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Impact of prenatal and neonatal nutrition on metabolism and future performance in dairy heifers.

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Certifiquen:

Que la memòria titulada **"Impact of prenatal and neonatal nutrition on metabolism and future performance in dairy heifers."** presentada per **Cristina Yunta Bernal** per optar al grau de Doctor en Veterinària, ha estat realitzada sota la direcció del Dr. Àlex Bach Ariza i, considerant-la acabada, autoritza la seva presentació perquè sigui jutjada per la comissió corresponent.

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En los últimos años se ha demostrado que existe una conexión entre las primeras etapas de la vida y la futura productividad y salud de los animales. En esta tesis se desarrollaron 3 estudios para evaluar, por un lado estrategias de manejo de los terneros durante las primeras semanas de vida que podrían mejorar su rendimiento, y por otro estrategias que podrían ser implementadas en el futuro para mejorar el desarrollo fetal durante la gestación. En el primer estudio se compararon parámetros de crecimiento, reproducción y salud en terneros alimentados con leche de tanque y lactorremplazante. No se encontraron diferencias en el crecimiento entre los tratamientos aunque sí una reducción en el cociente insulina/glucosa y un menor número de terneros tratados con antibióticos en los terneros alimentados con leche de tanque. Estos resultados sugieren un mejor metabolismo de la glucosa y un posible beneficio sanitario en los terneros alimentados con leche de tanque comparados con los alimentados con lactorremplazante. En el segundo estudio se quiso evaluar el rendimiento de crecimiento a corto y medio plazo así como el metabolismo de glucosa e insulina en terneros alimentados con 2, 3 o 4 L de lactorremplazante dos veces al día. Los terneros alimentados a base de 4 L de lactorremplazante dos veces al día tuvieron una mayor ganancia media diaria pre-destete, pero no fueron capaces de suplir la falta de lactorremplazante durante el destete, lo que produjo un descenso de la ganancia media diaria en comparación con los terneros alimentados con 2 L dos veces al día. La edad en la primera inseminación fue mayor en los terneros que recibieron sólo 2 L dos veces al día pero no se encontraron diferencias en la edad a la primera inseminación fecundante ni en el ratio de concepción. Por otro lado, ofrecer 4 L dos veces al día de lactorremplazante provocó efectos negativos transitorios en el metabolismo de la glucosa, aunque éstos desaparecieron con la edad. En el tercer estudio se evaluaron los efectos de la suplementación vía peritoneo de arginina en la hemodinámica de la arteria uterina en novillas entre 40 y 140 días de gestación. El flujo sanguíneo uterino y otros parámetros hemodinámicos se determinaron por ultrasonido Doppler. Las medidas incluyeron ritmo cardíaco, flujo sanguíneo, índices de pulsatilidad y resistencia. También se analizaron la concentración en plasma de aminoácidos (AA), óxido nítrico, glucosa, factor de crecimiento insulínico, progesterona, hormona de crecimiento y prolactina. La suplementación con arginina no causó el incremento del flujo sanguíneo de la arteria uterina pero modificó otros parámetros que podrían

influir en el crecimiento fetal como el ritmo cardiaco, la concentración materna de AA y la síntesis de progesterona.

A connection between early stages of life and health and performance in the adult life has been lately demonstrated. In this thesis three studies were conducted in order to evaluate on the one hand rearing strategies that may improve calves performance, and on the other hand strategies that may be implemented in the future to ameliorate fetal development during pregnancy. In the first study, differences in growth, reproductive and health performance were evaluated between calves fed milk replacer (MR) and calves fed whole milk (WM). No differences were found in milk or solid feed intake and growth performance between treatments. But, the reduced insulin to glucose ratio and the decrease in the number of antibiotic treatments in calves fed WM compared with those fed MR suggested an improvement in glucose metabolism, and a potential benefit on calf health when feeding WM. The second study aimed at evaluating short- and medium-term growth performance and glucose metabolism of dairy calves fed 2, 3 or 4 L of MR twice a day. Calves fed 4 L of MR twice daily had greater ADG during the preweaning period, but were unable to compensate the lack of MR during the weaning process resulting in a decreased ADG compared with calves fed 2 L twice daily. Age at first breeding was greater when calves received 2 L twice daily, but no differences were found on age at pregnancy and conception rate. Also, offering 4 L twice daily of MR had transitory negative effects on glucose metabolism, but those disappeared with age. The third study evaluated the effects of Arg supplementation on uterine artery hemodynamics between 40 and 140 days of gestation as it could foster placental vascularization. Uterine blood flow volume (FV) and other hemo-dynamics were determined using Doppler ultrasonography. The measurements included heart rate, FV, pulsatility index, and resistance index. Plasma concentrations of amino acids (AAs), nitric oxide, glucose, insulin growth factor, progesterone (P4), growth hormone, and prolactin were analyzed. Contrary to our hypothesis, Arg supplementation did not increase blood flow to the uterus but did change other parameters that could influence placental and fetal growth such as heart rate, maternal concentration of plasma AAs, or P4 synthesis.

