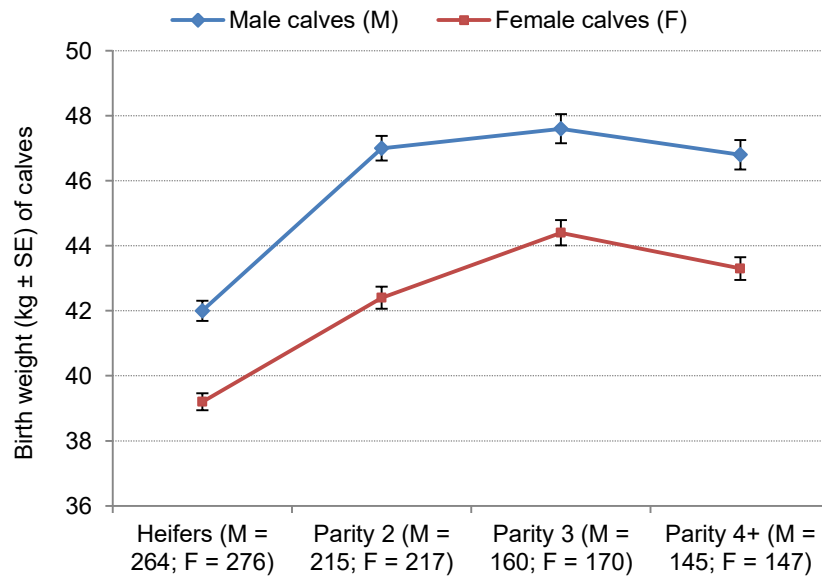
Figure 8. Birth weight of the calves in relation to their gender and parity of their dam.

4.3.2 Factors Influencing Offspring BW in Heifers

Of the variables offered to the offspring BW model in heifers, calf gender, season of calving, GL, HG, WH, DL and age at calving were significant (Table 6). The proposed model explains 29.5% of the variation in BW of the calves born to heifers. The mean BW of the calves born to heifers was estimated to be 41.3 ± 1.01 kg. Male calves were estimated to have a 2.57 kg higher BW than female calves, and the calves born in Summer and Fall were estimated to be 2.23 kg lighter than the calves born in Winter and Spring (P < 0.001). Calves born following a short (265 - 275 d) and medium (276 - 285 d) GL were estimated to be 5.01 and 2.18 kg lighter, respectively, in comparison to the calves born following a long (286 - 295 d) GL (P < 0.001). The BW of the calves was estimated to increase by 0.52, 0.54 and 0.73 kg with each centimeter increase in HG, WH and DL of the heifers at calving, respectively (P < 0.01). The BW was estimated to be significantly lower in the heifers that calved at an old age (25.5 to 37.3 mo) (P < 0.001). The calves born to very young (20.3 to < 22 mo) and standard aged (23.5 to < 25.5 mo) heifers were estimated to be lighter in comparison to calves born to young (22 to < 23.5 mo) heifers (Table 6).

Table 6. Significant predictors of birth weight (kg) of calves born to heifers

Predictors	Comparison	No.	Estimate (kg)	P-value
Intercept		540	41.3	< 0.001
Calf gender	Male	264	2.57	< 0.001
	Female	276	Referent	
Season of calving	Summer and Fall	325	- 2.23	< 0.001
	Winter and Spring	215	Referent	
Gestation length (d)	Short (265 to 275)	136	- 5.01	< 0.001
	Medium (276 to 285)	379	- 2.18	0.011
	Long (286 to 295)	25	Referent	
Heart girth (cm)	Linear	540	0.52	0.016
Wither height (cm)	Linear	540	0.54	0.007
Diagonal length (cm)	Linear	540	0.73	< 0.001
Age at calving (mo)	Very young (20.3 to < 22)	98	2.75	< 0.001
	Young (22 to < 23.5)	145	3.29	< 0.001
	Standard (23.5 to < 25.5)	198	2.35	< 0.001
	Old (25.5 to 37.3)	99	Referent	

4.3.3 Factors Influencing Offspring BW in Cows

Of the variables offered to the offspring BW model in cows, calf gender, season of calving, GL, parity, DP and MGEST were significant (Table 7). The present model explains 26.2% of the variation in BW of the calves delivered by the cows. The mean BW of the calves born to cows was estimated to be 44.1 ± 0.99 kg. Male calves were estimated to be 3.51 kg heavier than the female calves, and the calves born in Summer and Fall were estimated to be 1.12 kg lighter than the calves born in Winter and Spring (P < 0.001). Calves born after a short (265 - 275 d) and medium (276 - 285 d) GL, were estimated to be 4.96 and 2.52 kg lighter, respectively, in comparison to those born after a long (286 - 295) GL (P < 0.001). Cows in their second and third parity gave birth to calves that were estimated to be 1.02 kg heavier in comparison to calves born to older cows (P = 0.005). For cows having an identical HG, the BW of the calves was estimated to be 0.97 and 1.11 kg higher in cows with low (1,400 to < 5,400 kg) and high (6,500 to < 7,200) MGEST, respectively, in comparison to cows with very high (7,200 to 11,600 kg) MGEST (P < 0.05). The BW of the calves was estimated to be 1.14 and 1.60 kg higher in cows that had passed a long (55 to 275 d) and medium (45 to 54 d) DP

respectively, in comparison to cows having passed a short (< 45 d) DP (P = 0.002) (Table 7). There was a weak negative correlation between cow's heart girth and MGEST (r = -0.10, P < 0.001)

Table 7. Significant predictors of birth weight (kg) of calves born to cows

Predictors	Comparison	No.	Estimate (kg)	P-value
Intercept		1,054	44.1	< 0.001
Calf gender	Male	520	3.51	< 0.001
	Female	534	Referent	
Season of calving	Summer and Fall	570	- 1.12	< 0.001
	Winter and Spring	484	Referent	
Gestation length (d)	Short (265 to 275)	173	- 4.96	< 0.001
	Medium (276 to 285)	765	- 2.52	< 0.001
	Long (286 to 295)	116	Referent	
Parity of the cows	2 and 3	762	1.02	0.005
	4 to 9	292	Referent	
Heart girth (cm)	Linear	1,054	0.19	0.633
MGEST[1] (kg)	Low (1,400 to < 5,400)	270	- 1.16	0.021
	Intermediate (5,400 to < 6,500)	334	- 0.74	0.083
	High (6,500 to < 7,200)	222	- 0.43	0.337
	Very high (7,200 to < 11,600)	228	Referent	
Heart girth*MGEST	Low (1,400 to < 5,400)	270	0.97	0.039
	Intermediate (5,400 to < 6,500)	334	0.10	0.826
	High (6,500 to < 7,200)	222	1.11	0.034
	Very high (7,200 to 11,600)	228	Referent	
Dry period (d)	Long (55 to 275)	336	1.14	0.021
	Standard (45 to 54)	469	1.60	< 0.001
	Short (3 to 44)	249	Referent	

[1]MGEST = Cumulative milk production during gestation from conception to drying off.

4.4 Discussion

The present study provides new insight about some factors that are significantly associated with birth size of Holstein calves. Several studies have been published on the impact of specific environmental factors in relation to birth size (Kocak et al. 2007; Swali and Wathes 2006; Zhang et al. 2002). However, what is lacking is an evaluation of the combined effect of all of these variables including the age at calving in heifers and the amount of milk produced during specific moments in gestation in cows. Age at calving and milk production during gestation are identified as important determinants of calf birth size in heifers and cows, respectively. Moreover, calf gender, season of calving, GL, parity and morphometrics (HG, WH, DL) of the dam and DP are reaffirmed as factors being significantly associated with calf birth size. In addition, we found a weak correlation between sire breeding value for calving ease and BW of the calves. This result support previous investigation (Swali and Wathes 2006) in suggesting that the uterine environment has a greater influence on size at birth than the paternal genotype.

As in previous studies (Kocak et al. 2007; Swali et al. 2008), BW was retained as the most consistent and objective measure to describe the size of the calves at birth. The HG and BCI are highly positively correlated with BW and have been used as important descriptors of birth size in comparison with other measures (Lundborg et al. 2003; Swali and Wathes 2006). This means that the calves with lower BW have smaller HG and BCI, and are proportionately thinner (Swali and Wathes 2006). Logically, the factors that influence BW of the calves are expected to influence their HG and BCI. Therefore, the remaining of the discussion is focused on the factors related to BW, the most important descriptor of birth size in calves. As in other studies (Gutierrez et al. 2012; Sharma et al. 2012; Swali et al. 2008), BW estimates mentioned in the present study are based on the applied statistical models and are therefore conditional taking into account the included fixed and random effects, and somewhat different from the originally measured BW. Furthermore, in some other species including humans, it has been shown that detrimental environmental factors experienced by the dam during gestation (e.g. starvation or illness) do have a negative effect on the embryonic or fetal development which became clear by a lower weight of the offspring at birth (Sharma et al. 2012; Stein et al. 2004; Tao et al. 2012).

4.4.1 Calf Gender and Dam Parity

Calf gender is significantly associated with BW, male calves are heavier than female ones as previously reported (Dhakal et al. 2013; Gutierrez et al. 2012; Kertz et al. 1997). The male calves are carried 1.3 d longer than the female ones, which may partially explain their higher BW (Dhakal et al. 2013). Moreover, gender-specific genes affecting insulin sensitivity such as mutations in glucokinase gene may be responsible for the gender difference in BW. The genetically more insulin resistant female fetus is less responsive to the trophic effects of insulin and is therefore lighter (Wilkin and Murphy 2006). Calves born to heifers are 4.6 kg lighter than the calves delivered by cows, which is in line with results of previous studies (Dhakal et al. 2013; Johanson and Berger 2003). As a fetal calf has an average daily weight gain of 0.5 kg in the last week of gestation (Dhakal et al. 2013; Norman et al. 2009), the 1.9 d shorter GL in heifers in comparison to the cows in the current study, accounts for approximately 1 kg of the BW difference. Another plausible reason why heifers give birth to lighter calves is that heifers usually are still growing during their first gestation and preferentially use nutrients for their own growth rather than for the growth and development of their fetus (Funston and Summers 2013). The latter is furthermore visualized by a reduced development of the placenta (Funston and Summers 2013; Symonds et al. 2010). Moreover, the differences in nutrient partitioning in heifers versus cows are probably controlled by developmental changes in the somatotrophic axis of the dam (Brickell et al. 2009). Mature cows also become more resistant to the anabolic effects of growth hormone and placental lactogen (Weber et al. 2007), resulting in a degree of insulin resistance, which allows more glucose to be transferred to the fetus in gestating cows (Bell et al. 1995). Therefore, when compared to heifers, mature cows give birth to heavier calves (Dhakal et al. 2013; Johanson and Berger 2003). The pattern of increased BW from first to third parity followed by a decrease in subsequent parities is consistent with previous investigations (Kertz et al. 1997; Linden et al. 2009). Both IGF-I and insulin concentrations remarkably decrease as cows get older (Taylor et al. 2004). Reductions in these endocrine signals likely contribute to the lower BW of the calves delivered by older cows (Swali and Wathes 2006).

4.4.2 Season of Calving and GL

The later part of gestation is known to be critical for fetal growth. Suboptimal growth during this period predisposes to a lower BW of the calves (Symonds et al. 2010; Zhang et al. 2002). Cows calving in Summer and Fall have completed their last part of gestation during the hotter

seasons Spring and Summer, respectively. The negative effect of higher temperature on fetal growth and BW of calves born to both heifers and cows has been reported earlier (Linden et al. 2009; Tao et al. 2012). Several factors may contribute to the lower BW of the calves born in the Summer and Fall seasons. One possible factor is reduced dry matter intake (DMI) of the dam during the last part of gestation due to heat stress (Umphrey et al. 2001), contributing to decreased nutrient availability and consequently a lower BW (Linden et al. 2009; Norman et al. 2009). Similarly, a longer photoperiod during the second part of gestation leads to increased plasma prolactin concentrations, supporting higher milk production and thereby limiting nutrient availability for intrauterine calf growth (Garcia-Ispierto et al. 2009). Heat stress during late gestation is furthermore associated with decreased uterine blood flow and reduced placental function giving rise to an impaired dam-to-fetal exchange of glucose and amino acids (Reynolds et al. 1985). Another plausible factor is the shorter GL of the pregnancies during the hotter seasons (Tao et al. 2012), which has been shown to be associated with the large variation in temperature and humidity at that time (Dhakal et al. 2013; Norman et al. 2009). However, the threshold of seasonal conditions that affect BW of calves are largely attributed to the thermoneutral threshold of cow comfort being associated with geographical locations throughout the globe.

4.4.3 Morphometrics and Age at Calving in Heifers

Young age has been shown to affect neonatal BW in humans where growth of teenage mothers during gestation is associated with increased risks for low BW babies (Chen et al. 2007). Gestation at a young age necessitates not only partition of energy towards the developing fetus, but also towards the dam's own growth. In the current dairy industry, gestation in heifers usually occurs while animals are physically immature and still growing (Kertz et al. 1997). However, the impact of age at calving in heifers on BW of their calves has, to the best of our knowledge not been extensively documented earlier. We hypothesized that heifers that become pregnant while still growing, would give birth to significantly lighter calves. We found in the present study that heifers that are larger in HG, WH and DL at calving deliver significantly heavier calves, which is in concordance with previous studies (Linden et al. 2009; Swali and Wathes 2006). However, in the present study, the calf BW is curvilinear with the age of the heifers at calving. Calves born to very young (20.3 to < 22 mo) heifers have a comparatively lower BW in comparison to calves born to young (22 to < 23.5 mo) heifers suggesting that the intrauterine environment may limit fetal calf growth due to competition for nutrients with dam growth (Wathes et al. 2008). A suboptimal growth

associated with low IGF-I concentrations results in heifers that conceive at an older age (Brickell et al. 2009; Wathes et al. 2008). As the IGF system can modulate the delivery of substrates to the fetus, we speculate that the lower IGF-I in older heifers during gestation, may be responsible for the lighter BW of their calves (Brickell et al. 2009; Gutierrez et al. 2012; Wathes et al. 2008). Moreover, the GL in heifers is curvilinear, giving rise to a shorter GL in both young and old animals in comparison to the intermediate aged group, which further contributes to the lower BW of calves born to relatively young and old first parity dams (Norman et al. 2009; Simerl et al. 1991). The incidence of dystocia is also illustrated to be affected by age in a curvilinear manner, the young and older heifers being more affected (Johanson and Berger 2003; Simerl et al. 1991). Higher calving difficulties in young and older heifers are, however, possibly due to immaturity of the dam and consequently relatively oversized calves in young heifers and excessive fat deposition in the pelvis of old heifers, respectively (Raboisson et al. 2013).

4.4.4 Milk Production and Length of the DP

Genetic selection in Holstein cattle has resulted in larger cows with a high milk production potential. We hypothesized that the fetal calf growth and thereby BW of the newborn calves, is compromised in cows with high milk production during gestation (MGEST). The rationale behind this hypothesis originates from studies done in a variety of other species including humans, in which it was found that fetal growth is significantly retarded in case of a lack of nutrients during gestation (Sharma et al. 2012; Symonds et al. 2010). This lack of nutrients can be either caused by a famine (Stein et al. 2004) or adverse environmental conditions (Tao et al. 2012). We found that among the cows with identical HG, very high MGEST ($\geq$ 7,200 liter) negatively affects BW of the newborn calves. The Scale, MPEAK and MPCONC are highly correlated (> 60%) with MGEST in the present study, which is consistent with previous investigations where the lactation curves were depicted by the Wilmink function (Yamazaki et al. 2011). Similar negative effects of the other production variables as calculated by the MilkBot model, on BW of the calves, are observed when they are replacing MGEST in the mixed model (data not shown). The depletion of energy reserves in lactation overlapping with gestation negatively affects the BW, as reported in humans (Sengul et al. 2013). However, in contrast to the present study, milk production level has been shown to be indistinguishable among cows that had given birth to low-, average- and high-BW calves (Swali and Wathes 2006). This may be due to the fact that in the latter study, M305 was used as a proxy for milk production during fetal growth and to the relatively small (n = 65) sample

size. Typically, dairy cows conceive and are therefore pregnant at the time they still produce large quantities of milk. The latter implies they partition high amounts of glucose and amino acids towards their udder while there is an embryo or fetus growing in their uterus. Glucose, insulin and IGF-I concentrations in cows are negatively correlated with their level of milk production during the production phase of the lactation (Ingvartsen and Friggens 2005; Taylor et al. 2004). This is furthermore evidenced by a depletion of body reserves during lactation, as shown by a negative correlation between BCS and the level of milk production (Yamazaki et al. 2011). These adaptations associated with high milk production in turn affect glucose availability and concentrations of insulin and IGF-I for the embryo and thereby affect the growth of both the early embryo and the placenta (Green et al. 2012). Although the nutrient requirements for the embryo may be relatively low, metabolic activity is high and this represents a critical period for epigenetic control and organogenesis of subsequent fetal development (Van Soom et al. 2013; Wu et al. 2006). A poor intrauterine nutritional environment associated with lower glucose, insulin and IGF-I levels in the cows may even occur in later phases of lactation due to the high persistency of milk production as is typically noticed in modern high yielding dairy cows (Ingvartsen and Friggens 2005). These phases of lactation often coincide with the mid and late part of gestation which are known to be most crucial in terms of fetal growth. Therefore, the effects of variation in nutritional environment at that time may even have greater effects than in early gestation (Stein et al. 2004; Wu et al. 2006). For example, significant reductions in BW have been shown to be caused by low plasma glucose concentrations during the mid and late gestation (Zhang et al. 2002). The close relationship between fetal glucose uptake and dam glucose levels would explain a large part of the mechanism whereby the nutritional level of the dam affects fetal growth (Bell et al. 1995). Therefore, selection for greater milk production and high persistency in dairy cows may lead to reduced glucose availability for the developing embryo and fetus with subsequent deleterious repercussions for BW of the calves including their survival and life time performance (Banos et al. 2007; Berry et al. 2008; Gonzalez-Recio et al. 2012).

In the current study, the calf BW was higher in cows with a medium (45 to 54 d) and long (55 to 275 d) DP than in cows with a short DP (3 to 44 d). Similar findings have been reported earlier, the average offspring BW is lower in cows having passed a DP of 51 to 60 d in comparison to those having gone through a longer DP (Atashi et al. 2013). On the other hand, in the latter study calf BW did not differ for cows with DP of 0 to 35 d, 36 to 50 d, or 51 to 60 d (Atashi et al. 2013). Longer DP is associated with heavier calves and subsequently with a

higher incidence of dystocia (Atashi et al. 2013). In our study, the cows with higher milk production tended to have a shorter subsequent DP, which is in line with previous reports (Atashi et al. 2013). In less productive cows, daily milk production often decreases to low levels before the planned drying off date; therefore, farmers no longer keep these cows in production, which results in longer DP in comparison with higher yielding cows (Atashi et al. 2013). Although there is no difference in serum glucose, insulin and IGF-I levels in cows with short versus longer DP (Pezeshki et al. 2007), we speculate that cessation of partitioning nutrients towards milk for extended duration in case of a longer DP, provides higher amounts of energy and amino acids toward fetal growth giving rise to higher BW of the calves.

The present study was undertaken to investigate the impact of environmental and dam factors on the BW of Holstein calves. Conclusively, results of the present study reaffirm calf gender, season of calving, GL, parity, morphometrics of the dam and length of the DP to be significantly associated with calf birth size. Furthermore, age at calving in heifers and level of milk production during gestation in cows, were indicated as decisive determinants of calf birth size. These novel findings may provide a basis for developing managerial interventions to improve long-term health and productivity of the offspring.