ABBREVIATIONS USED

AA: amino acids

ADG: average daily gain

AFB: age at first breeding

AFC: age at first calving

$ALLOWANCE_{MR}$: milk replacer allowance

Arg: arginine

AUC: area under the curve

BW: body weight

C_b: basal concentration

CI: calving interval

$COST_{MR}$: cost of milk replacer

$COST_{SIKNESS}$: cost of veterinary treatments

$COST_{STARTER}$: cost of starter

$COST_{WM}$: cost of whole milk

CP: crude protein

CR: clearance rate

CRG: glucose clearance rate

DE: digestible energy

DM: dry matter

DMI: dry matter intake

DNA: deoxyribonucleic acid

EAA: essential amino acids

EDV: end diastolic velocity

EE: ether extract

fd: doppler shift

FEAI: first effective insemination

FF: feeding frequency

ft: transmitted frequency

fr: received frequency

FV: flow volume

GE: gross energy

GH: growth hormone

GIT: gastrointestinal tract

GLUT4: glucose transporter type 4

GtoF: gain to feed

GTT: glucose tolerance test

HR: heart rate

I: comparison index

IAUC: insulin area under the curve

IGF: insulin-like growth factor

IgG: immunoglobulins

IS: insulin sensitivity

ItoG: insulin to glucose

IUGR: intra uterine growth retardation

JI: jugular infusion

LPS: lipopolysaccharide

ME: metabolizable energy

ME_g: metabolizable energy for gain

ME_i: metabolizable energy ingested

ME_m: metabolizable energy for maintenance

MR: milk replacer

MV: mean velocity

NAHMS: national animal health monitoring system

NE: net energy

NE_m: net energy for maintenance

NO: nitric oxide

NRC: national research council

NSWM: non saleable whole milk

P4: progesterone

PI: pulsatility index

PrI: peritoneal infusion

PRL: prolactin

PSV: peak systolic velocity

RI: resistance index

ST: starter intake

$t_{1/2}$: half life

t_b: time to reach basal level

TNF-α: tumor necrosis factor alpha

UPL: ultra performance liquid
    chromatograph

WM: whole milk

Y: expected milk yield

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Chapter 1

LITERATURE REVIEW

INTRODUCTION

In dairy cattle, two of the most important goals are optimizing milk production performance, and minimizing metabolic problems of the lactating cow. Efforts in this direction have been done for years through nutritional models. However, most of those models omit nutrient requirements during the beginning of pregnancy and the connection of different rates of neonatal body weight (BW) gain and future milk production.

In the last years, a connection between early stages of life and disease and performance in the adult life has been demonstrated. Thus, it is logical to think that metabolic problems and an optimal production performance could be partially achieved via an adequate management in the first stages of life.

1.1. FETAL PROGRAMMING

Every multicellular organism is the result of several synchronized events driven by genetic instructions, however, nowadays, it is known that fetal organs and systems are sensitive to the environment of the uterus and that the influence of this environment can lead to long term consequences (Barker, 2004) such as changes in performance and metabolic function of the offspring (Wu et al., 2006). These processes are usually called fetal programming and occur because of modifications in the chromatin structure through acetylation of histones or methylation of DNA resulting in modulation of gene expression without altering gene sequence (Bach, 2012).

The concept of metabolic programming was originally established by Barker et al. (1995). The authors conducted epidemiological studies and suggested that maternal undernutrition was highly correlated with an increased risk of health problems (hypertension, type II diabetes, and cardiovascular diseases) of children born experiencing intrauterine growth retardation (IUGR) at adulthood. Indeed, standardized mortality ratios diminish as birth weight increases. Also, low birth weight has been linked in human studies to increased incidence of type II diabetes (Hales et al., 1991; Forsen et al., 2000).