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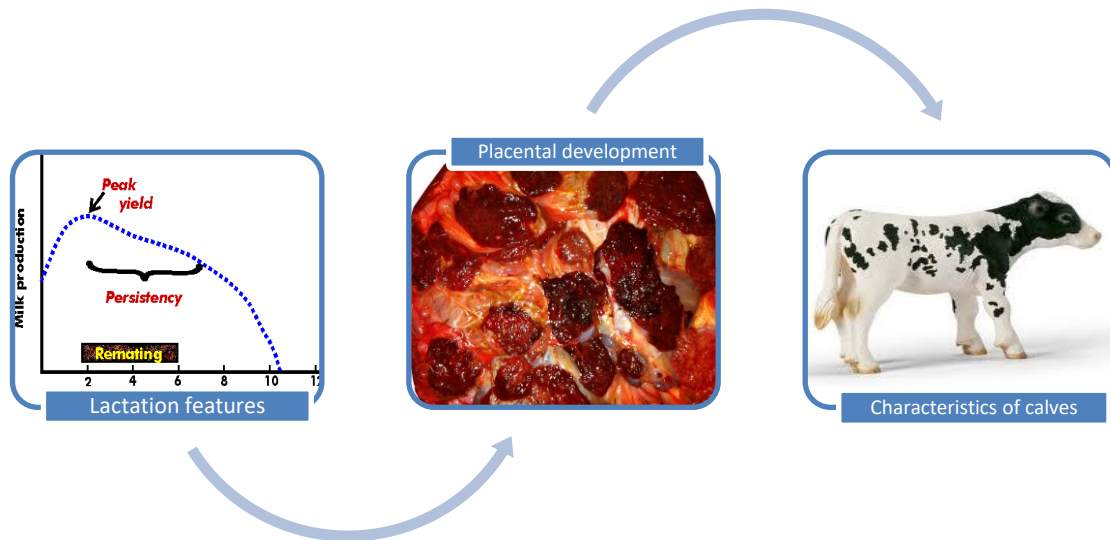
FACTORS ASSOCIATED WITH GROSS PLACENTAL MORPHOLOGY IN DAIRY CATTLE

Abstract

This paper reports on a study of gross placental morphology of 282 expelled placentas from 89 primi- and 193 multiparous Holstein dams immediately after calving and examines associations with environmental factors such as typical herd features and season of calving, and maternal factors such as age at calving, level of milk yield at conception and cumulative amount of milk produced during gestation. The highest correlation between calf measurements and placental characteristics was found between the weight of the calf and the total cotyledonary surface ($r = 0.643$; $P < 0.001$), confirming the high importance of the cotyledonary surface available for nutrient transfer to the developing fetus. Younger age in adolescent and smaller heart girth in multiparous dams were associated with a higher cotyledon number, suggesting placental compensation in dams with lower capacities in terms of dry matter intake. No significant association between milk yield during gestation in multiparous animals and gross placental morphometrics could be detected, indicating that factors such as the amount of milk produced during gestation affect placental development less than fetal weight close to term. Therefore, placental growth may be sustained at the expense of other tissues in an attempt to maintain pregnancy and minimize the adverse consequences for the fetus. The present study offers evidence concerning factors affecting the placental surface size for nutrient transfer from dam to calf in dairy cattle based on gross morphometrics, but needs confirmation from studies in which this surface size is more profoundly assessed by measuring the branches present in the cotyledonary villi.

5.1 Introduction

In eutherian mammals, pregnancy is an apparently highly co-operative interaction between the mother and the fetus which, all being well, leads to delivery of viable offspring with little detriment to future health or fecundity of the mother (Fowden and Moore 2012). In these mammals, the organ through which respiratory gases, nutrients, and wastes are exchanged between the maternal and fetal systems is the placenta. This organ therefore plays an important role in the competition between mother and fetus for resource allocation. Placental development responds to both fetal signals of nutrient demand and maternal signals of nutrient availability and, by adapting its phenotype, regulates the distribution of available resources (Fowden and Moore 2012; Fowden et al. 2006). Placental adaptation may occur by a change in its surface area for nutrient transfer, the thickness of its interhaemal barrier separating maternal and fetal circulations, its density of nutrient transporters, metabolic rate

and blood flow as well as its synthesis and metabolism of specific hormones (Fowden and Moore 2012; Vaughan et al. 2012).

The conflict in nutrient allocation between mother and offspring during pregnancy is especially intriguing in modern dairy cattle, in which it has been calculated that the aim should be for heifers to start calving at around 24 months of age and to calve thereafter at intervals of <385 days in order to optimize their lifetime productivity (Inchaisri et al. 2010). The former implies that gestation in primiparous animals should take place when dams are still growing, while the latter implies that in multiparous animals gestation should to a large extent coincide with lactation. Recently, we have demonstrated that young age at first gestation and a high level of milk yield during gestation in multiparous dams both reduce intrauterine fetal development, probably by diverting a significant proportion of nutrients to dam growth and milk production (Kamal et al. 2014). Subsequently, we have demonstrated that both growth and lactation during gestation have a significant impact on gross placental morphology in cattle (Van Eetvelde et al. 2016).

Fetal intrauterine growth retardation due to nutritional constraints is closely linked to placental development and function (Cetin and Alvino 2009; Fowden et al. 2008). Basically, under conditions of impaired nutrient supply that may limit normal fetal growth, the placenta has shown to adapt so as to increase its transport capacity (Burton and Fowden 2012). Earlier studies in beef cattle and sheep have demonstrated that placentas may adapt their phenotype to be able to accommodate fetal nutrient demand and that this process may include either morphological or functional mechanisms acting during different stages of feto-placental development (Fowden et al. 2010; Long et al. 2009; Sullivan et al. 2009). To the best of our knowledge, there are currently no studies on the impact of maternal factors such as age at conception and level of milk yield during gestation on gross morphology of the placenta in dairy cattle.

5.2 Materials and Methods

All experimental procedures were approved by the Ethical Committees of ILVO (Institute of Agricultural and Fisheries Research, Melle, Belgium) and the Faculty of Veterinary Medicine, Ghent University, Belgium (Approval numbers 2011/166 and EC2012/189– ILVO).

5.2.1. Farms, Animals, and Management

The calves and their dams involved in the study reported here also used in a larger study reported in Chapter 4 of this thesis (Kamal et al. 2014). Briefly, two small dairy herds (on average 70 lactating cows) in Flanders (Belgium) and one large herd (>2000 lactating cows) in Rostock (Germany) were selected based on their long history of successful collaboration with the Ghent University. All three herds were subject to an official milk recording system and a veterinary herd health programme to closely monitor both productivity and health. In all herds, only Holstein cattle were raised and the average 305 d milk production of the cows was ~9,200 kg. The heifers and cows were housed in free-stall barns. They were fed according to their requirements for maintenance and growth (heifers) and production (cows) as indicated by the results of the monthly production tests. The rations comprised high quality roughages (maize silage, grass silage, sugar beet pulp and fodder beets) supplemented with concentrates. The cows were generally milked twice a day but some high producing cows in the German herd were milked 3 times daily. Heat detection was performed by the herdsmen and their employees at least three times daily at regular time intervals. Observed heats, as well as all other observations concerning health and fertility were recorded in a herd-health computer software program. Heifers were generally inseminated at an age of 15 months, the cows at the first estrus occurring after 50 d postpartum. Semen from different bulls was used randomly. The heifers and cows approaching parturition were separated in a maternity pen and closely monitored by farm employees.

5.2.2. Measurements and Data Collection

A retrospective cohort study design was used. In Belgium, the study took place from August 2011 through April 2013. In Germany, the measurements were taken only in January and February of 2012 and 2013. Fetal membranes were collected after spontaneous expulsion and immediately checked for completeness by careful visual inspection. Placental measurements were made within 24 hours after expulsion. Membranes expelled after 12 hours were classified as retained and excluded from further analysis, as they were damaged and incomplete. Also, excluded were both membranes and calves related to twin births, stillborn calves, births following insemination from non-Holstein bulls and births following an abnormally short (<265 d) or long (>295 d) gestation. Microcotyledons (less than 1.0 cm in diameter) were classed as accessory cotyledons and not included in cotyledon number and surface calculations (Facciotti et al. 2009; Miglino et al. 2007).

The entire fetal placenta was weighed using digital scales with an accuracy of 0.1 kg. Cotyledons were then manually removed one by one, after which the remaining membranes and the cotyledons were weighed separately. Each cotyledon was laid flat and the major and minor diameters were measured using calipers with an accuracy of 1 mm. The individual cotyledonary surface area was calculated using the formula to calculate the area of an ellipse: Area (ellipse) = πab; where a = half of the major diameter and b = half of the minor diameter. Total cotyledonary surface area was calculated as the sum of all individual cotyledonary surface areas. Placental efficiency was defined as calf weight/placental weight, while the surface efficiency was defined as calf weight/total cotyledonary surface.

Immediately after birth, the gender of the calf was recorded and its birth weight was measured in kilograms on a digital weighing scale. On the 1st day, the heart girth, wither height and diagonal length of both dams and calves were measured in centimeters following the procedure described in our earlier study (Kamal et al. 2014). Dams were given a body condition score on a 1 to 5 scale (1 = emaciated, 5 = obese) with 0.25 increments (Edmonson et al. 1989). For each dam, date of birth, previous calving date, date of conception, monthly milk weights of the previous lactation (i.e. that of the calving preceding the one included in the study) and the dry-off dates were extracted from the herd databases.

5.2.3. Calculations and Estimations

The gestation length of both heifers and cows was derived from the last recorded insemination of the dam to the date of calving and was restricted between 265 to 295 d as mentioned above. Days open, lactation length, length of the dry period and calving interval in the cows were calculated based on the data gathered from the herd database. The days open was calculated as the time from when a cow calves until when she conceives. Monthly milk weights were fitted to the MilkBot model (Ehrlich 2011) to summarize the magnitude and shape of each lactation curve. The cumulative 305 d milk production, cumulative milk production during gestation from conception to drying off, milk production at lactation peak, cumulative milk production from 15 d before to 15 d after conception, time to peak milk and time to half of the peak milk were calculated directly from MilkBot parameter values (Ehrlich 2011).

5.2.4. Statistical Analysis

All statistical analyses were performed using the SAS software (SAS Institute Inc., Cary, North Carolina, USA). Normality of the data and error terms of the models were checked

using the Kolmogorov-Smirnov test (P < 0.01). Descriptive data are expressed as mean ± SD or as median (min-max) if not normally distributed. Pearson correlation coefficients were calculated to assess relationships between variables using PROC CORR. Data were log transformed before performing statistical analysis when it was not normally distributed. Linear mixed models were built using PROC MIXED to identify variables associated with gross placental morphometrics (i.e. cotyledon number, total cotyledonary surface and placental efficiency). Since surface efficiency was lognormally distributed, PROC GLIMMIX was used to detect variables associated with this parameter. In all models, for cows that were measured twice, the repeated statement within cow was used. All fixed effects (country and dam characteristics) and their 2-way interactions were included in the initial models, after which nonsignificant variables (P > 0.05) were omitted using the backwards approach. Results are presented as model estimates, with the intercept presenting the overall estimated mean. For continuous variables, the parameter estimate refers to the estimated alteration in the outcome when the parameter is increased by one unit. For categorical variables, the estimate refers to the estimated alteration in outcome for a category when compared to the reference category (Ref.). For example in Table 10, the model-based estimated number of cotyledons in heifers is 112, while with per month increase in age of the dam, the number of cotyledons is estimated to decrease by 3.6.

5.3 Results

5.3.1 Descriptives

Tables 8 and 9 represent the descriptive data distributed by parity, including basic statistical analyses.

5.3.2 Correlations between placental characteristics and both calf and dam measurements

The highest correlation between calf measurements and placental characteristics was found between the weight of the calf and the total cotyledonary surface (r = 0.643; P < 0.001).

5.3.3 Model building to explain the variation in placental characteristics

Separate multivariable regression models were built to detect factors significantly associated with gross placental characteristics of primi- and multiparous dams respectively (Tables 10 and 11). Age at conception in heifers and heart girth in cows were negatively associated with the number of cotyledons. In cows, none of the data indicating level of milk yield during gestation were found to be significant contributors.

Table 8. Descriptive statistics (mean ± SD) of the dam characteristics by parity.

Characteristics	Primiparous (n = 89)	Multiparous (n = 193)	P-value
Wither height (cm)	139.5 ± 4.48	144.0 ± 4.94	<0.001
Diagonal length (cm)	158.4 ± 6.27	170.7 ± 6.33	<0.001
Heart girth (cm)	202.0 ± 7.63	214.2 ± 9.78	<0.001
Age at calving (months)	24.1 ± 2.37	51.1 ± 18.03	<0.001
Gestation length (days)	278 ± 4.4	280 ± 4.7	<0.001
Days open	-	115 ± 59	-
Lactation length (days)	-	338 ± 54.0	-
Dry off period (days)	-	57 ± 25.5	-
Calving interval (days)	-	395 ± 60.3	-
Milk at conception (kg)	-	34.4 ± 7.7.94	-
Milk first trimester of gestation (kg)	-	2868.6 ± 617.51	-
Milk second trimester of gestation (kg)	-	2315.4 ± 481.28	-
Milk third trimester of gestation (kg)	-	859.3 ± 488.04	-
Milk during total gestation (kg)	-	6043.2 ± 1438.71	-

Table 9. Descriptive statistics (mean ± SD) of calf weight and fetal placenta by parity.

Characteristics	Primiparous (n = 89)	Multiparous (n = 193)	P-value
Calf weight (kg)	41.3 ± 4.71	45.1 ± 5.35	<0.001
Placental weight (kg)	5.2 ± 1.23	5.5 ± 1.26	0.060
Membrane weight (kg)	2.8 ± 0.87	2.8 ± 0.82	0.540
Total cotyledonary weight (kg)	2.5 ± 0.51	2.7 ± 0.62	0.003
Cotyledon number	112 ± 29.5	108 ± 28.6	0.209
Average cotyledonary weight (kg)	0.023 ± 0.008	0.027 ± 0.009	0.005
Total cotyledonary surface (m²)	0.51 ± 0.084	0.61 ± 0.108	<0.001
Placental efficiency (calf weight/placental weight)	8.2 ± 1.57	8.5 ± 1.63	0.127
Surface efficiency (calf weight/total cotyledonary surface)	82.4 ± 13.99	74.8 ± 11.30	<0.001

Table 10. Factors associated with major morphometrics of the fetal part of placenta in primiparous dams (n = 89)*.

Variable	Cotyledon Number	Total cotyledonary surface (m²)	Placental efficiency	Surface efficiency (kg/m²)
Intercept	112	0.53	7.9	81.3
Age at conception (m)	-3.6 $^{p=0.006}$			1.01 $^{p=0.045}$
Country				
Belgium (n = 37)		-0.043 $^{p=0.005}$	0.74 $^{p=0.027}$	
Germany (n = 52)		Ref.	Ref.	
Cotyledon number		0.001 $^{p<0.001}$		1.00 $^{p<0.001}$
Gestation length (d)		0.003 $^{p=0.08}$		

Superscripts represent the actual p-values.

*Average weight of calves in Belgium and Germany was 40.2 ± 4.85 kg and 42.0 ± 4.51 kg, respectively. Average weight of placenta in Belgium and Germany was 4.9 ± 1.32 kg and 5.4 ± 1.12 kg, respectively.

Table 11. Factors associated with major morphometrics of the fetal part of placenta in multiparous dams (n = 193)*.

	Cotyledon Number	Total cotyledonary Surface (m²)	Placental efficiency	Surface efficiency (kg/m²)
Intercept	108	0.64	8.4	74
Heart girth (cm)	-0.5 $^{p=0.04}$			
Days open (d)	0.1 $^{p=0.04}$			
Country				
Belgium (n = 58)		-0.083 $^{p<0.001}$	0.53 $^{p=0.038}$	
Germany (n = 135)		Ref.	Ref.	
Cotyledon number		0.001 $^{p<0.001}$		1.00 $^{p<0.001}$
Gestation length (d)		0.004 $^{p=0.034}$		

Superscripts represent the actual p-values.

*Average weight of calves in Belgium and Germany was 43.5 ± 5.54 kg and 45.8 ± 4.66 kg, respectively. Average weight of placenta in Belgium and Germany was 5.2 ± 1.15 kg and 5.6 ± 1.22 kg, respectively.

5.4 Discussion

The aim of the study reported here was to examine typical environmental and maternal factors associated with gross placental morphology in dairy cattle. Since dairy cattle management is characterized by features such as pregnancy at a young age in primiparous animals and lactation during gestation in multiparous animals, we focused on these typical maternal factors. These features are particularly important since both growth and lactation have recently been shown to significantly affect gross placental traits when coinciding with gestation (Van Eetvelde et al. 2016). Since only the fetal portion of the placental tissue was available for examination, the potential impact of certain maternal factors on the maternal placental tissue could not be assessed. Our study was also restricted to the gross phenotypic morphology of the placenta (placental weight and the number and size of the cotyledons).

A major feature of the chorioallantoic placenta is an enlargement of the surface area at the feto-maternal junction by the formation of chorionic villi within the cotyledonary plaques. These villi interdigitate with vascular folding of the uterine caruncular endometrium and eventually serve as the functional unit for feto-maternal exchange (Jainudeen and Hafez 1993). Obviously, the villous surface area depends upon the length and branch pattern of each villus. The villous surface probably grows in parallel with the increased need for energy from fetus and placenta (Baur 1972). Therefore, a given volume of fetus and placenta tends to have an optimal size of villous surface for metabolic exchange at its disposal (Baur 1972). The assumption in our study is that the total base area of the cotyledons is proportional to the villous surface area available for nutrient transfer. Accordingly, it is expected that the placental exchange area may be related to the cotyledonary base area (Kannekens et al. 2006). Therefore, the highest correlation between calf measurements and placental characteristics was found between the weight of the calf and the total cotyledonary surface, confirming the high importance of the cotyledonary surface availability for nutrient transfer to fetal development (Sullivan et al. 2009).

The study reported here estimated the cotyledonary surface area to be 0.53 m^2 in primiparous and 0.64 m^2 in multiparous dams. This extent is comparable with similar studies of the bovine (Bertolini et al. 2002; Sullivan et al. 2009) where the feto-maternal interface is measured at the base of the cotyledons upon manual separation from the caruncle. In the study by Baur (1972), on the other hand, the total cotyledonary area of the term bovine placenta was determined to possess a total nutrient exchange area of 120 m^2; that is, about 190 times higher

than in our study. This gross discrepancy probably arises from the fact that Baur's study of the exchange surface included an assessment of the villous surface of the cotyledons. In 2006, Kannekens and collaborators introduced the surface amplification factor (SAF) based on a detailed stereological study (Kannekens et al. 2006). The SAF represents an accurate estimation of the degree of villous branching at a given time during gestation (Kannekens et al. 2006). At 135 days of gestation, the SAF estimate of 108.0 suggests that underneath every square millimeter of placentome surface there is 108.0 mm^2 of feto-maternal interface (Kannekens et al. 2006). Therefore, the feto-maternal interface for any size of placentome can be estimated by measuring its total surface area and multiplying that by the SAF correction factor. In this context, the total villous surface area of the feto-maternal interface at day 135 of gestation is estimated to be 18.5 m^2 (Kannekens et al. 2006). As the SAF increases over time (Kannekens et al. 2006), the villous surface area increases to 69.8 m^2 at term (Ribeiro et al. 2008). This last estimation is very similar to our findings.

The number of uterine caruncles in the dam is determined prior to her birth (Atkinson et al. 1984). Usually, there are 75 to 120 of them. A corresponding number of fetal cotyledons attach to the maternal caruncles to form approximately 70-120 placentomes that serve as the functional unit for feto-maternal exchange. Studies have noted the presence of spare caruncles not covered by or attached to a cotyledon throughout gestation (Long et al. 2009). Hence, the cotyledon number seems to be more dynamic in nature and probably dependent on specific modulating factors. In *Bos Indicus* cattle, a maximum of 20 cotyledons are initially present while from day 40 to 50 post-insemination, the number triples, and gradually increases further, averaging about 80 up to day 70 post-insemination (Assis Neto et al. 2010). Usually there is no change in the number of bovine cotyledons from day 90 of gestation, while the mean weight and surface of each placentome may continue to increase (Laven and Peters 2001), resulting in their characteristic mushroom-like shape (Leiser et al. 1997).

In the light of the above, we hypothesized for the present study, that factors occurring during early pregnancy, like growth during adolescent pregnancy in primiparous animals and a high level of milk production in multiparous animals, might affect the number of cotyledons, while factors mainly occurring later in gestation affect the size of the cotyledons and hence the total cotyledonary surface. It is difficult to compare studies of the size and characteristics of the bovine placenta due to the large variety of definitions and study designs used. For example in some studies it is not always clear how the weight of the placenta was measured (wet or after drying), making large variations between studies in results for placental efficiency inevitable.

Based on our models, the factors contributing the most to gross placental morphology are age at conception in heifers and heart girth (as a proxy for body weight) in multiparous animals. Both are significantly but negatively associated with the number of caruncles occupied by cotyledons. Caution should, however, be taken with the results of the present study. Although statistical analyses revealed significant associations, the actual numbers indicate only relatively small differences between the different subjects.

The findings of the present study are in concordance with our hypothesis that the fetal part of the placenta compensates in times of nutrient restriction. Fetuses of both younger heifers and lighter multiparous animals, both presumably having a lower dry matter intake, compensate by occupying significantly more caruncles in order to guarantee survival and further development. Similar placental compensation in terms of number of cotyledons has also been reported in sheep (Wallace et al. 1999). Also in cattle, more cotyledons are expected in heifers which have been fed low dietary protein as their fetuses may have increased their caruncular occupancy to compensate and to meet their nutritional demands (Sullivan et al. 2009). Negative correlations between insulin-like growth factor binding protein (IGFBP) concentrations throughout gestation and cotyledon number at expulsion suggest that in the bovine, lower IGFBP concentrations in times of nutrient restriction may increase the bioavailability of IGF-I, resulting in the formation of more cotyledons occupying the available caruncles (Sullivan et al. 2009).

In comparison to young growing animals in our study, multiparous and hence lactating dams gave birth to heavier calves, although with an even higher increase in total cotyledonary surface and hence less efficient placentas, which suggests lactation to be associated with a more pronounced placental compensation, effectuated by an increased size of the cotyledons. However, the study reported here was not able to detect direct effects of the level of milk yield, such as the number of liters at top production or the total amount of milk produced during gestation, on gross placental morphometrics. The lack of evidence for the the effect of the latter may be caused by several factors. For one thing it is well known that selection towards high milk yield gives rise to several other compensation mechanisms in order to first safeguard the dam and the newly born calf in preference to investing in a subsequent generation. Modern high-producing dairy cows, for example, unlike less selected and hence lower-producing animals, have been shown to significantly postpone ovarian activity and hence a subsequent pregnancy after calving (Opsomer et al. 2000).

At least some of the otherwise normal placentas in our study also presented microcotyledons (accessory cotyledons). These have a diameter of less than 1.0 cm and are usually found at the extremities of chorioallantois. Overall, they were very few in number and most often formed a cluster in association with a neighboring cotyledon. Most likely, the observations above are not affected by the relatively small role of these structures, which do not significantly contribute to overall placental function and conceptus development (Facciotti et al. 2009; Miglino et al. 2007).

The results of the study reported here partly confirm the conclusion drawn by Vaughan and colleagues (Vaughan et al. 2012) that many environmental factors like the level of milk production affect placental development less severely than fetal weight close to term, suggesting that placental growth may be sustained at the expense of other tissues in an attempt to maintain pregnancy and minimize the adverse consequences for the fetus. Ultimately, the ability of the placenta to balance the competing interests of mother, milk production and fetus in terms of resource allocation may determine not only the success of pregnancy in producing viable neonates but also the health and productivity of the offspring in later life.

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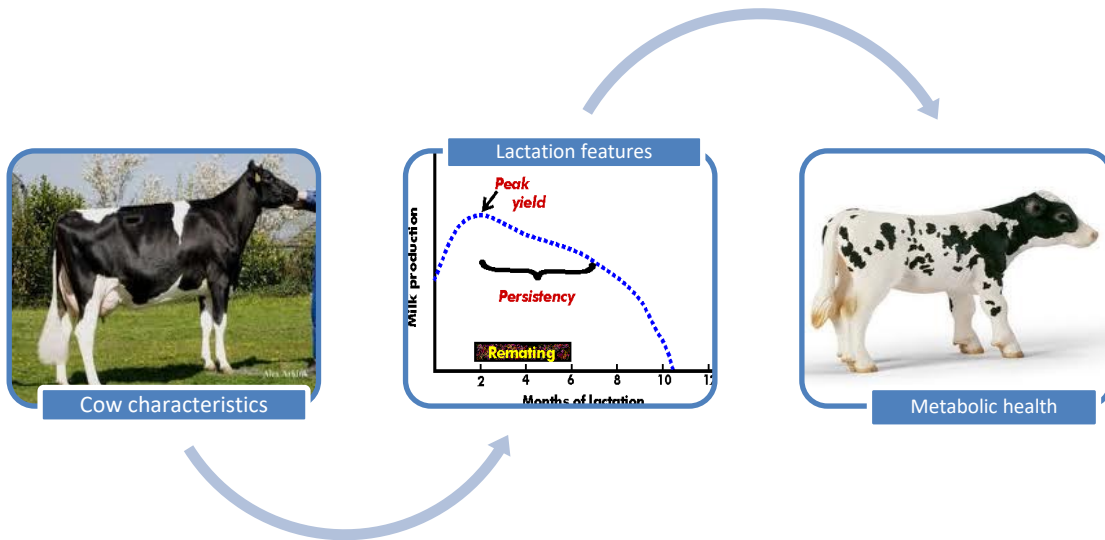
FACTORS ASSOCIATED WITH INSULIN TRAITS IN HOLSTEIN CALVES

Adapted from:

Abstract

The objective of the present retrospective cohort study was to evaluate potential associations between environmental factors and dam characteristics, including level of milk production during gestation, and insulin traits in newborn Holstein calves. Birth weight and gestational age of the calves at delivery were determined. On the next day, heart girth, wither height and diagonal length of both the calves and their dams were measured. Parity, body condition score and age at calving were recorded for all dams. For the cows, days open prior to last gestation, lactation length (LL), length of dry period (DP) and calving interval were also calculated. The magnitude and shape of the lactation curve both quantified using the MilkBot model based on monthly milk weights, were used to calculate the amount of milk produced during gestation. Using the same procedure, cumulative milk production from conception to drying off (MGEST) was calculated. A blood sample was collected from all calves (n = 481; 169 born to heifers and 312 born to cows) at least five hours after a milk meal on day three of life to measure basal glucose and insulin levels. In addition, an intravenous glucose-stimulated insulin secretion test was performed in a subset of the calves (n = 316). After descriptive analysis, generalized linear mixed models were used to identify factors that were significantly associated with the major insulin traits (Ins_b, basal insulin level; QUICKI, quantitative insulin sensitivity check index; AIR, acute insulin response; DI, disposition index) of the newborn calves. The overall average birth weight of the calves was 42.7 ± 5.92 kg. The insulin traits were significantly associated with gender and season of birth when data of all calves were analyzed. In addition, the insulin traits in calves born to cows were significantly associated with MGEST, DP and LL. The Ins_b was estimated to be higher in calves born to the cows having passed a higher MGEST (P = 0.076) and longer DP (P = 0.034). The QUICKI was estimated to be lower in calves born to the cows having passed a higher MGEST (P = 0.030) and longer DP (P = 0.058). Moreover, the AIR (P = 0.009) and DI (P = 0.049) were estimated to be lower in male compared to female calves. Furthermore, the AIR (P = 0.036) and DI (P = 0.039) were estimated to be lower in calves born to cows having passed a longer LL. The decisive effects of MGEST, DP and LL in cows on the insulin traits of their calves may provide a basis for developing managerial interventions to improve metabolic health of the offspring.

Keywords: insulin traits, dairy calves, risk factors

6.1 Introduction

Dam nutrition during gestation in mammals has been recognized as a key to metabolic programming *in utero*. An impaired nutrient supply during intrauterine development leads to metabolic adaptations to enhance survival in the short term but may become detrimental in postnatal life (Fleming et al. 2012). The unique metabolic adaptations to reduced nutrient supply *in utero* are well coordinated in an attempt to maximize the uptake and use of nutrients by the most vital organs like the brain (Fleming et al. 2012). As a consequence, these adaptations often occur at the expense of those organs that are less critical for survival (e.g. the pancreas) (Gutierrez et al. 2012; Limesand et al. 2013; Long et al. 2010). Hence, suboptimal intrauterine nutrition may end up in birth of growth restricted newborns with an impaired β-cell mass, visualized by a lowered insulin secretion when challenged with a standard glucose bolus (Limesand et al. 2013; Owens et al. 2007; Rozance et al. 2006). Epigenetic mechanisms such as DNA methylation and histone modification of the fetal genome are likely mediators of such postnatal expression of intrauterine events (Wu et al. 2006). The environmental sensitivity of the epigenome is viewed as an adaptive mechanism by which the developing organism adjusts its metabolic and homeostatic systems to suit the anticipated postnatal environment. The long-term effects of these adaptations predispose the offspring to adiposity and metabolic diseases in later life if nutrition in postnatal life does not match that foreseen by the fetus on the basis of its intrauterine environment (De Rooij et al. 2006; Micke et al. 2011).

Relatively high incidence of metabolic disorders and reduced longevity in high-yielding cows present major challenges in today's dairy industry (Berry et al. 2008; Gutierrez et al. 2012; Wu et al. 2006). Decreases in insulin responsiveness and insulin secretion are considered to be etiologic key mechanisms in the association between high yield and transition metabolic disorders (De Koster and Opsomer 2013). Recently, we have described the adaptations that take place in the transition period both in terms of insulin responsiveness as well as of insulin secretion (De Koster and Opsomer 2013), while we also have demonstrated (epi)genetic backgrounds to be involved herein (Bossaert et al. 2009). However, very little is known about the epigenetic background of the insulin-dependent glucose metabolism in calves, although their dams may vary immensely in nutritional milieu both at conception and later during gestation. Moreover, the level of milk production during gestation in cows has been suggested to be an important contributor to the nutritional milieu for the developing embryo and fetus, since available nutrients need to be partitioned between milk production by the

dam and the intrauterine growth of the offspring (Berry et al. 2008; Funston and Summers 2013). Earlier studies examining the effect of milk production at different stages of gestation revealed that higher production levels during embryonic and fetal development are associated with reduced performance and longevity of the offspring (Berry et al. 2008; Funston and Summers 2013; Gonzalez-Recio et al. 2012). The reduced performance and longevity of the offspring have been linked to an impaired endocrine profile during their postnatal life as a consequence of poor intrauterine environment (Gutierrez et al. 2012; Micke et al. 2011). Moreover, abundant postnatal nutrition can challenge the homeostatic mechanisms of the offspring adapted to a poor intrauterine environment and lead to the development of deleterious metabolic conditions. Hence, evidence exists to suggest that an increasing proportion of transition disorders occur in cows for which intrauterine programming of the endocrine pancreas may be a causative factor. Although the underlying causes of this rather new phenomenon are not yet fully elucidated, managerial factors typically associated with a high level of milk production during gestation are often referred to as potential candidates. Moreover, while several papers report on the effect of the maternal environment and nutrition on the metabolic and endocrine profiles of their calves (Gutierrez et al. 2012; Long et al. 2010; Radunz et al. 2012), information on parameters such as milk production during gestation in cows and their effect on insulin traits in their newborn calves has, to the best of our knowledge not been documented yet. Furthermore, despite the multifactorial nature of intrauterine growth, the current literature is lacking studies in which a multifactorial approach has been used to identify factors associated with insulin traits in Holstein calves.

The objective of the present study was to evaluate potential associations between environmental factors and dam characteristics and major insulin traits in newborn Holstein calves. Our main interest went to the hypothesis that the level of milk production during gestation in cows affects the intrauterine development of the fetal endocrine pancreas and hence programs the level of insulin secretion in newborn calves.

6.2 Materials and Methods

6.2.1 Farms, Animals, and Management

Calves and their dams involved in the current study also participated in a larger study reported in Chapter 4 of this thesis (Kamal et al. 2014). Briefly, data were collected from four small dairy herds (on average 70 lactating cows) in Flanders (Belgium) and one large herd (> 2000 lactating cows) in Rostock (Germany). Herds were selected based on their long history

of successful collaboration with the Ghent University Ambulatory Clinic. All herds participated in an official milk recording, artificial insemination, and veterinary herd health program to closely monitor both health and productivity. In all herds, only Holstein cows were milked and average 305 day milk production was ~9300 kg. The heifers and cows were housed in free-stall barns. They were fed according to their requirements for maintenance and growth (heifers) and production (cows) based on the results of the monthly production tests. Generally, the ration consisted of high quality roughages (maize silage, grass silage, sugar beet pulp and fodder beets) supplemented with concentrates. The cows were generally milked twice a day; whereas in Germany some high producing cows were milked 3 times. Heat detection was performed by the herdsmen at least three times daily at regular time intervals. In some herds aids were used to optimize heat detection. Observed heats, as well as all other observations concerning health and fertility were carefully recorded using a herd health software program. Heifers were generally inseminated at an age of 15 months whereas the cows were generally inseminated at the first estrus occurring after 50 days postpartum. Heifers and cows approaching parturition were separated in a maternity pen and were closely monitored by the herd employees. After calving, the calves were immediately removed from the maternity pen into individual calf pens. Calves were fed 4 L of colostrum within the first 10 h after birth either by bottle feeding or by esophageal tube. For first 3 days, the calves were fed whole milk in equal amounts.

6.2.2 Measurements and Data Collection

A retrospective cohort study design was used. The outline of the measurements, data collection and blood sampling is shown in Figure 9. The calves were enrolled at birth between August 2011 and April 2013. Twin calves, calves born dead or that died during the first three days of life, born following inseminations with semen from non-Holstein bulls or born following an abnormally short (< 265 days) or long (> 295 days) gestation length were not enrolled. Immediately after birth, the gender of the calves was recorded and its birth weight (**BW**) was measured in kilograms. On the next day, the heart girth (**HG**), wither height (**WH**) and diagonal length (**DL**) of both dams and calves were measured in centimeters following the procedure described in our earlier study (Kamal et al. 2014). Body condition score of the dams was determined on a 1 to 5 scale (1 = emaciated, 5 = obese) with 0.25 increments (Edmonson et al. 1989). The dam's date of birth, the previous calving date, the date of conception, together with the monthly milk weights of the lactation that started at

the calving preceding the one that was included in the study and the dry off date were extracted from the herd databases.
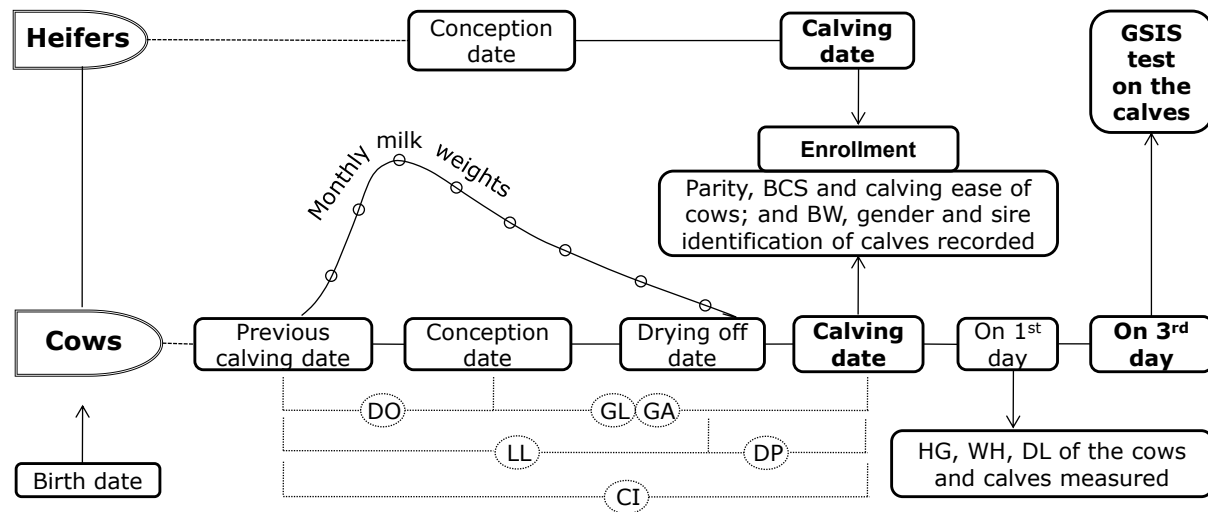

Figure 9. Outline of the measurements, data collection and blood sampling. BCS, body condition score; DO, days open; GL, gestation length; GA, gestational age at delivery; LL, lactation length; DP, dry period; CI, calving interval; BW, birth weight; HG, heart girth; WH, wither height; DL, diagonal length; GSIS, glucose-stimulated insulin secretion

6.2.3 Sampling and Laboratory Analyses

All procedures were approved by the concerned Ethical Committee where appropriate. On day three of life at least five hours after a milk meal (in morning for the Belgian herds and in afternoon for the German herd to be compatible with herd management), a basal blood sample was collected from all calves (n = 481; 169 born to heifers and 312 born to cows). Briefly, after adequate fixation of the calf, a small surface of the skin at the height on the left jugular vein was disinfected with alcohol. A catheter with 18-gauge needle was inserted into the left jugular vein. Stress was avoided as much as possible. Blood samples (± 10 ml) were obtained from the catheter in evacuated gel-coated and fluorinized tubes (Vacutainer®, Terumo Europe NV, Leuven, Belgium) for isolation of serum and plasma, respectively. In addition to the basal blood sample, an intravenous glucose-stimulated insulin secretion (**GSIS**) test was performed in a subset of the calves (n = 316; 109 born to heifers and 207 born to cows). In performing the GSIS test, a glucose solution (Glucose 30%®, Eurovet Animal Health, Heusden-Zolder, Belgium) at 150 mg/kg BW was infused immediately after the basal sampling (Bossaert et al. 2009). Exactly 10 minutes after the glucose bolus had been

administered, a second blood sample was taken from the right jugular vein in evacuated tubes as described above using a Venoject® (Terumo Europe NV, Leuven, Belgium) needle. In a previous study, maximum levels of serum insulin were observed at 10 minutes after the glucose infusion (Bossaert et al. 2009). Therefore, blood samples were taken just prior and exactly 10 minutes after the calves had been given an intravenous glucose bolus to objectively measure the insulin secretion capacity of the newborn calves. All blood samples were centrifuged at 2460g for 20 minutes to isolate plasma and serum that were stored at -20°C until analysis. Plasma glucose was measured using the hexokinase method (GLUC3 Cobas®, Roche Diagnostics GmbH, D-68305 Mannheim, Germany). The intra- and inter-assay coefficients of variation for glucose measurements were 0.8% and 1.3%, respectively. Insulin in serum was measured using the bovine insulin ELISA test kit (Mercodia®, Uppsala, Sweden), with intra- and inter-assay coefficients of variation 2.9% and 2.7%, respectively.