Thus, it seems clear that the organs and systems of the body go through sensible periods when they are plastic and responsive to the environment during development.

For most organs and systems, the sensible period occurs in utero. To illustrate this plasticity, Brooks et al. (1995) pointed that small women have small babies even in pregnancies after ovum donation when the woman donating the egg is large. And calves born to heifers are smaller than calves born to adult cows. Indeed, fetal growth in late gestation is normally limited by the maternal capacity to supply nutrients to the fetus and also by maternal size (Harding 2001).

The consequences for the offspring when the utero environment is not optimal depend on the stage of gestation when the shortages occur. Data from people who were in utero during the Dutch famine in 1944 were collected by Ravelli et al. (1998). During that time, official rations were around 700 calories. People who were conceived during the famine had, in adult life, raised serum cholesterol and incidence of coronary disease. And those exposed to the famine during mid or late gestation were insulin resistant and had impaired glucose tolerance (Ravelli et al., 1998; Roseboom et al., 2001). Thus, it seems that some risk factors for the different diseases have their origins in utero but are programmed at different times depending upon organs/system related. Those differences reflect when each organ is sensitive during the different stages of pregnancy.

Apart from maternal nutrition, other factors such as stress, hypoxia or excessive fetal exposure to glucocorticoids, have also been proposed as possible influencers in fetal development. As example, lambs born from stressed ewes perform worse spatial learning cognitive abilities than lambs born from control ewes (Coulon et al., 2014). In addition, the detrimental effect was not alleviated by enriched housing conditions after weaning. However, this thesis will be focus on nutrition as it is thought to be the primary programming stimulus (Harding, 2001).

1.1.1. Influences of maternal nutrition to the offspring

It is clear that the genes of a fetus influence its growth, however, studies in humans and animals pointed that this growth is also dictated by the nutrients it receives (Harding, 2001).

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1.1.1.1. Intrauterine growth retardation

During pregnancy, when an impaired growth and development of the mammalian embryo/fetus or its organs occur it is called intrauterine growth retardation (IUGR) (Wu et al., 2006). The first observation that researchers captured was that altering maternal nutrition in pregnancy leads to changes in birth weight. The second observation was the link existing between a low birth weight and later incidence of disease risk (Barker et al., 1995). The consequences of maternal malnutrition induced by a low protein diet and/or caloric restriction have been described in both animals and humans. But, why should fetal responses to undernutrition lead to disease later in life? According to Barker (2004), people who are small at birth are vulnerable to later disease through three main reasons: First, because they have fewer cells in key organs, second, because of their hormonal profile and metabolism, and last, people who are small at birth are more vulnerable to adverse environmental influences in later life because of their unique responses to stress.

Studies involving animal models have shown that pregnant rats fed low protein diets produced pups with alterations in pancreatic islets (Berney et al., 1997). Altered pancreatic islets have lifelong effects and predispose the animal to glucose intolerance and diabetes. Moreover, rats fed a low protein to energy ration during pregnancy, produced pups with altered glucose synthesis and insulin secretion (Desai et al., 1995). In livestock species, undernutrition often occurs during the first two trimesters of gestation. In pigs, nutrient deficiencies during early pregnancy that compromise placental development had consequences for growth potential and carcass quality (Foxcroft et al., 2006). In ruminant species, it has been demonstrated that lambs from restricted dams have altered glucose metabolism (Effertz et al., 2007). Furthermore, hyperglycemia and altered insulin secretion appeared in lambs born to ewes that were nutrient restricted in early gestation (Ford et al., 2007). Also, restricted ewes in late gestation gave birth to insulin resistant lambs that had also a reduction in GLUT4 expression in adipose tissue with increased adipose mass (Gardner et al., 2005). In beef cattle, placental angiogenesis, cotyledon weight and fetal development was compromised when dams were fed diets providing 75 % of recommended nutrient allowances (Vonnahme et al., 2007; Long et al., 2009).