6.2.4 Calculations and Estimations

The gestational age at delivery of the calves was derived from the last recorded insemination of the dam to the birth date of the calves and was restricted to 265 to 295 days as mentioned above. The season of birth of the calves was grouped into four: Winter (21 December to 20 March), Spring (21 March to 20 June), Summer (21 June to 20 September) and Fall (21 September to 20 December). The body condition index (**BCI**) of the calves was calculated as BW/(WH*DL) (Kamal et al. 2014). The basal glucose (**Glu_b**) and insulin (**Ins_b**) levels, and the stimulated (10 minutes after the glucose infusion) glucose and insulin levels were used to calculate the indices of insulin sensitivity and insulin secretion. The Ins_b has been described as an important insulin trait in calves (Hammon et al. 2012). Mathematical handling of the basal levels of glucose and insulin concentration has been used for the calculation of the quantitative insulin sensitivity check index [**QUICKI**, $1/(\log Glu_b + \log Ins_b)$] to measure insulin sensitivity in ruminants (Limesand et al. 2013) including newborn calves (Bossaert et al. 2009). The acute insulin response (**AIR**, increment of insulin above the baseline following the administration of a standard glucose bolus) was used as a measure of first-phase insulin secretion (Limesand et al. 2013; Radunz et al. 2012). We also calculated the disposition index (**DI**, the product of the QUICKI and AIR), an index of first-phase insulin secretion that takes insulin sensitivity into account (Bergman et al. 2002), and which has commonly been used in animal studies (De Blasio et al. 2007). Age at calving in heifers was calculated as the interval from the date of birth to the date of calving. Days open (**DO**), lactation length (**LL**), length of the dry period (**DP**) and calving interval (**CI**) of all cows were calculated. Monthly milk

weights were fitted to the MilkBot model (Ehrlich 2011) to summarize the magnitude and shape of each lactation curve. Using the model, cumulative 305 days milk (**M305**), cumulative milk production during gestation from conception to drying off (**MGEST**), milk production at lactation peak (**MPEAK**), cumulative milk production from 15 days before to 15 days after conception (**MPCONC**), time to peak milk (**TPEAK**) and time to half of the peak milk (**THALF**) were calculated (Ehrlich 2011; Kamal et al. 2014).

6.2.5 Statistical Analysis

Statistical analyses were performed using the SAS Enterprise Guide program (version 6.1; SAS Institute Inc, Cary, NC). Data are presented as means ± standard deviation, unless otherwise specified. Differences were declared significant when $P < 0.05$ and tendencies when $P \leq 0.10$. Student's *t*-test was used to examine differences between means. Pearson correlation coefficients (r) were estimated to describe relationships between variables using the PROC CORR procedure. Generalized linear mixed models were built in PROC GLIMMIX with Lognormal distributions and Identify link functions. Separate models were built for the Ins_b, QUICKI, AIR and DI. First, the model was fitted for each of the independent variables separately. In each model, herd was included as random effect. Dam parity, calf gender and season were considered as class variables. Table 13 lists the quantitative independent dam variables described above. The BW, HG, BCI and gestational age of the calves were quantitative independent calf variables. Those variables that had an effect on the insulin traits ($P \leq 0.2$ in the Wald statistic for type 3 analysis) were selected for further multivariable analysis. The selected fixed effects and their 2-way interactions were included in the initial model, but removed if found non-significant, after which the model was refitted. Modeling was performed using a forward-backward stepwise algorithm with a selection criterion based on Bayesian Information Criterion. The DO and CI were not included in the initial models because of their very high correlation (94% each) with LL. MPCONC was also not included in the initial model because of its high correlation (86%) with MGEST. Separate similar models were built with these variables by replacing LL and MGEST, respectively (data not shown).

6.3 Results

6.3.1 Subject Characteristics

Calf characteristics. The characteristics of the calves are presented in Table 12. The overall average BW of the calves was 42.7 ± 5.92 kg. The calves born to the heifers (n = 169) were significantly smaller in BW, HG, WH, DL, and BCI compared with the ones born to the cows (n = 312) (P < 0.001). Male calves (n = 238) were on average 3.5 kg heavier than their female counterparts (n = 243; 44.5 ± 6.10 vs. 41.0 ± 5.21 kg; P < 0.001). The Glu_b and Ins_b were positively correlated (r = 0.37, P < 0.001) in the calves. There was a negative correlation between log QUICKI and log Ins_b (r = -0.97, P < 0.001) and between log QUICKI and log AIR (r = -0.25, P < 0.001) (Figure 10). The insulin traits were not correlated with BW of the calves and they were also not significantly different between the calves born to heifers versus the ones born to cows (P > 0.05).
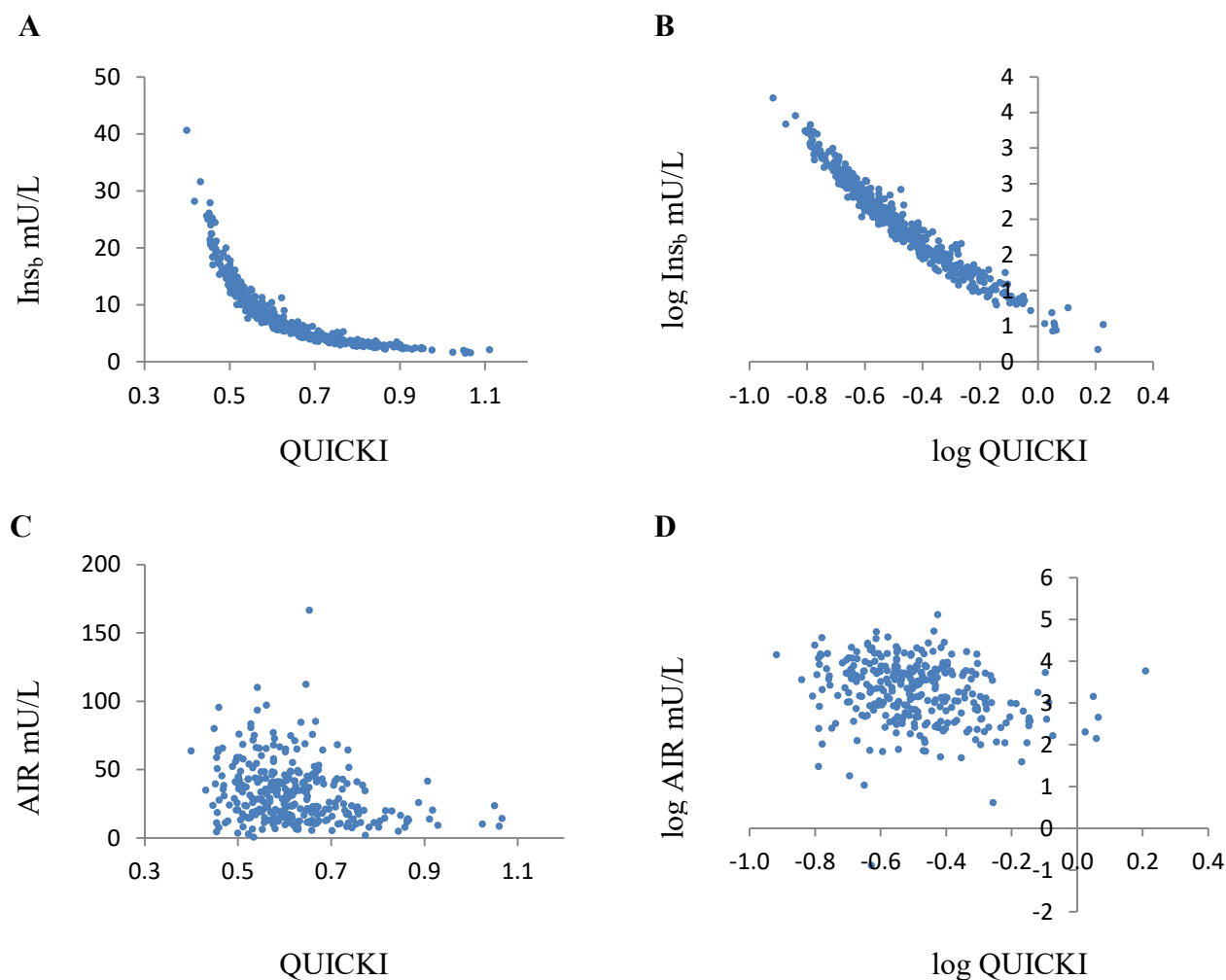

Figure 10. Hyperbolic relationships between insulin sensitivity and insulin secretion are linearised by log transformation. QUICKI, quantitative insulin sensitivity check index; Ins_b, basal insulin; AIR, acute insulin response.

Table 12. *Characteristics of the calves (mean ± SD)*

Characteristics	All calves (n)	Calves born to heifers (n)	Calves born to cows (n)
Birth weight (kg)	42.7 ± 5.92 (481)	40.0 ± 5.05[a] (169)	44.2 ± 5.86[b] (312)
Heart girth (cm)	80.7 ± 4.05 (481)	79.0 ± 3.96[a] (169)	81.6 ± 3.81[b] (312)
Wither height (cm)	76.2 ± 3.42 (481)	75.5 ± 3.41[a] (169)	76.6 ± 3.36[b] (312)
Diagonal length (cm)	70.6 ± 4.08 (481)	69.3 ± 4.21[a] (169)	71.2 ± 3.86[b] (312)
Body condition index	79.4 ± 8.46 (481)	76.5 ± 7.22[a] (169)	81.0 ± 8.67[b] (312)
Gestational age at delivery (day)	279.0 ± 4.74 (481)	277.8 ± 4.39[a] (169)	279.6 ± 4.81[b] (312)
Basal glucose level (mM/L)	6.2 ± 0.88 (481)	6.3 ± 0.84 (169)	6.2 ± 0.91 (312)
Basal insulin level (mU/L)	8.2 ± 5.21 (481)	8.2 ± 4.46 (169)	8.2 ± 5.59 (312)
Quantitative insulin sensitivity check index	0.6 ± 0.13 (481)	0.6 ± 0.12 (169)	0.6 ± 0.13 (312)
Stimulated glucose level (mM/L)	8.1 ± 0.92 (316)	8.2 ± 1.00 (109)	8.1 ± 0.87 (207)
Stimulated insulin level (mU/L)	41.6 ± 24.07 (316)	42.6 ± 23.87 (109)	41.1 ± 24.21 (207)
Acute insulin response (mU/L)	32.9 ± 22.15 (316)	34.4 ± 22.86 (109)	32.1 ± 21.78 (207)
Disposition index	19.8 ± 13.03 (316)	20.8 ± 13.50 (109)	19.2 ± 12.77 (207)

Body condition index = birth weight/(wither height*diagonal length).

[a,b]Values within a row with different superscript letters are significantly different (all at P < 0.001).

Dam characteristics. The characteristics of the dams are presented in Table 13. Heifers (n = 169) calved at an average age of 24.9 ± 2.64 months. Cows (n = 312) completed 2.0 ± 1.30 lactations and aged 52.1 ± 18.33 months when they were measured. The M305, MPEAK and MGEST in the cows were 9284.8 ± 1650.48, 37.3 ± 8.17 and 6034.6 ± 1351.13 kg, respectively. Among the morphometric measures of the dams, HG was the one that showed the highest correlation with their M305 (r = 0.29) and MPEAK (r = 0.40), respectively (P < 0.001). The M305 was positively correlated with the MPEAK (r = 0.92, P < 0.001) and negatively correlated with TPEAK (r = -0.40, P < 0.001). The M305 tended to be positively correlated with LL (r = 0.09, P = 0.09). Moreover, the LL in cows was correlated with their TPEAK (r = 0.25, P < 0.001) and THALF (r = 0.17, P = 0.003), respectively.

Table 13. *Characteristics of the dams (mean ± SD)*

Characteristics	All dams (n = 481)	Heifers (n = 169)	Cows (n = 312)
Heart girth (cm)	209.0 ± 10.08	202.0 ± 7.13[a]	212.8 ± 9.40[b]
Wither height (cm)	143.1 ± 5.35	140.3 ± 4.44[a]	144.6 ± 5.20[b]
Diagonal length (cm)	166.5 ± 8.35	159.6 ± 6.32[a]	170.3 ± 6.74[b]
Lactation number	1.3 ± 1.42	0.0 ± 0.00[a]	2.0 ± 1.30[b]
Body condition score	3.0 ± 0.46	3.0 ± 0.44	3.0 ± 0.46
Age at calving (m)	42.5 ± 19.70	24.9 ± 2.64[a]	52.1 ± 18.33[b]
M305 (kg)	—	—	9284.8 ± 1650.48
TPEAK (day)	—	—	59.9 ± 17.96
THALF (day)	—	—	360.9 ± 198.60
MPEAK (kg)	—	—	37.3 ± 8.17
MPCONC (kg)	—	—	1010.6 ± 220.83
MGEST (kg)	—	—	6034.6 ± 1351.13
Days open (day)	—	—	120.2 ± 64.58
Lactation length (day)	—	—	342.6 ± 60.53
Length of dry period (day)	—	—	57.2 ± 22.11
Calving interval (day)	—	—	399.8 ± 64.29

M305, cumulative 305 days milk production; TPEAK, time to peak milk; THALF, time to half of the peak milk; MPEAK, milk production at lactation peak; MPCONC, cumulative milk production from 15 days before to 15 days after conception; MGEST, cumulative milk during gestation from conception to drying off.

[a,b]Values within a row with different superscript letters are significantly different (all at P < 0.001).

6.3.2 Factors Significantly Associated with Major Insulin Traits in Holstein Calves

Calves Born to All Dams. When data of all calves were analyzed (Table 14), insulin traits were significantly associated with gender and season of birth. The Ins_b was estimated to be lower (P = 0.001) and the QUICKI was estimated to be higher (P < 0.001) in male versus female calves. The Ins_b was estimated to be lower in calves born in Spring (P < 0.001) and Summer (P = 0.016) compared with calves born in Winter. The QUICKI was estimated to be higher in calves born in Spring (P < 0.001) and Summer (P = 0.002) compared with the

calves born in Winter. Moreover, the AIR (P = 0.003) and DI (P = 0.031) were estimated to be lower in male compared to female calves. The insulin traits in calves born to heifers were not associated with any factor (P > 0.05), except the QUICKI which was estimated to be higher in calves born in Spring (P = 0.038) compared with those born in Winter (Table 15).

Table 14. *Factors significantly affecting insulin traits in calves born to all dams*

Insulin traits	Predictors	Comparison (n)	Effect*	P-value
Basal insulin level	Calf gender	Male (238)	-	0.001
		Female (243)	Referent	
	Season of birth	Fall (148)	+	0.755
		Spring (54)	-	<0.001
		Summer (56)	-	0.016
		Winter (223)	Referent	
Quantitative insulin sensitivity check index	Calf gender	Male (238)	+	<0.001
		Female (243)	Referent	
	Season of birth	Fall (148)	+	0.898
		Spring (54)	+	<0.001
		Summer (56)	+	0.002
		Winter (223)	Referent	
Acute insulin response	Calf gender	Male (157)	-	0.003
		Female (159)	Referent	
Disposition index	Calf gender	Male (157)	-	0.031
		Female (159)	Referent	

*Referent means all other level(s) of the variable are compared to it; '+' is positive influence; '-' is negative influence

Table 15. *Factors significantly affecting insulin traits in calves born to heifers*

Insulin traits	Predictors	Comparison (n)	Effect*	P-value
Basal insulin level	Calf gender	Male (78)	-	0.143
		Female (91)	Referent	
Quantitative insulin sensitivity check index	Calf gender	Male (78)	+	0.109
		Female (91)	Referent	
	Season of birth	Fall (61)	-	0.695
		Spring (15)	+	0.038
		Summer (26)	+	0.561
		Winter (67)	Referent	
Acute insulin response	Calf gender	Male (48)	-	0.199
		Female (61)	Referent	
Disposition index	Calf gender	Male (48)	-	0.162
		Female (61)	Referent	

*Referent means all other level(s) of the variable are compared to it; '+' is positive influence; '-' is negative influence

Calves Born to the Cows. The insulin traits in calves born to cows were significantly associated with gender, season, MGEST, DP and LL (Table 16). The Ins_b was estimated to be lower (P = 0.008) and the QUICKI was estimated to be higher (P = 0.004) in male compared with female calves. The Ins_b was estimated to be lower in calves born in Spring (P < 0.001) and Summer (P = 0.003) compared with calves born in Winter. The QUICKI was estimated to be higher in calves born in Spring (P < 0.001) and Summer (P < 0.001) compared with calves born in Winter. The Ins_b was estimated to be higher in calves born to cows having passed a higher MGEST (P = 0.076) and longer DP (P = 0.034). QUICKI was estimated to be lower in calves born to cows having passed a higher MGEST (P = 0.030) and longer DP (P = 0.058). Moreover, the AIR (P = 0.009) and DI (P = 0.049) were estimated to be lower in male compared with female calves. Furthermore, the AIR (P = 0.036) and DI (P = 0.039) were estimated to be lower in calves born to cows having passed a longer LL.

Table 16. *Factors significantly affecting insulin traits in calves born to cows*

Insulin traits	Predictors	Comparison (n)	Effect	P-value
Basal insulin level	Calf gender	Male (160)	-	0.008
		Female (152)	Referent	
	Season of birth	Fall (87)	+	0.974
		Spring (39)	-	<0.001
		Summer (30)	-	0.003
		Winter (156)	Referent	
	MGEST[1] (kg)	Linear (312)	+	0.076
	Dry period (day)	Linear (312)	+	0.034
Quantitative insulin sensitivity check index	Calf gender	Male (160)	+	0.004
		Female (152)	Referent	
	Season of birth	Fall (87)	+	0.596
		Spring (39)	+	<0.001
		Summer (30)	+	<0.001
		Winter (156)	Referent	
	MGEST[1] (kg)	Linear (312)	-	0.030
	Dry period (day)	Linear (312)	-	0.058
Acute insulin response	Calf gender	Male (109)	-	0.009
		Female (98)	Referent	
	Lactation length (day)	Linear (207)	-	0.036
Disposition index	Calf gender	Male (109)	-	0.049
		Female (98)	Referent	
	Lactation length (day)	Linear (207)	-	0.039

[1]MGEST = cumulative milk production during gestation from conception to drying off.

6.4 Discussion

Milk production per cow is increasing annually on behalf of long-term genetic selection and improved management practices. High milk production in cows gives rise to reduced blood glucose, insulin and IGF-I levels and thereby favors a lower energy status (Albarrán-Portillo and Pollott 2013; Bossaert et al. 2014). We hypothesized that high milk production during gestation in cows affects the intrauterine development of the fetal endocrine pancreas and hence programs reduced insulin secretion in the newborn calves. The rationale behind this hypothesis originates from studies done in a variety of other species including humans (De Rooij et al. 2006), in which it is found that insulin secretion in newborns is significantly reduced in case of a lack of nutrients during intrauterine development (Limesand et al. 2013; Owens et al. 2007). This lack of nutrients can either be caused by a famine (De Rooij et al. 2006), a specific physiologic status like lactation (Berry et al. 2008; Funston and Summers 2013; Wu et al. 2006) or adverse environmental conditions (Tao et al. 2014). In a previous study (Bossaert et al. 2009), we have shown that in comparison to beef calves, dairy calves show signs of peripheral insulin resistance already early after birth. In that study, we used a limited number of calves in which we performed more extensive tests like the intravenous glucose tolerance test (IVGTT) and the intravenous insulin tolerance test (IVITT). However, the present epidemiological study was performed using a larger number of subjects. The latter however limits the use of sophisticated tests. Therefore, we used tests that are easier to perform under field conditions and which had been proven to be satisfying in comparable field studies performed in several other species including ruminants (Bergman et al. 2002; Limesand et al. 2013; Radunz et al. 2012). The present study is to the best of our knowledge one of the first to specifically address the direct effects of environment and dam characteristics including the level of milk production during gestation, on insulin traits in newborn Holstein calves. A diagrammatic representation of the relationship between insulin traits of the calves and the environmental factors associated with them is presented in Figure 11.
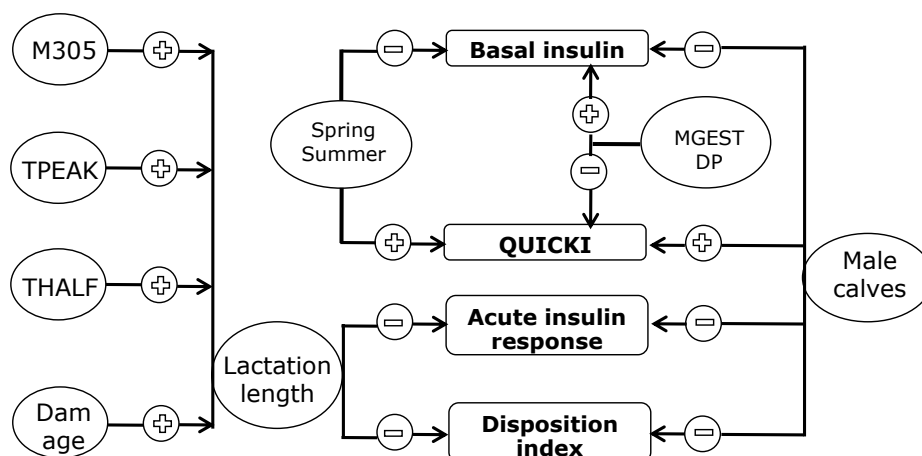
Figure 11. Diagrammatic representation of the relationship between insulin traits of calves (illustrated in rectangles) and environmental and dam variables (in ovals). Signs on the arrows represents the direction of the influence of the variable. M305, cumulative 305 days milk production; TPEAK, time to peak milk; THALF, time to half of the peak milk; MGEST, cumulative milk production during gestation from conception to drying off; DP, length of the dry period; QUICKI, quantitative insulin sensitivity check index; ⊕, positive influence; ⊖, negative influence

6.4.1 Calf Characteristics Including Insulin Traits

The BW and other size parameters of the calves recorded in the present study are comparable with previous investigations (Bossaert et al. 2014; Muri et al. 2005). However, BW represents fetal growth only at one point in time (= at the end of gestation), and does not provide an insight into the pattern of fetal growth throughout gestation. Therefore it is not surprising that BW and other size parameters are not correlated with any insulin traits of the calves in the present study. This result is consistent with previous studies where the impact of poor fetal growth on insulin secretion was not related to birth weight (Jaquiery et al. 2012; Limesand et al. 2013). The Ins_b is positively correlated with Glu_b in the present study, which is consistent with a previous investigation (Muri et al. 2005). Furthermore, in the present study, a relatively narrow range of Glu_b is maintained in the calves with a wide range of Ins_b. This indicates that the calves are able to control glycaemia effectively at the expense of variable concentrations of insulin (De Blasio et al. 2007). Basal insulin concentrations may depend on the ontogenic development of the endocrine pancreas, the insulin sensitivity of the peripheral tissues, as well as on the amount, time point, and frequency of colostrum intake (Hammon et al. 2012). However, the calves in the present study had all been fed 4 L of

colostrum within the first few hours after birth by esophageal tube. They were also fed equal amount of milk during first days of life. Besides, insulin concentration may depend on the diurnal variation (Lee et al. 1992), however, we did not find a significant difference in insulin concentration between Belgian and German herds. Moreover, insulin sensitivity and insulin levels are mutually related in such a way that reduced insulin sensitivity is compensated for by an increased insulin production. The latter is illustrated by the fact that Ins_b is related to QUICKI in a hyperbolic fashion in the present study, with insulin production increasing as insulin sensitivity decreases, as occurs in young lambs (De Blasio et al. 2007) and human beings (Bergman et al. 2002). When QUICKI was plotted against AIR, the data points also approximated a hyperbolic curve, as occurs in young lambs (De Blasio et al. 2007). The hyperbolic relationship between log QUICKI and log Ins_b, and between log QUICKI and log AIR in the present study is confirmed by a significant inverse linear relationship. This implies that the product of insulin secretion and sensitivity which is represented by the DI (Bergman et al. 2002) is nearly constant implicating that DI may be a good surrogate measure of β-cell function in relation to the peripheral insulin sensitivity since DI reflects the ability of the endocrine pancreas to compensate for reduced insulin sensitivity. Therefore, this index has been increasingly used in metabolic studies in animals. In the present study, the insulin traits calculated for the calves born to heifers were not significantly different from the ones calculated for the calves born to cows. This finding implies that the calves born to heifers do not display altered intrauterine development of the endocrine pancreas compared with the calves born to lactating cows although significant differences exist in blood metabolite concentrations between heifers and cows during gestation (Bossaert et al. 2014).

6.4.2 Factors Significantly Associated with Major Insulin Traits in Holstein Calves

Calf Gender. We observed sexual dimorphism in insulin sensitivity and insulin secretion in the Holstein calves. Although both sexes showed similar fasting plasma glucose concentrations, serum insulin levels are higher in female than in male calves. In other words, female calves produce more insulin to maintain similar glycaemic levels than male calves. Insulin sensitivity correlates positively to the percentage of type I muscle fibers in calves (Sternbauer and Essen-Gustavsson 2002). The male calves in the present study are heavier than the female calves. We speculate that a higher amount of muscle fibers may partially explain higher QUICKI and thereby lower Ins_b in the male calves (Sternbauer and Essen-Gustavsson 2002). The latter is furthermore confirmed by the results of our previous study in which we showed that calves of the double muscled Belgian Blue breed had significantly

lower Ins_b and QUICKI values in comparison to Holstein calves (Bossaert et al. 2009). Similarly, female babies have higher insulin concentrations at birth (Wilkin and Murphy 2006) and they are less responsive to the trophic effects of insulin owing to mutations in the glucokinase gene (Wilkin and Murphy 2006). Furthermore, our data show a significantly lower insulin secretory response following a standard glucose bolus (as indicated by lower AIR and DI) in male calves in comparison to female calves. The gender-specific programming of insulin secretion and action identified in the present study is important in relation to earlier studies showing increased vulnerability of male calves, compared with females, to the later development of adiposity as a consequence of early growth restriction (Jaquiery et al. 2012). Also in human, men are generally known to have a higher risk to suffer from insulin resistance, diabetes mellitus type 2 and cardiovascular disease (Geer and Shen 2009; Sattar 2013). The latter further illustrates the lower capacity of the endocrine pancreas of male individuals to compensate for peripheral insulin resistance in adult life (De Rooij et al. 2006; Fleming et al. 2012; Morrison 2008). In the bovine, a disease complex characterized by insulin resistance, hyperglycemia and glucosuria is well known in intensively fed veal calves (Hugi et al. 1997). While the origin of this metabolic disease complex is generally attributed to the specific nutritional characteristics of these calves, it is remarkable that the disease is only mentioned in bull calves and not in replacement heifer calves. This may be attributed by the level of nutrition offered to young calves (Bach et al. 2013) in addition to the insulin sensitivity and the ability of the endocrine pancreas to secrete insulin at that stage (Jaquiery et al. 2012; Sternbauer and Essen-Gustavsson 2002).

Season of Birth. Our previous research has shown that heifers and cows completing their late gestation during the hotter seasons deliver smaller calves, which reflects a compromised fetal development *in utero* (Kamal et al. 2014). The latter is very similar to studies in sheep in which heat stress during gestation has been shown to be associated with the birth of smaller lambs (Morrison 2008). In addition, the Ins_b is lower and QUICKI is higher in the calves born in Spring and Summer in comparison to the calves born in Winter in the present study. Our result is consistent with a previous study where reduced insulin levels and enhanced insulin sensitivity have been observed in calves born following maternal heat stress during late gestation (Tao et al. 2014). This implies that calves born in Spring and Summer are more efficient in moving glucose from the circulation into insulin dependent tissues due to increased insulin sensitivity, leading to a lower pool of circulating glucose (Tao et al. 2014). We speculate that calves born to dams that were subjected to higher ambient temperatures

during late gestation have impaired pancreatic maturation *in utero* (Limesand et al. 2013). The late gestation heat stress compromises placental development, which results in fetal hypoxia, undernutrition, and eventually fetal growth retardation (Morrison 2008). However, the extent of stress among dairy cows in response to heat may vary according to geographical locations across the globe. Moreover, a decrease in dry matter intake is a hallmark of the heat stress response in animals and undernutrition is a factor in the reduced pancreatic growth (Wu et al. 2006). Furthermore, a longer photoperiod during hotter seasons leads to increased plasma prolactin concentrations in cows, supporting higher milk production and thereby limiting nutrient availability for intrauterine growth (Accorsi et al. 2005). Our data indicate that the basal metabolism of calves is affected by the seasonal stress imposed on the mother during late gestation.

Lactation characteristics in cows. We have tested the hypothesis that intrauterine growth of fetal endocrine pancreas is compromised when the dam is targeting high levels of nutrients into milk production during gestation. However, the effect is opposite to our hypothesis with the view that the Ins_b is higher and QUICKI is lower in the calves born following a greater MGEST in cows in the present study. Such association between the insulin traits and MGEST is rather difficult to explain. However, the energy status during lactation in cows may be affected by cow factors irrespective of the level of MGEST (Piccardi et al. 2013; Sensosy et al. 2012). The cows having a higher MGEST had a shorter DO (r = -0.47, P < 0.001) and DP (r = -0.52, P < 0.001) in the present study, which is in line with previous reports (Atashi et al. 2013). Perhaps cows having higher MGEST are in a better energy status and, therefore, also provided a better maternal environment for the intrauterine development of the endocrine pancreas giving rise to higher insulin levels and lower insulin sensitivity in the calves. In less productive cows, daily milk production often decreases to low levels before the planned drying off date; therefore, farmers no longer keep these cows in production, which results in longer DP compared with higher producing cows (Atashi et al. 2013). In our study, the Ins_b is higher and QUICKI is lower in the calves born to the cows having passed a longer DP. Although no difference exists in serum glucose, insulin, and IGF-I levels in cows with short versus longer DP (Pezeshki et al. 2007), we speculate that cessation of partitioning nutrients toward milk for extended duration in cows experiencing a longer DP, provides higher amounts of energy and amino acids toward growth and maturation of the pancreatic β-cell, giving rise to higher insulin levels in the calves (Long et al. 2010). However, the MGEST and DP are not significantly associated with the insulin secretion at the stimulated state in the

present study. This may be due to the fact that only a subset of the calves was employed for the GSIS test.

A longer LL in cows leads to a longer partitioning of energy and nutrients towards the udder and less towards the uterus. The lower partitioning of nutrients to the gravid uterus in cows may lead to less glucose crossing the placenta and hence a retardation in the development of the embryo (Green et al. 2012; Sensosy et al. 2012). Although the energy requirements for the embryo may be relatively low, metabolic activity is high and this represents a critical period for epigenetic control and organogenesis during fetal development (Fleming et al. 2012; Wu et al. 2006). Certainly, β-cells in pancreatic islets develop during the embryonic and early fetal period of the intrauterine development in cattle (Carlsson et al. 2010). Exposure of dams to reduced nutrient intake during early gestation is associated with reductions in insulin concentrations in sheep fetuses (Limesand et al. 2013) and in calves (Long et al. 2010). The adaptations to nutrient restriction that limit insulin concentrations are not fully known, but a morphological study demonstrated smaller pancreatic islets and a decrease in β-cell mass following early growth restriction in sheep (Limesand et al. 2013). Chronic hypoglycemia during later stages of fetal development leads to an intrinsic islet defect that is responsible for the decreased insulin secretion in sheep offspring without significantly decreasing their β-cell mass (Rozance et al. 2006). In the present study, the AIR is lower in calves born following longer LL in cows. The lower insulin secretion in the calves born to the cows with longer LL is even evident after adjusting for the sensitivity, as indicated by a lower DI. We speculate that chronic glucose partitioning towards the udder during the entire period of gestation is present in cows with longer LL which potentially contributes to a reduced β-cell mass due to slower proliferation rates and subsequently a reduced insulin secretion in the newborn calves (Limesand et al. 2013; Lucy et al. 2014; Rozance et al. 2006). Considerable evidence suggests that energy restriction before birth is associated with enhanced insulin sensitivity in early postnatal life. However, this dysfunction appears to emerge in case the nutrient restriction in earlier parts of gestation is followed by abundance of nutrients in late part of gestation (Long et al. 2010; Radunz et al. 2012). In our study, the QUICKI of the calves was not associated with the LL in cows. This observation is consistent with a study in sheep where nutritional restriction throughout gestation was imposed to the fetal lambs by removal of the majority of the endometrial caruncles from the uterus of the dams (Owens et al. 2007). The latter gave rise to an impaired insulin secretion without any enhancement of insulin sensitivity in the fetal lambs (Owens et al. 2007). These

findings invoke that elongation of LL in cows, or factors associated with the longer LL, impair β-cell responsiveness to glucose stimulation, hence insulin secretion in the newborn calves (Gutierrez et al. 2012; Owens et al. 2007; Rozance et al. 2006).

Major finding of the present study is that major insulin traits in newborn Holstein calves are significantly associated with gender and season of birth. Subsequently, in calves born out of multiparous dams, insulin traits are significantly associated with the amount of milk produced during gestation, lactation length and length of the dry period (being related to the intrauterine nutritional environment) prior to their birth. Whether the changes in insulin traits early in life exert long-term effects on the metabolic function in later life is unknown. However, several reports have linked lactation during gestation in the dam with impaired future performance and longevity of the offspring (Berry et al. 2008; Gonzalez-Recio et al. 2012). If the alterations in insulin traits of the calves persist in later life, as they do in newborns confronted with intrauterine growth restriction in other species, they may contribute to adverse metabolic outcomes in later life.

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GENERAL DISCUSSION

Dam nutrition during gestation in mammals has been recognized as a key to metabolic programming *in utero*. In humans, there has been an explosion of data showing that perturbations like undernutrition during prenatal life program fetal development in the sense that survival in the short term is maximised, but significantly increases the risk of suffering from a diverse range of health problems in later life (Eriksson 2016; Marciniak et al. 2017). Intrauterine programming of postnatal physiological function has been demonstrated experimentally in several species using a range of techniques to compromise the intrauterine environment and alter fetal development (Sferruzzi-Perri et al. 2017; Sferruzzi-Perri and Camm 2016; Smits et al. 2012). Such intrauterine programming is obvious in high-producing dairy cattle, which evolved from 2000 to 8000 kg milk production per year over a period of 50 years. To maximise milk production, farmers breed their stock at young age in order to have a first calf at 24 months of age (Wathes et al. 2014), and subsequently have their cows calved at intervals no longer than 385-400 days (Inchaisri et al. 2010). The latter implies dairy cattle are rather atypical because they have to manage the compatibility of optimal reproductive performance and (early) gestation with continued growth or the production of large quantities of milk. Rather than being an absolute shortage of energy substrates *per se*, this metabolic priority for growth and lactation (after calving) is known to generate adverse conditions hampering optimal ovarian function, follicular growth, oocyte maturation and early embryonic development (Leroy et al. 2008). We speculate that the atypical fact that adolescent animals are still growing significantly while being pregnant and that adult cows have to partition large quantities of nutrients to the udder during gestation both affect the prenatal development of the calf and potentially the development of its metabolic features, jeopardising its health, fertility and productivity in later life. The association of young age in heifers and high milk production during gestation in cows with reduced longevity and productivity of their offspring (Banos et al. 2007; Berry et al. 2008; Gonzalez-Recio et al. 2012), furthermore emphasizes the potential impact of these features on metabolic programming *in utero*. Therefore, we focused our study on the feto-placental development in relation to growth in heifers and level of milk production in cows, and we specifically addressed the direct effects of other environmental and dam characteristics on feto-placental development. In this study, growth and milk production during gestation have been identified as important determinants for the feto-placental development in heifers and cows, respectively. Additionally, calf gender, season of calving, gestation length, parity and morphometrics of the dam and length of the dry period are reaffirmed as factors being significantly associated with calf birth size and placental traits. Subsequently, insulin traits in

calves born to multiparous dams are significantly associated with the amount of milk produced during gestation, lactation length and length of the dry period prior to their birth.

7.1 General Findings

Birth weight: Birth weight of calves has been used as important descriptor of birth size in comparison with other measures (Graham et al. 2010; Lundborg et al. 2003; Swali and Wathes 2006). It is a complex trait influenced by the interaction between genetic and non-genetic intrauterine factors (Kocak et al. 2007). Therefore, the use and interpretation of birth weight in relation to fetal programming assumes that it indeed reflects specific fetal responses to particular variations in the nutritional status of the dam. Several studies have been published on the impact of specific environmental factors in relation to birth weight of calves (Kocak et al. 2007; Swali and Wathes 2006; Zhang et al. 2002). In consistence with these studies, BW of calves is retained as the most consistent and objective measure to describe the size of the calves at birth (Kamal et al. 2014). In other species including humans, it has also been shown that detrimental environmental factors experienced by the dam during gestation (e.g. starvation or illness), do have a negative effect on embryonic or fetal development which became clear by a lower weight of the offspring at birth (Sharma et al. 2012; Stein et al. 2004; Tao et al. 2012). Conclusively, animal experiments and human epidemiological data show that a wide range of individual tissues and whole-organ systems can be programmed *in utero* with adverse consequences for their physiological function later in life. This programming occurs across the normal range of birth weights with the worst prognoses at the extremes (Fowden et al. 2006a).

Birth weight is often used as a measure of fetal growth and development in pregnancy, but it is also understood to be an imprecise measure because it represents fetal growth at one point in time at the end of gestation and does not provide any insight into the pattern of fetal growth throughout full gestation (Wilcox 2001). For example, intrauterine growth restriction (IUGR) may result from poor placental development early in pregnancy, leading to reduced substrate supply for optimal fetal growth throughout full gestation. Alternatively, placental growth may have occurred normally in early pregnancy, but an insult in late gestation may result in a severe but short-lived reduction in placental substrate delivery to the fetus, leading to IUGR due to acute substrate restriction. Similarly, there may have been several short but severe reductions in substrate supply to the fetus over late gestation. Animal studies have demonstrated that the timing, duration and exact nature of the insult during pregnancy are all

important determinants of the pattern of intrauterine growth and the specific physiological outcomes. It is clear, for example, that the period of pregnancy during which nutrient restriction is experienced determines which of the cell types and hence also organs is most affected (Bertram and Hanson 2001; Fowden et al. 2006a). The effects of all these determinants have been confirmed by the fact that maternal and environmental insults with little if any effect on birth weight can alter subsequent cardiovascular and metabolic function.

Placental Development: The placenta is pivotal in the competition between mother and fetus for resource allocation. In the bovine, the caruncular-cotyledonary unit is called a placentome and is the primary functional area of physiological exchange between the mother and her fetus (Jainudeen and Hafez 1993). Cotyledonary growth progressively increases throughout gestation, in combination with an increase in capillary area, villous surface and number (Baur 1972; Funston et al. 2010; Vonnahme et al. 2007). Obviously, it is expected that the placental exchange area may be related to the cotyledonary base area (Kannekens et al. 2006). Indeed, the highest correlation between calf measurements and placental characteristics is found between the weight of the calf and the total cotyledonary surface (Kamal et al. 2017), confirming the high importance of the cotyledonary surface availability for nutrient transfer to fetal development (Sullivan et al. 2009). Basically, under conditions of impaired nutrient supply that may limit normal fetal growth, the placenta has shown to adapt so as to increase its transport capacity (Burton and Fowden 2012). Earlier studies in beef cattle and sheep demonstrated that placentas may adapt their phenotype to accommodate fetal nutrient demand and that this process may include either morphological or functional mechanisms acting during different stages of fetoplacental development (Fowden et al. 2010; Long et al. 2009; Sullivan et al. 2009).

Placental development responds to both fetal signals of nutrient demand and maternal signals of nutrient availability and, by adapting its phenotype, regulates the distribution of available resources (Fowden et al. 2006b; Fowden and Moore 2012). Placental adaptation may occur as a result of changes to the placental surface area for nutrient transfer, the thickness of the interhaemal barrier separating the maternal and fetal circulations, the placental abundance of nutrient transporters, metabolic rate and blood flow, as well as placental synthesis and metabolism of specific hormones (Fowden and Moore 2012; Vaughan et al. 2012). Once placentation has begun, the programming effects of environmental signals may be mediated via changes in placental development, and therefore, fetal IUGR due to nutritional constraints is closely linked to placental development and function (Fowden et al. 2008; Cetin and Alvino

2009). Usually, there are 70-120 placentomes to serve as the functional unit for feto-maternal exchange. In *Bos Indicus* cattle, the number gradually increases averaging about 80 up to day 70 post-insemination (Assis Neto et al. 2010). Usually there is no change in the number of bovine cotyledons from day 90 of gestation, while the mean weight and surface of each placentome may continue to increase (Laven and Peters 2001), resulting in their characteristic mushroom-like shape (Leiser et al. 1997). Obviously, nutritional effects on cotyledonary growth may occur earlier during gestation, but compensatory cotyledonary growth occurs in the third trimester to meet the rapid increase in nutritional demands by the fetus (Zhu et al. 2007b). The birth weight of the calves is positively correlated with placental weight and cotyledonary size and weight (Kamal et al. 2017; Shah et al. 2007; Zhang et al. 1999). Moreover, calf gender has been reported to affect cotyledonary parameters in sheep (Dwyer et al. 2005) and cattle (Zhang et al. 1999). Therefore, the cotyledons are of great biological significance for the development and growth of the fetus.

Insulin Traits: In dairy cattle, pregnancy and lactation are two physiological states very important for the survival of the species, both significantly increasing the demands for glucose. During peak milk production, it is impossible to meet glucose requirements by increasing dry matter intake and thus cows go into a period of negative energy and glucose balance. To support the partitioning of glucose towards the pregnant uterus and the lactating mammary gland, substantial metabolic adaptations have to occur at the level of the carbohydrate, protein and lipid metabolism. The latter is especially the case at the end of pregnancy and the beginning of lactation to fully support the challenging physiological environment at that specific time. The cow shifts her metabolism towards increased glucose production, decreased glucose use by non-mammary tissues and increased use of lipids to provide energy. These changes occur through increased hepatic glucose production, glucose sparing by non-mammary tissues, increased glucose uptake by the mammary gland, increased hepatic glycogen mobilisation, decreased lipogenesis, increased lipolysis, increased proteolysis, increased ketogenesis and increased use of ketone bodies (Bell 1995; De Koster and Opsomer 2013a).

We have measured peripheral glucose and insulin concentrations in neonatal calves as a proxy for their glucose metabolism (Kamal et al. 2015). The calves are able to control glycaemia effectively at the expense of variable concentrations of insulin (De Blasio et al. 2007; Muri et al. 2005). Basal insulin concentrations may depend on the ontogenic development of the endocrine pancreas, the insulin sensitivity of the peripheral tissues, as well as on the amount,

time point, and frequency of colostrum intake (Hammon et al. 2012). Moreover, insulin sensitivity and insulin levels are mutually related in such a way that reduced insulin sensitivity is compensated for by an increased insulin secretion. The latter is illustrated by the fact that basal insulin level and the acute insulin response are related to the insulin sensitivity in a hyperbolic fashion, with insulin production increasing as insulin sensitivity decreases, as occurs in young lambs (De Blasio et al. 2007) and human beings (Bergman et al. 2002). Therefore, the product of insulin secretion and sensitivity which is represented by the disposition index (DI) (Bergman et al. 2002) is nearly constant, implicating that the DI may be a good surrogate measure of β-cell functions in relation to the peripheral insulin sensitivity. Whether the glucose and insulin traits early in life exert long-term effects on metabolic function in later life is not known. However, when the alterated glucose-insulin traits of the calves persist in later life, as they do in newborns confronted with IUGR in other species, they may contribute to adverse metabolic outcomes in later life.

Parity of Dam: In general, the reproductive capacity of nulliparous heifers is higher than that of multiparous cows. This can likely be attributed to the fact that oocytes and embryos of nulliparous heifers have not been challenged by the metabolic stress of milk production. Furthermore, uteri of nulliparous heifers have not been confronted yet with the consequences of a calving event, which, in most cases, is associated with bacterial contamination. In addition to the better reproductive performance of nulliparous heifers, significant differences have been noted in terms of production, reproductive capacity, longevity and resilience against metabolic challenges between the offspring of first- versus higher-parity animals (Banos et al. 2007; González-Recio et al. 2012), with, in most cases, the offspring of first-parity animals being in a more favourable condition. The latter may be interpreted as an indication of the deleterious effect of lactation during conception and early pregnancy because, in contrast with multiparous dairy cows, first-parity animals do not lactate. Therefore, all too often researchers use first-parity heifers as non-lactating and hence 'negative' controls when examining the effects of lactation and its concomitant metabolic consequences on the prenatal development of offspring. However, researchers should be aware that when pregnancy coincides with continued growth of the first parity dam, the fetus may face intense competition for nutrients for its mother's own metabolic needs while still growing (Kamal et al. 2014).

In comparison to young growing animals, multiparous and hence lactating dams gave birth to heavier calves (Kamal et al. 2014), which is in line with results of previous studies (Dhakal et

al. 2013; Johanson and Berger 2003). The shorter gestation length in heifers in comparison to cows at least accounts for the birth weight difference (Dhakal et al. 2013; Norman et al. 2009). Another plausible reason is that the heifers usually are still growing during their first gestation and preferentially use nutrients for their own growth rather than for the growth and development of their fetus (Funston and Summers 2013). The latter is furthermore visualized by a reduced development of the placenta in heifers (Funston and Summers 2013; Symonds et al. 2010) and a higher increase in total cotyledonary surface and hence less efficient placentas in cows (Kamal et al. 2017). The latter suggests lactation to be associated with a more pronounced placental compensation which is effectuated by an increased size of the cotyledons. Moreover, cows also become more resistant to the anabolic effects of growth hormone and placental lactogen (Weber et al. 2007), resulting in a degree of insulin resistance, which allows more glucose to be transferred to the fetus in gestating cows (Bell et al. 1995). Therefore, when compared to heifers, mature cows give birth to heavier calves (Dhakal et al. 2013; Johanson and Berger 2003). However, the insulin traits of the calves born to heifers are not different from the calves born to cows (Kamal et al. 2015). This implies that the calves born to heifers do not display an altered intrauterine development of the endocrine pancreas compared with the calves born to lactating cows although significant differences exist in blood metabolite concentrations between heifers and cows during gestation (Bossaert et al. 2014). Nevertheless, several reports have linked lactation during gestation in the dam with impaired future performance and longevity of the offspring (Berry et al. 2008; González-Recio et al. 2012).

Gender of the Calves: Male calves are generally heavier than female ones. Calf gender has been reported to affect cotyledonary parameters in sheep (Dwyer et al. 2005) and cattle (Zhang et al. 1999). Male calves are carried longer than the female ones, which may partially explain their higher birth weight (Dhakal et al. 2013). Moreover, gender-specific genes affecting insulin sensitivity such as mutations in the glucokinase gene may be responsible for the gender difference in birth weight. The genetically more insulin resistant female fetus is less responsive to the trophic effects of insulin owing to mutations in the glucokinase gene and is therefore lighter (Hattersley et al. 1998; Wilkin and Murphy 2006). The gender insulin hypothesis is also inspired by observations of the reduced effects of fetal insulin concentrations on placental growth in individuals affected by mutations in the glucokinase gene (Shields et al. 2008). Female calves produce more insulin to maintain similar glycaemic levels than male calves (Kamal et al. 2015). A higher amount of muscle fibers in the male

calves may partially explain higher insulin sensitivity and thereby lower basal insulin levels (Sternbauer and Essen-Gustavsson 2002). The latter is furthermore confirmed by the results of a study in which it is shown that calves of the double muscled Belgian Blue breed had significantly lower basal insulin and a higher sensitivity in comparison to Holstein calves (Bossaert et al. 2009). Similarly, female babies have higher insulin concentrations at birth (Wilkin and Murphy 2006). Furthermore, a significantly lower insulin secretory response following a standard glucose bolus is usual in male calves in comparison to female calves.

The gender-specific programming of insulin secretion and action is important, showing an increased vulnerability of male calves, compared with females, to the later development of adiposity as a consequence of early growth restriction (Jaquiery et al. 2012). In humans, men are generally known to have a higher risk to suffer from insulin resistance, diabetes mellitus and cardiovascular disease (Geer and Shen 2009; Sattar 2013). The latter further illustrates the lower capacity of the endocrine pancreas of male individuals to compensate for peripheral insulin resistance in adult life (De Rooij et al. 2006; Fleming et al. 2012; Morrison 2008). In cattle, a disease complex characterized by insulin resistance, hyperglycemia and glucosuria is well known in intensively fed veal calves (Hugi et al. 1997). While the origin of this metabolic disease complex is generally attributed to the specific nutritional characteristics of these calves, it is remarkable that the disease is only mentioned in bull calves and not in replacement heifer calves. This may be attributed by the level of nutrition offered to young calves (Bach et al. 2013) in addition to the insulin sensitivity and the ability of the endocrine pancreas to secrete insulin at that stage in bull calves (Jaquiery et al. 2012; Sternbauer and Essen-Gustavsson 2002).

Season: The later part of gestation is known to be critical for fetal growth. Heifers and cows completing the last trimester during a hot season give birth to smaller calves (Kamal et al. 2014; Linden et al. 2009; Tao et al. 2012). This finding is also similar to a study in sheep in which heat stress during gestation has been shown to be associated with the birth of smaller lambs (Morrison 2008). One potential factor is reduced dry matter intake of the dam during the last part of gestation due to heat stress (Umphrey et al. 2001), contributing to decreased nutrient availability and consequently a lower birth weight of calves (Linden et al. 2009; Norman et al. 2009). Similarly, a longer photoperiod during the second part of gestation leads to increased plasma prolactin concentrations, supporting higher milk production and thereby limiting nutrient availability for intrauterine calf growth (Accorsi et al. 2005; Garcia-Ispierto et al. 2009). Another plausible factor is the shorter gestation length of the pregnancies during

the hotter seasons (Tao et al. 2012), which has been shown to be associated with the large variation in temperature and humidity at that time (Dhakal et al. 2013; Norman et al. 2009). Furthermore, heat stress during late gestation is associated with decreased uterine blood flow and reduced placental function giving rise to an impaired dam-to-fetal exchange of glucose and amino acids (Reynolds et al. 1985).

The energy metabolism of calves is affected by the seasonal stress imposed on the mother during late gestation. Reduced insulin levels and enhanced insulin sensitivity have been observed in calves born following maternal heat stress during late gestation (Guo et al. 2016; Kamal et al. 2015; Tao et al. 2014). The calves are more efficient in moving glucose from the circulation into insulin dependent tissues due to increased insulin sensitivity, leading to a lower pool of circulating glucose (Tao et al. 2014). So, the calves born to dams that were subjected to higher ambient temperatures during late gestation, have impaired pancreatic maturation *in utero* (Limesand et al. 2013). The late gestation heat stress compromises placental development, which results in fetal hypoxia, undernutrition, and eventually fetal growth retardation (Morrison 2008). Moreover, a decrease in dry matter intake is a hallmark of the heat stress response in animals and undernutrition is a factor in the reduced pancreatic growth (Wu et al. 2006).

Metabolic alteration in calves born to heat-stressed cows persists until the preweaning period (Tao et al. 2014; Monteiro et al. 2016) and has been associated with reduced milk production during the first lactation (Monteiro et al. 2013). Similar effects are also observed in sheep where intrauterine growth restriction caused by maternal heat stress during early to mid-gestation resulted in compromised insulin synthesis and secretion in the lambs compared with those that develop under thermoneutrality (Limesand et al. 2006). Moreover, it is observed that the calves born to heat-stressed dry cows had a lower plasma concentration of cortisol immediately after birth compared with calves born to cooled cows, indicating that maternal heat stress alters the fetal development of hypothalamus-pituitary-adrenal axis and related stress responses during the postnatal period (Tao et al. 2012). The metabolism of an animal is of importance in growth and body composition, which in turn alters her future productive and reproductive performance. Therefore, metabolic adaptation of heat-stressed calves during the transition from intra- to extrauterine life might affect the future performance of the calves. However, since there are significant differences in climatological relative humidity and diurnal temperature range across the globe, metabolic programming of dairy cattle owing to heat stress varies not only seasonally, but also geographically.

7.2 Programming in Relation to Young Age in Dams

The fetus may experience intense competition for nutrients for its mother's own metabolic needs in the case she is still growing, resulting in lower birth weight of calves (Kamal et al. 2014). In adolescent animals, the 'normal' hierarchy of nutrient partitioning between maternal body growth and fetal growth has been shown to be altered (Wallace et al. 2006). In sheep, rapid maternal growth seems to result in placental growth restriction and often premature delivery of low birth weight lambs compared with moderately nourished ewes of equivalent age (Wallace et al. 2006). Young age has also been shown to affect neonatal birth weight in humans where growth of teenage mothers during gestation is associated with increased risks for low birth weight babies (Chen et al. 2007). In the current dairy industry, gestation in heifers usually occurs while animals are physically immature and still growing (Kertz et al. 1997). It is found that heifers that are larger in body frame at calving deliver significantly heavier calves, which is in accordance with previous studies (Linden et al. 2009; Swali and Wathes 2006). However, the calf birth weight is curvilinear with the age of the heifers at calving (Kamal et al. 2014). Calves born to very young (20.3 to < 22 mo) heifers have a comparatively lower birth weight in comparison to calves born to young (22 to < 23.5 mo) heifers suggesting that the intrauterine environment may limit fetal calf growth due to competition for nutrients with dam growth (Wathes et al. 2008). Moreover, a suboptimal growth associated with low IGF-I concentrations results in heifers that conceive at an older age (25.5 to 37.3 mo) (Brickell et al. 2009; Wathes et al. 2008). Because the IGF system can modulate the delivery of substrates to the fetus, we speculate that the lower IGF-I in older heifers during gestation, may be responsible for the lighter birth weight of their calves (Brickell et al. 2009; Gutierrez et al. 2012; Wathes et al. 2008). Moreover, the gestation length in heifers is curvilinear, giving rise to a shorter gestation length in both young and old animals in comparison to the intermediate aged group, which further contributes to the lower birth weight of calves born to relatively young and old first parity dams (Norman et al. 2009; Simerl et al. 1991).

We aimed to detect maternal factors associated with placental development in cattle. The main finding of the study is that maternal growth during gestation significantly challenges placental development (Kamal et al. 2017). In our model, the factor contributing the most to gross placental morphology is age at conception in heifers. This is significantly but negatively associated with the number of caruncles occupied by cotyledons (Kamal et al. 2017). The latter indicates that the placenta compensates in times of nutrient restriction so that in

younger, more extensively growing heifers, the placenta compensates by occupying significantly more caruncles in order to guarantee the development of the offspring. More cotyledons are expected in placentas of heifers fed low dietary protein as these may have increased their caruncular occupancy to compensate and to meet the nutritional demands of the fetus (Sullivan et al. 2009). Similar placental compensation in terms of number of cotyledons has also been reported in sheep (Wallace et al. 1999). Negative correlations between insulin-like growth factor binding protein (IGFBP) concentrations throughout gestation and cotyledon number at expulsion in cattle suggests that lower IGFBP concentrations in times of nutrient restriction may increase the bioavailability of IGF-I, resulting in the formation of more cotyledons over caruncular sites (Sullivan et al. 2009).

Overall, our results suggest two potential compensatory mechanisms in the placenta. First, in early pregnancy, the placenta may cope by developing more cotyledons over the available caruncles, enabling pregnancy to survive. Second, at the end of pregnancy, an expansion of the cotyledonary surface is suggested to meet the increasing nutrient demands of the fetus. Although the placenta seems to compensate for nutritional challenges by adapting cotyledonary characteristics, it is suggested that placental efficiency remains lower in these pregnancies, with potential consequences for the developing fetus. However, the contribution of maternal tissue in this compensatory mechanism and the microscopic characteristics of the fetomaternal interface should be more deeply investigated.

A summary of effects of the dam characteristics on the newborn calves is presented in Figure 12. Conclusively, age at calving in heifers is indicated as decisive determinant of the calf's birth size and placental development. This novel finding may provide a basis for developing managerial interventions to improve long-term health and productivity of the offspring.
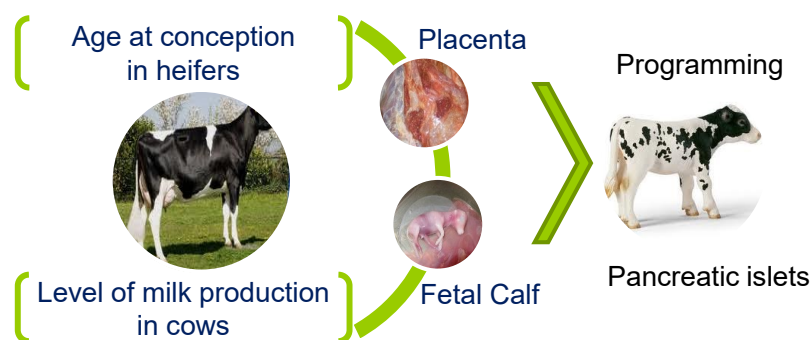

Figure 12. Summary of effects of the dam characteristics on the newborn calves.

7.3 Metabolic Programming in Relation to Lactation in Dam

The genetic drive to produce large quantities of milk makes modern dairy cows more vulnerable to factors generally known to impair overall health and fertility. Besides, the reproductive capacity of modern dairy cows is under extreme pressure especially because of very high rates of (early) embryonic mortality (Wiltbank et al. 2016). The latter may be a reflection of the high number of insults the gametes and early embryos are confronted with during the periconceptual period (Leroy et al. 2015; Ribeiro et al. 2016). In these cows, lactation during gestation leads to a significant loss of nutrients (like proteins and glucose) for the fetus, because these are diverted towards the udder instead of to the gravid uterus. We have calculated that modern multiparous dairy cows, on average, produce 6193.1 kg milk during their 278-day gestation (Kamal et al. 2014). This implies that the calf developing *in utero* in the lactating cow 'misses' in total 446 kg glucose (on average 72 g glucose per kg milk produced) and 217 kg proteins compared with a calf developing in a non-lactating dam. Consequently, for a cow with similar heart girth, the birth weight of calves born to cows producing more than 7200 kg milk during gestation was, on average, 1 kg lower than the weight of calves born to lower-producing cows (Kamal et al. 2014). The depletion of energy reserves in lactation overlapping with gestation negatively affects the birth weight in humans (Sengul et al. 2013). Glucose, insulin and IGF-I concentrations in cows are negatively correlated with their level of milk production during the production phase of lactation (Ingvartsen and Friggens 2005; Taylor et al. 2004), as shown by a negative correlation between BCS and the level of milk production (Yamazaki et al. 2011). These adaptations associated with high milk production in turn affect glucose availability and concentrations of insulin and IGF-I for the embryo and thereby affect the growth of both the early embryo and the placenta (Green et al. 2012). Though the nutrient requirements for the embryo may be relatively low, metabolic activity is high and this represents a critical period for epigenetic control and organogenesis of subsequent fetal development (Van Soom et al. 2013; Wu et al. 2006).

In modern high yielding dairy cows, a poor intrauterine nutritional environment associated with lower glucose, insulin and IGF-I levels in the cows may even occur in later phases of lactation due to the high persistency of milk production (Ingvartsen and Friggens 2005). These phases of lactation often coincide with the mid and late part of gestation which are known to be most crucial in terms of fetal growth. Therefore, the effects of variation in nutritional environment at that time may even have greater effects than in early gestation

(Stein et al. 2004; Wu et al. 2006). For example, significant reductions in birth weight have been shown to be caused by low plasma glucose concentrations during mid and late gestation (Bell et al. 1995; Zhang et al. 2002). The cows with higher milk production tended to have a shorter subsequent dry period (Atashi et al. 2013; Kamal et al. 2014). The shortening and omitting of the dry period shifts milk yield from the postcalving to the precalving period; resulting in an improvement in the body weight and energy balance in early lactation (van Hoeij et al. 2017; van Knegsel et al. 2014). In less productive cows, daily milk production often decreases to low levels before the planned drying off date; therefore, farmers no longer keep these cows in production, which results in a longer dry period in comparison with higher yielding cows (Atashi et al. 2013). Although there is no difference in serum glucose, insulin and IGF-I levels in cows with short versus longer dry period (Pezeshki et al. 2007), cessation of partitioning nutrients towards milk for extended duration in case of a longer dry period, provides higher amounts of energy and amino acids toward fetal growth giving rise to higher birth weight of the calves. These results suggest that concomitant selection for greater milk yield and higher persistency (resulting in a shorter dry period) in dairy cows may lead to reduced glucose availability for the developing embryo and fetus, with subsequent deleterious repercussions for the birth weight of calves and potentially their survival and life time performance (Banos et al. 2007; Berry et al. 2008; González-Recio et al. 2012).

We aimed to detect maternal factors associated with placental development in cows (Kamal et al. 2017). The main finding of that study was that maternal lactation during gestation significantly challenges placental development. In comparison to young growing animals, multiparous and hence lactating dams were associated with an even higher increase in total cotyledonary surface and hence less efficient placentas (Kamal et al. 2017). The latter suggests lactation to be associated with a more pronounced placental compensation which is effectuated by an increased size of the cotyledons. Based on the study however, we were not able to detect direct effects of the level of milk yield like the amount of liters at top production nor the total amount of milk produced during gestation on gross placental morphometrics. The latter may be caused by several factors. First of all it is well known that selection towards high milk yield gives rise to several other compensation mechanisms in order to first safeguard the dam and the newly born calf in preference of investing in a subsequent generation. Modern high producing dairy cows for example have been shown to significantly postpone ovarian activity and hence a subsequent pregnancy after calving in comparison to less selected and hence lower producing animals (Opsomer et al. 2000).

Therefore, environmental factors such as the level of milk production affect placental development less severely than fetal weight close to term, suggesting that placental growth may be sustained at the expense of other tissues in an attempt to maintain pregnancy and minimize the adverse consequences for the fetus (Vaughan et al. 2012). To further elucidate the mechanisms underlying placental compensation, more research on factors affecting placental development, with a special interest on milk production level and moment of drying off, is warranted.

We have measured the lactation features in cows during pregnancy and peripheral glucose and insulin concentrations in neonatal calves as a proxy for their glucose metabolism (Kamal et al. 2015). The major finding of that study was that major insulin traits in newborn Holstein calves are significantly associated with the amount of milk produced during gestation, lactation length and the length of the dry period before their birth (Kamal et al. 2015). However, the effect is oddsopposite to our hypothesis with the view that the basal insulin level is higher and insulin sensitivity is lower in the calves born following a greater cumulative milk production during gestation in cows. Such association between the insulin traits and milk production is rather difficult to explain. However, the energy status during lactation in cows may be affected by cow factors irrespective of the level of production (Piccardi et al. 2013; Sensosy et al. 2012). In less productive cows, daily milk production often decreases to low levels before the planned drying off date; therefore, farmers no longer keep these cows in production, which results in longer dry period compared with higher producing cows (Atashi et al. 2013). Interestingly, the basal insulin level is higher and insulin sensitivity is lower in the calves born to the cows having passed a longer dry period in our study (Kamal et al. 2015). Although no difference exists in serum glucose, insulin, and IGF-I levels in cows with short versus longer dry period (Pezeshki et al. 2007), cessation of partitioning nutrients toward milk for extended duration in cows experiencing a longer dry period, provides higher amounts of energy and amino acids toward growth and maturation of the pancreatic β-cell, giving rise to higher insulin levels in the calves (Long et al. 2010).

Lower partitioning of nutrients to the gravid uterus in cows having a longer lactation length may lead to less glucose crossing the placenta and hence a delay in the development of the embryo (Green et al. 2012; Sensosy et al. 2012). Although the energy requirements for the embryo may be relatively low, metabolic activity is high and this represents a critical period for epigenetic control and organogenesis during fetal development (Fleming et al. 2012; Wu et al. 2006). Certainly, β-cells in pancreatic islets develop during the embryonic and early

fetal period of the intrauterine development in cattle (Carlsson et al. 2010). Exposure of dams to reduced nutrient intake during early gestation is associated with reductions in insulin concentrations in sheep fetuses (Limesand et al. 2013) and in calves (Long et al. 2010a). The adaptations to nutrient restriction that limit insulin concentrations are not fully known, but a morphological study demonstrated smaller pancreatic islets and a decrease in β-cell mass following early growth restriction in sheep (Limesand et al. 2013). Chronic hypoglycemia during later stages of fetal development leads to an intrinsic islet defect that is responsible for the decreased insulin secretion in sheep offspring without significantly decreasing their β-cell mass (Rozance et al. 2006). The acute insulin response is lower in calves born following longer lactation length in cows (Kamal et al. 2015). The lower insulin secretion in the calves born to the cows with longer lactation length is even evident after adjusting for the sensitivity, as indicated by a lower disposition index. The chronic glucose partitioning towards the udder during the entire period of gestation is present in cows with longer lactation length which potentially contributes to a reduced β-cell mass due to slower proliferation rates and subsequently a reduced insulin secretion in the newborn calves (Limesand et al. 2013; Lucy et al. 2014; Rozance et al. 2006). Substantial evidence suggests that energy restriction before birth is associated with enhanced insulin sensitivity in early postnatal life. However, this dysfunction appears to emerge in case the nutrient restriction in earlier parts of gestation is followed by abundance of nutrients in late part of gestation (Long et al. 2010a; Radunz et al. 2012). Nevertheless, the insulin sensitivity of the calves is not associated with the lactation length in cows (Kamal et al. 2015). This observation is consistent with a study in sheep where nutritional restriction throughout gestation was imposed to the fetal lambs by removal of the majority of the endometrial caruncles from the uterus of the dams (Owens et al. 2007). The latter gave rise to an impaired insulin secretion without any enhancement of insulin sensitivity in the fetal lambs (Owens et al. 2007). These findings invoke that elongation of lactation length in cows, or factors associated with the longer lactation length, impair β-cell responsiveness to glucose stimulation, hence insulin secretion in the newborn calves (Gutierrez et al. 2012; Owens et al. 2007; Rozance et al. 2006).

A summary of effects of the dam characteristics on the newborn calves is presented in Figure 12. Conclusively, selection for greater milk production and high persistency in dairy cows may lead to reduced glucose availability for the developing embryo and fetus with subsequent deleterious repercussions for birth weight of the calves. Insulin traits in the calves are also significantly associated with the amount of milk produced during gestation, lactation length

and length of the dry period prior to their birth. Whether the changes in birth weight and insulin traits early in life exert long-term effects on the metabolic function in later life is unknown. However, several reports have linked lactation during gestation in the dam with impaired future performance and longevity of the offspring (Berry et al. 2008; Gonzalez-Recio et al. 2012). When the alterations in insulin traits of the calves persist in later life, as they do in newborns confronted with intrauterine growth restriction in other species, they may contribute to adverse metabolic outcomes in later life.

7.4 Metabolic Programming in Different Species

Birth weight is the principal predictor associated with the growth of body in Holstein calves (Graham et al. 2010). Exposure of bovine dams to reduced nutrient intake during early gestation may not influence birth weight or postpartum growth but may cause alterations in muscle fiber development and synthesis of adipose tissue in offspring (Long et al. 2010b). Therefore, birth weight by itself is not sufficient to identify fetal growth pattern, in particular when birth weight is within the normal range for gestational age. Hence, no adverse effects for the lower birth weight calves have been mentioned in terms of their fertility or productivity in their first lactation, indeed, the trend for fertility was even in the opposite direction (Swali and Wathes 2006; Wathes et al. 2008). This may accord with the 'thrifty phenotype' hypothesis that animals which are nutritionally growth retarded *in utero* become metabolically adapted to this situation and are thus better able to cope with a low energy environment postnatally (Gluckman and Hanson 2004). In addition to gestational age and gender, other pregnancy characteristics, such as maternal height and weight before pregnancy, parity, and ethnicity account for a large part of variation in fetal growth velocity and weight at birth (Mongelli and Gardosi 1995). It has been shown that customized fetal growth estimation, adjusting for maternal and fetal characteristics, allows a precise evaluation of fetal growth restriction by identifying newborns who have failed to reach their genetic potential of growth and who are at a high risk of adverse neonatal outcome (Clausson et al. 2001; de Jong et al. 1998). It is postulated that fetal growth pattern would induce changes in body dimension and metabolic parameters at birth irrespective of birth weight itself.

Human studies provide substantial evidence for relationships between low birth weight and metabolic syndromes resulting from β-cell dysfunction (Green et al. 2010). In humans, the worst prognosis for adult health is when fetal nutrient restriction is followed by an excess food supply in childhood, so that the adults become proportionately more obese (Barker

2001). In sheep, experimental nutrient restriction *in utero* increases the neonatal growth rate of soft and skeletal tissues and eventually leads to visceral adiposity in the young lamb without altering the body mass index (De Blasio et al. 2007). The latter is, consistent with observations in IUGR infants (Ong et al. 2000), probably reflecting the decrease in muscle mass that occurred concomitant with increased adiposity (De Blasio et al. 2006). Furthermore, nutrient restriction *in utero* increases insulin sensitivity of the glucose and free fatty acid metabolism in the young lamb (De Blasio et al. 2007), rather than enhancing insulin production, which is also consistent with observations in IUGR infants (Bazaes et al. 2003; Mericq et al. 2005). This enhanced insulin action directly contributes to their visceral adiposity (De Blasio et al. 2007). The latter has been observed in IUGR children that are small at birth (Greenwood and Bell 2002; Ong et al. 2000). If this excessive accretion and storage of fat persists, as is evident in adult human beings who were IUGR (Ong et al. 2000), it may partly account for their adverse metabolic and cardiovascular outcomes at adult age (Eriksson et al. 1999; Rasmussen 2001). Catch-up growth, but not size at birth, independently predicted increased visceral adiposity in the juvenile lamb (De Blasio et al. 2007). This is consistent with observations in children and adults who were IUGR, and in whom small size at birth is predictive of reduced lean tissue mass, while catch-up growth is predictive of overall obesity in later life (Kensara et al. 2005; Rogers and Grp 2003).

Insulin controls glucose homeostasis by both suppressing hepatic glucose production and stimulating glucose uptake into skeletal muscle and adipose tissues. Resistance to insulin is recognized as a common underlying feature of metabolic syndrome both in human and domestic animals. Maternal food restriction results in early developmental alteration in pancreatic β-cell in terms of both number and function (Garofano et al. 1998). This induces adaptive alterations in body composition and metabolism suggestive of higher insulin sensitivity independently from birth weight itself (Beltrand et al. 2008). IGF1 and 2, determinants for β-cell growth, are elevated in the pancreas of intrauterine growth restricted lambs, and these growth factors are postulated to be associated with neonatal catch-up growth (Gatford et al. 2008). The increased exposure to cortisol prenatally in the nutrient restricted lamb may increase the abundance of insulin targets and, hence, the response to insulin in late gestation, which might persist in early postnatal life (De Blasio et al. 2007). Later in life, the mismatch between postnatal and fetal environment would favor postnatal changes in body composition during catch-up growth and the development of insulin resistance (Leunissen et al. 2008) to reset the child's growth to follow his/her initial and genetic growth trajectory.

Exposure of bovine dams to reduced nutrient intake during early gestation exerts a modulating effect on regulation of postnatal plasma glucose and tissue development (Long et al. 2010a). It may not influence birth weight or postpartum growth but may cause alterations in muscle fiber development and synthesis of adipose tissue in offspring and have a negative effect on beef quality and efficiency of production (Long et al. 2010b). Placental restriction does not affect the fasting plasma insulin but reduces insulin secretion in response to a glucose load in the young lamb (De Blasio et al. 2007). This is consistent with observations in human IUGR infants (Bazaes et al. 2003) and suggests that increased insulin abundance in the fasting state at least was not contributing to increased insulin action and catch-up growth or increased adiposity. This finding implies that in the sheep, as shown in the rat and observed in human beings, prenatal restriction impairs β-cell responsiveness to glucose stimulation or secretory capacity and, hence, insulin secretion in the post-prandial state in early postnatal life (Bazaes et al. 2003; Simmons et al. 2001).

Undernutrition during early to mid pregnancy has been stated to affect the early construction and development of a diverse range of foetal tissues and organs (Fowden et al. 2006b). In humans, the latter has been illustrated by an intra-uterinely programmed pancreas leading to an impaired insulin secretion and hence higher risk to suffer from diabetes mellitus 2. Hence, persisting effects of early malnutrition become translated into pathology, thereby determining chronic risk for developing glucose intolerance and diabetes (Aerts et al. 1990). Earlier studies have shown that insulin secretion is compromised in high yielding dairy cows suffering from fertility problems like cystic ovarian disease (Opsomer et al. 1999; Vanholder et al. 2005). Impaired insulin secretions seem to be at least partly caused by the elevated blood levels of non-esterified fatty acids (NEFAs) in cows during the periparturient period (Bossaert et al. 2008). At least in some cows, the (endocrine) pancreas seems to be highly sensitive for these elevated NEFAs, rendering those cows at a higher risk to suffer from lower peripheral insulin concentrations. Hence, as in humans where an impaired insulin secretion is a decisive factor in the development of diabetes type 2 (Kahn 2003), also in modern dairy cows an impaired insulin secretion seems to be associated with some of the so-called production diseases (De Koster and Opsomer 2013b), and may be related to the existence of a negative energy balance during early pregnancy while *in utero*.

7.5 Mismatch of Nutritional Environment

Because farmers are motivated to maximise daily growth in their growing young stock in order to maximise milk production in the first and subsequent lactations, they accentuate the mismatch between the milieu the offspring is prepared for and the milieu the neonates actually arrive in, which may lead to even more deleterious effects. Examples of this are well known in human medicine, where it has been shown that babies who experienced IUGR and thereafter experienced catch-up growth are more prone to reproductive disorders, such as polycystic ovarian syndrome (Ibáñez et al. 2008). Indeed, epidemiological studies in both beef (Funston and Deutscher 2004; Funston et al. 2012) and dairy (Swali and Wathes 2007; Brickell et al. 2009) cattle have shown that heifers growing fast in the first months of life have a significantly earlier pubarche but need more inseminations to become pregnant, ending up with a similar age at first calving compared with their slower-growing peers. In this light, we may refer to the 'thrifty phenotype hypothesis', which proposes that the epidemiological associations between poor fetal and infant growth and the subsequent development of Type 2 diabetes and metabolic syndrome results from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism (Hales and Barker 2001). This hypothesis may also apply to high-producing dairy cattle and may contribute to the high occurrence of metabolic and fertility problems currently noted in high-yielding dairy cows.

The focus of human biomedical research into prenatal programming has been on the predisposition to adult-onset diseases, such as hypertension and diabetes (Eriksson 2016; Marciniak et al. 2017). The latter may not seem relevant to the performance and well-being of productive livestock like dairy cattle. However, the efficiency of the production of meat, wool and milk, and the susceptibility to disease of domestic livestock continue to vary widely among and within similarly managed herds and flocks of relatively uniform genetic background. At least some of this hitherto unexplained variation is suggested to be attributed to carryover effects of metabolic perturbations during different phases of embryonic and fetal development. The best-described effects are those on early muscle and adipose tissue development, with putative consequences for the capacity for lean tissue growth, propensity for fattening and therefore feed efficiency in meat animals (Bell 2006). In livestock, there is increasing evidence that production characteristics are significantly affected by environmental factors like maternal diet during gestation. In addition, it seems likely that the growing body of evidence for nutritional modulation of immune function and susceptibility to infectious and parasitic diseases will eventually implicate prenatal predisposing factors.

7.6 General Conclusions

Data from dairy heifers and cows confirm the results from other species in showing that young maternal age and lactation during pregnancy can affect feto-placental development. The nutritional environment experienced by the embryo during its development from a zygote to a blastocyst can for example also influence the expression of genes important for normal foetal growth and development. This knowledge attributes very interesting and innovative information for application to practical ruminant production. For example, genetic selection to enhance production in the form of early puberty, increased ovulation rates and higher milk yields may place more stringent demands on maternal nutrition during key periods of embryonic and foetal development than hitherto realized. Therefore, confronting animals that have been bred for high levels of production with extensive farming systems and lower feed inputs in developing countries may compromise foetal development, neonatal viability and adult health and production. Additionally, insulin is a key metabolic hormone that plays a crucial role in regulating energy homeostasis in the body. In addition, insulin-dependent signaling has important functions in reproduction and early embryo development. As metabolism and reproduction are closely linked, metabolic challenges may be the source of reproductive disorders and decreased fertility. This is known for the dairy cow and for other species including the human. Although metabolic disorders in the dairy cow often derive from a failure to adapt to a high milk production, the situation in the human is often linked to emerging conditions and associated diseases in our modern society such as obesity and diabetes, where an excessive energy intake causes decreased fertility in women. Moreover, in a population where malnutrition is prevalent, nutritional interventions during pregnancy may modify the metabolic phenotype in the young child that could have consequences for later chronic disease risks.

Because of the incomplete knowledge about the mechanisms of intrauterine growth restriction, attempts to alleviate the detrimental effects of undernutrition on postnatal growth performance in livestock have so far achieved only limited success. The recognition of fetal programming suggests that strategies to promote postnatal growth and health of livestock should be initiated at the key stages of prenatal development (Finch et al. 2004). Thus, targeting an effective window of opportunity during a specific period of pregnancy would be most beneficial for preventing intrauterine growth restriction. Despite much failure, the largely trial-and-error approaches to treating pregnant dams have generated some promising results. These approaches include hormonal therapy (Costine et al. 2005; Wallace et al. 2004);

dietary supplementation of energy, protein concentrates, or both (Patterson et al. 2003; Zhang et al. 2002); adequate nutritional support for immature pregnant dams (Swali and Wathes 2006; Wallace et al. 2005); extended lactation to avoid insemination at peak yield (Douhard et al. 2014; Lehmann et al. 2017); provision of antioxidant nutrients (Castillo et al. 2005; Fang et al. 2002); and manipulations of the arginine-nitric oxide/polyamine pathway (Mateo et al. 2006). Although these methods are diverse in nature, they appear to directly or indirectly promote feto-placental growth and uteroplacental blood flow via increasing the availabilities of arginine, nitric oxide, or both.

7.7 Limitations and Future Perspectives

Limitations. There were several limitations in our study. **1)** The study herds were not selected randomly. Data were collected from four small dairy herds (on average 70 lactating cows) in Flanders (Belgium) and one large herd (> 2000 lactating cows) in Rostock (Germany). They were selected based on their long history of successful collaboration with the Ghent University Ambulatory Clinic. **2)** Measurements and data collection was not done year round for all herds. Especially in the German herd, the measurements and data collection were only done in the month January and February. **3)** Blood sampling was not done at the same moment during the day for all herds under study. The blood sampling from the calves at the Belgian herds was done in the mornig after an overnight fasting. However, the blood sampling in the German herd was done in afternoon at least five hours after a milk meal to be compatible with the herd management practices. **4)** Less extensive tests were used. The study was performed using a larger number of subjects which limits the use of sophisticated tests. Therefore, we used tests that are easier to perform under field conditions and which had been proven to be satisfying in comparable field studies performed in several other species including ruminants

Future Perspectives. Animal scientists can learn much from modern studies in human medicine, especially those dealing with the related metabolic diseases. Clearly, however, much remains to be learned about the underlying molecular and physiological mechanisms of prenatal programming in dairy animals, as well as the quantitative importance of this phenomenon relative to the modulating effects of postnatal nutrition and other environmental factors. From a practical point of view, in terms of animal production, greater knowledge about the underlying mechanisms will allow the incorporation of concepts of prenatal

programming into management systems with the ultimate goal of improving food production and eventually farm profitability.

- Epigenetics and its applications in developmental programming are relatively innovative sciences and it is thought that their application will bring about significant extra value in animal production.

- A major challenge now is to capitalize and build on this knowledge to improve dairy animal and public health through appropriate recommendations to young growing and lactating individuals, and definition of suitable intervention strategies.

- Arginine-derived signaling and regulatory molecules (nitric oxide and polyamines) are crucial for placental and fetal growth. New knowledge on the mechanisms regulating fetal growth and development will be beneficial for designing new, rational, and effective strategies to prevent and treat intrauterine growth retardation in livestock.

- Because intrauterine growth retardation remains a major problem in mammalian pregnancies, innovative interdisciplinary research in the areas of nutrition, reproductive physiology, and vascular biology are critical to design the next generation of nutrient-balanced gestational diets and develop new tools for livestock management, which will enhance the efficiency of animal production and improve the well being of animals.

- In our study, the basal insulin level is higher and insulin sensitivity is lower in the calves born following a greater cumulative milk production during gestation in cows. Such association between the insulin traits and milk production is opposite to our hypothesis and therefore rather difficult to explain. Hence, further in-depth study is required to clarify this fact

Further advances in this field have the potential to combat the burden of common metabolic diseases in dairy cattle as well as in human, which represent major health care issues of the 21st century.

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SUMMARY

Dam nutrition during gestation in mammals has been recognized as a key to metabolic programming *in utero*. The conflict in nutrient allocation between mother and offspring during pregnancy, is especially intriguing in modern dairy cattle. In these animals, age at first calving of 24 months and calving intervals of 365 to 385 days are strived for to ascertain the income of the farmer (Inchaisri et al. 2010). The latter however implies that gestation in primiparous animals should take place when dams are still growing, while in multiparous animals gestation should coincide with lactation. The heifers' age and cows' level of milk production during gestation have both been suggested to be important contributors to the nutritional environment for the developing embryo and fetus (Berry et al. 2008; Brickell et al. 2009; Funston and Summers 2013), since available nutrients need to be partitioned between growth (in heifers) and milk production (in cows) of the dam and the intrauterine growth of the offspring. In **Chapter 2** a general review was presented to provide the currently available knowledge about metabolic programming in dairy cattle.

For our own research, we hypothesized that young age in heifers and high milk production during gestation in cows affect the intrauterine feto-placental development and subsequent development and organ function of the calf. As mentioned in **Chapter 3,** these hypotheses lead to the following scientific aims:

1. To evaluate environmental and dam factors, including age at calving in heifers and level of milk production during gestation in cows, that might be associated with birth size in Holstein calves (Chapter 4).
2. To study the impact of maternal factors like age at conception and level of milk production during gestation on gross morphology of the placenta in dairy cattle (Chapter 5).
3. To evaluate potential associations between environmental factors and dam characteristics and major insulin traits in newborn Holstein calves (Chapter 6).

The study presented in **Chapter 4** was undertaken to investigate the impact of environmental and dam factors on the birth weight of Holstein calves, the latter being a proxy for intra-uterine development. Results of the study reaffirmed calf gender, season of calving, gestation length, parity, morphometrics of the dam and length of the dry period to be significantly associated with calf birth size. Furthermore, age at calving in heifers and level of milk production during gestation in cows, were indicated as decisive determinants of calf birth size.

Results of the study presented in **Chapter 5** partly confirm that many of the environmental insults such as the level of milk production affect placental development less severely than fetal weight close to term, suggesting that placental growth may be sustained at the expense of other tissues in an attempt to maintain pregnancy and minimize the adverse consequences for the fetus. Ultimately, the ability of the placenta to balance the competing interests of mother, milk production and fetus in terms of resource allocation may determine not only the success of pregnancy in producing viable neonates but also the health and productivity of the offspring in later life.

Major finding of the study presented in **Chapter 6** is that major insulin traits in newborn Holstein calves are significantly associated with gender and season of birth. Subsequently, in calves born out of multiparous dams, insulin traits are significantly associated with the amount of milk produced during gestation, lactation length and length of the dry period prior to their birth. Whether the changes in insulin traits early in life exert long-term effects on the metabolic function in later life is unknown. However, several reports have linked lactation during gestation in the dam with impaired future performance and longevity of the offspring (Berry et al. 2008; Gonzalez-Recio et al. 2012). When the alterations in insulin traits of the calves persist in later life, as they do in newborns confronted with intrauterine growth restriction in other species, they may contribute to adverse metabolic outcomes in later life.

The general discussion on metabolic programming in dairy cattle in relation to birth size, placental development and insulin secretion is presented in **Chapter 7.** Based on the results and discussion presented in this thesis, the following conclusion can be drawn: data of the epidemiological studies provide clear indications for environmental events like young age in heifers and (heavy) lactation in multiparous cows taking place during gestation to have a significant impact on both the size as well as the metabolism of newborn dairy calves. The latter warrants for more fundamental studies to decipher the underlying mechanisms which at their turn may indicate preventive and curative strategies in order to increase life expectancy in dairy cattle. These novel findings may provide a basis for developing managerial interventions to improve long-term health and productivity of the offspring.

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SAMENVATTING

Bij zoogdieren zijn de voeding en de voedingstoestand van het moederdier erkend als belangrijke factoren voor wat betreft het intra-uterien metabool programmeren van de nakomeling. Het conflict inzake de voorziening van voedingsstoffen aan het moederdier enerzijds versus aan de nakomeling anderzijds, is vooral uitdagend bij moderne melkkoeien. In het kader van economische optimalisatie is het bij dit type dieren immers van groot belang dat de pinken afkalven op een leeftijd van 24 maanden waarna idealiter een tussenkalftijd van 365 à 385 dagen moet worden nagestreefd (Inchaisri et al. 2010). Dit laatste impliceert evenwel dat de dracht bij pinken plaatsheeft terwijl het moederdier zelf nog volop aan het groeien is, terwijl bij de multipare dieren de intra-uteriene ontwikkeling van de vrucht samenvalt met de productie van grote hoeveelheden melk. In eerdere literatuur (Berry et al. 2008; Brickell et al. 2009; Funston and Summers 2013) werd dan ook gesuggereerd dat bij de pinken leeftijd bij conceptie en bij de multipare koeien het niveau van de melkproductie tijdens de dracht mogelijks een sleutelrol spelen bij de intra-uteriene ontwikkeling van het kalf aangezien zij een duidelijke weerslag kunnen hebben op de verdeling van de voorhanden zijnde voedingsmiddelen. In hoofdstuk 2 werd alvast dieper ingegaan op de literatuur die hieromtrent momenteel voorhanden is.

De belangrijkste hypothesen van ons onderzoek waren dat leeftijd bij pinken en het niveau van de melkproductie geproduceerd tijdens de dracht bij multipare koeien een significante weerslag hebben op de intra-uteriene ontwikkeling van de foetus en de placenta en op het intermediaire metabolisme van het pasgeboren kalf. In hoofdstuk 3 zijn dan ook de volgende 3 wetenschappelijke hoofddoelstellingen van deze thesis verwoord:

1. Het identificeren en evalueren van omgevings- en maternale factoren, zoals de leeftijd bij de eerste kalving en het niveau van de melkproductie tijdens de dracht, die mogelijks geassocieerd zijn met de grootte van neonatale Holsteinkalveren (Hoofdstuk 4).

2. Het onderzoeken van de impact van maternale factoren zoals leeftijd bij eerste conceptie en het niveau van de melkproductie tijdens de dracht op de macroscopische kenmerken van de placenta van pas gekalfde koeien (Hoofdstuk 5).

3. Het evalueren van de mogelijke associaties tussen omgevings- en maternale factoren enerzijds en de belangrijkste kenmerken van het insulinemetabolisme van neonatale Holsteinkalveren anderzijds (Hoofdstuk 6).

Uit de resultaten beschreven in hoofdstuk 4 blijkt dat de grootte van een pasgeboren Holsteinkalf, wat algemeen aanzien wordt als een indicator voor de intra-uteriene groei en ontwikkeling, geassocieerd is met het geslacht van het kalf, het seizoen van afkalven, de drachtduur, de pariteit en de lichaamsmaten van de moeder en de lengte van de droogstand voorafgaand aan de geboorte van het kalf. Bovendien bleek bij de pinken ook bij multipare koeien de leeftijd bij afkalven en de hoeveelheid tijdens de dracht geproduceerde melk een significante weerslag te hebben op de grootte van de kalveren.

Het onderzoek uitgevoerd in hoofdstuk 5 toonde aan dat het niveau van de melkproductie en de meeste van de onderzochte omgevingsfactoren een minder sterke invloed uitoefenen op de ontwikkeling van de placenta dan het gewicht van het kalf. Desalniettemin werd aangetoond dat er zich compensatoire ontwikkelingsmechanismen ter hoogte van de placenta voordoen teneinde de dracht te handhaven en negatieve effecten op de ontwikkeling van de foetus te minimaliseren. Door placenta's van pas gekalfde Holsteinvaarzen en –koeien te vergelijken met deze van vaarzen en koeien van het Belgisch Witblauwe ras, kon worden aangetoond dat de runderplacenta tijdens de vroege dracht compenseert door de ontwikkeling van meer cotyledonen, terwijl compensaties op het einde van de dracht vooral bestaan uit een toename in ontwikkeling van de individuele cotyledonen. Finaal speelt de placenta immers een doorslaggevende rol in het evenwichtig verdelen van de voedingsmiddelen tussen de zich ontwikkelende vrucht enerzijds en het groeiende of melkproducerende moederdier anderzijds. In deze context is de placenta van groot belang voor het in stand houden van de dracht enerzijds maar is zij tevens ook van belang voor de gezondheid en de productiviteit van de nakomelingen op lange termijn.

De meest in het oog springende conclusie van hoofdstuk 6 is dat de belangrijkste insuline karakteristieken bij een pasgeboren Holsteinkalf geassocieerd zijn met het geslacht van het kalf en het seizoen van geboorte. Bovendien zijn deze insuline-kenmerken bij kalveren geboren uit multipare koeien ook afhankelijk van de hoeveelheid melk geproduceerd tijdens de dracht, en de lengte van zowel de voorafgaande lactatie als de voorafgaande droogstand. Of deze typische neonatale insuline karakteristieken ook een invloed hebben op het metabolisme van het dier op latere leeftijd, is tot op heden nog niet volledig duidelijk. Eerder uitgevoerde onderzoeken hebben echter wel een verband gesuggereerd tussen lactatie tijdens de dracht en een verminderde productie en langleefbaarheid bij de nakomelingen (Berry et al. 2008; Gonzalez-Recio et al. 2012). Indien deze typische specificaties van het insuline metabolisme bij pasgeboren kalveren wel degelijk aangehouden blijven op latere leeftijd,

zoals het geval is bij neonati van andere diersoorten die tijdens de dracht worden geconfronteerd met een beperking van de intra-uteriene groei, gaan we er van uit dat ze ook bij melkvee een rol kunnen spelen in het reguleren van het metabolisme op latere leeftijd.

Finaal wordt in hoofdstuk 7 dieper ingegaan op de algemene discussie. Hierin wordt geconcludeerd dat we op basis van de epidemiologische studies die in deze thesis werden uitgevoerd, hebben aangetoond dat factoren als jonge leeftijd bij pinken en (doorgedreven) melkproductie bij multipare koeien welke beiden plaatshebben tijdens de dracht, een signifancte invloed uitoefenen op zowel de grootte als het meatbolisme van een pasgeboren Holsteinkalf. Dit laatste nodigt uit om meer fundamenteel onderzoek te doen naar de onderliggende mechanismen hiervan. Mogelijks kan dit vervolgens leiden tot het ontwikkelen van zowel preventieve als curatieve (managements) strategieën om zowel de gezondheid, de productie als ook de levensduur van onze melkkoeien in de toekomst verder te optimaliseren.

Referenties:

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and Marcel. If I forgot someone to mention please be assured that my gratitude is not less than for those listed above.

Special thanks go to the honorable Secretary, Ministry of Fisheries and Livestock and Director General, Department of Livestock Services, Bangladesh for their kind consent and permitting me to complete my PhD. I would also like to thank my colleagues at the Department of Livestock Services, Bangladesh for their assistance which enabled me to continue my PhD study.

I am also particularly grateful to all the talented students (Aart, Maayke, Audrey, Ruben, Elke, Inge, Geoff, Matheiu, Jan, Harm, Jolien, Birthe, Karolien, Lisette, Lies, Liesbeth, Marit, Hanna, Mieke, Wannes, Olivia, Stefi, Pieter, Timothy, Thomas and Brecht) for their kind help and support to measure cows and calves in the German herd. I am also grateful to Lars, Steven, Sandra, Leila, Els, Marnik and Ria for their help and support.

I also extend my special thanks and gratitude to my friend Anisur, bhabi Bipasha, brother Zaiful and sisters Kabita and Shajeda for their support and for taking care of my family throughout this study period and also for their invaluable and unconditional love, support and encouragement.

Finally, the entire process would not have been possible without the unending love and support of my beloved wife Nasreen Parveen, and my affectionate daughter Rifa Tasfia and son Basratul Rafid. I would like to express the most warmly thanks to them for their patience, support and love during all the years when I was far away from home.

Beste allemaal, hartelijk dank!

Md Mostofa Kamal

Ghent, Belgium

30-May-2017

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CURRICULUM VITAE

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Md Mostofa Kamal was born on 15th October 1973 at Maolana Bari in the village Sathalia under Saghata upazila in Gaibandha district. He is the second son of late Abdul Latif Akond and late Aleya Begum. Dr. Kamal started his primary education at Munshirhat Government Primary School and secured Junior Scholarship in class eight. He passed Secondary School Certificate (SSC) examination in 1989 from Saghata Pilot Boy's High School, Saghata, Gaibandha. He has passed Higher Secondary School Certificate (HSC) in 1991 from Gaibandha Government College, Gaibandha. He did his bachelor and masters in Veterinary Sciences from Bangladesh Agricultural University. He has completed Doctor of Veterinary Medicine (DVM) degree from Bangladesh Agricultural University, Mymensingh in 1995. He was awarded the 'University Prize' for securing first position out of 72 students in the bachelor studies. He also completed Master of Science (MS) in Microbiology from the same university in 1999. He was awarded the 'University Gold Medal' for securing First Class with distinction and highest marks in the Masters studies. Subsequently he took part in Bangladesh Civil Service (BCS) exam, a unique procedure of securing a position to the Government job in Bangladesh. In 1999, he succeeded the 19th BCS examination and he had started his career in October 1999 as Veterinary Surgeon in the Department of Livestock Services (DLS) under Government. He passed 10 years of field experience as the Veterinary Surgeon. He has been promoted to the rank of Upazila Livestock Officer since December 2009.

He started his collaborative research on cattle reproduction at the Department of Surgery and Obstetrics in Bangladesh Agricultural University (BAU) through a USDA (United States Department of Agriculture) funded BG-ARS-121 project. He has presented the results in national conferences including the 15th AAAP Animal Science Congress Scholarship in 2011 in Thailand. Consequently the research results have been published as full-length article in international journals: Asian Journal of Animal Science, Journal of Applied Animal Research and Turkish Journal of Veterinary and Animal Sciences. In 2011, he has got European Special Research Fund (BOF) scholarship to pursue his doctoral research on 'Metabolic Programming in Dairy Cattle' for two years in Ghent University, Belgium. He has successfully presented the results of his Belgian doctoral research in several international conferences organized in Belgium, Germany and Turkey. Subsequently the results have been published as full-length

article in 'Journal of Dairy Science' being published from America and in 'Animal' being published from Europe.

In addition to many national training, he has attended several international training courses organized at Ghent University in Belgium: Low Countries Studies, Presentation Skills in English, Communication Skills, Project Management, Laboratory Animal Science and Veterinary Epidemiology. He also attended several international conferences for oral presentation: Epigenetics and Periconception Environment, the 1st General Conference of EPICONCEPT, 24-25 April 2013, Antalya, Turkey; Improving Smallholder and Industrial Livestock Production for Enhancing Food Security, Environment and Human Welfare, the 15th AAAP Animal Science Congress of the Asian-Australasian Association of Animal Production Societies, 26-30 November 2012, Thammasat University, Bangkok, Thailand; VIBes in Biosciences, the 3rd International PhD Student Symposium, 5-7 September 2012, Ghent, Belgium; the 16th Annual Conference of the European Society for Domestic Animal Reproduction, 29 August to 01 September 2012, University College Dublin, Dublin, Ireland; Dairying for Food Security and Livelihood Development, International Dairy Conference, 03-05 April 2010, BAU, Mymensingh, Bangladesh; and Sustainable Animal Production, Health and Environment, 24-27 November 1999, CCS Haryana Agricultural University, Hisar, India. Dr. Kamal is a noted veterinarian with long experience in research on livestock development and active involvement in the research on livestock development. He has published >40 articles in reputed journals and proceedings. He has received a prestigious travel grant to attend the 15th AAAP Animal Science Congress in Bangkok, Thailand. Currently he has been working as the Upazila Livestock Officer (leave reserve post) at Artificial Insemination Activities Extension and Embryo Transfer Technology Implementation project under DLS. He has published a number of books both at home and abroad. He is married to Nasreen Parveen and blessed with a daughter Rifa Tasfia and a son Basratul Rafid.

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