Despite of its possible importance because of the key role that fat reserves and mobilization play in early lactation, very few studies in dairy cattle have been conducted addressing this aspect. It is important to note that, in the adult cow, most of gestation takes place simultaneously with lactation. Thus, in dairy cows some problems could occur as embryonic development and maternal milk production compete for nutrients (Bell and Bauman, 1997). Thus, some detrimental effects of high milk production on fetal development can be expected in dairy cattle (Bach, 2012). However, Banos et al. (2007) did not find any relationship between maternal milk yield during pregnancy and offspring milking performance in the first lactation. In the same direction, Berry et al. (2008) concluded that offspring milk yield was due to factors other than maternal milk production. But more recently, González-Recio et al. (2012), analyzed data from 40,065 Holstein cows in a very interesting study in which the objective was to determine the impact that the co-existence of the fetal development with the maternal lactation has on the adult life performance. The authors found that the coincidence of pregnancy and maternal lactation reduces the future milk productivity of the fetus. Indeed, females that were conceived in absence of lactation produced more milk during its first lactation, had longer lifespan and had less metabolic problems than females conceived during the maternal lactation. Furthermore, these negative effects were more pronounced in high producing cows. As an example, cows that were conceived during their dam's lactation and belonged to the superior 10th percentile for genetic merit, produced 90 kg less milk than the females whose fetal development occurred in the absence of lactation (i.e., born to a heifer) (González-Recio et al., 2012). Also, embryos developed during maternal mastitis presented numerically shorter productive lifespan.

Not only intermediary metabolism can be influenced by maternal undernutrition. The first trimester of fetal life is when more number of follicles and oocytes are present in fetal ovaries and ovarian folliculogenesis is not complete until the end of gestation (Erickson, 1966). Studies in humans reporting an impaired reproductive fitness because of a diminution in fetal growth can be found (Cooper et al., 1996; Adair, 2001). It seems that pulsatile release of luteinizing and follicle-stimulating hormone is initiated in utero, continues through infancy, and thereafter ceases until it resumes at puberty. In beef cattle, a 60 % of energy restriction during the first trimester of

pregnancy resulted in heifers with 30% less high-quality follicles (Mossa et al., 2009). Furthermore, a protein deficiency during the last 100 days of pregnancy delayed age of puberty of the offspring (Corah et al., 1975). In dairy cattle, reproductive performance is nowadays economically very important for producers, thus it would be interesting to elucidate whether nourishing the fetus properly, would lead to heifers with an ameliorate reproductive performance.

Apart of metabolism and reproduction, the health of the offspring can also be programmed in the utero. Even though the transfer of immunity lies mainly in the quality and quantity of the colostrum offered to calves, the capacity that a calf may have to absorb the immunoglobulins (IgG) may be influenced by the nutrient intake of the dam while in utero. Indeed, intestinal tissues are responsive to IUGR. Offsprings that have been gestated under IUGR conditions have decreased small intestinal mass and length and altered intestinal villi morphology (Avila et al., 1989). The failure to absorb these IgG may predispose the calf to disease or/and decrease its future productivity (Blecha et al., 1981). Rat dams with a protein deficiency resulted in a 2-fold decrease in serum IgG2 through lactation and a 1.5-2 fold decrease in total serum IgG in the pups (Michalek et al., 1975). Even though it may be logical to expect that the colostrum from protein-restricted dams would have a lesser amount of IgG, also, the offspring may be programmed by the protein restriction of the dam impairing their ability to properly absorb IgG. Indeed, Blecha et al. (1981) found no significant correlations between concentration of immunoglobins (IgM, IgG1 and IgG2) in the colostrum of the cow and precalving crude protein consumption, but reported that a protein restriction during the last trimester of gestation affected the ability of calves to absorb IgG1 and IgG2. Loh et al. (1971) already described that protein restrictions on rat dams impaired protein absorption by the pups decreasing the development of absorptive cells in the pup's jejunum. In the same direction, Meyer et al. (2010) reported an alteration of the jejunal proliferation and total intestinal vascularization of the fetus when a moderate nutrient restriction was applied during early to mid-pregnancy of beef cattle.

In summary, the results from animal models indicate that shortage of nutrients during even short periods during pregnancy may have metabolical, reproductive, and

immunological implications in the offspring that sometimes become evident only at adulthood.

1.1.1.2. Maternal oversupply

Increasing energy intake typically increases the rate of ovulation in farm animals. Thus, increasing feed intake during a short period of time (flushing) around the time of conception has been employed by producers to increase the number of fetuses in sheep (Cole, 1990). In dairy cattle, the NRC (2001) recommends to provide the pregnant cow with an over-supply of energy during the pre-partum to minimize post calving upsets to the cow. However, how this high-energy diet affects the future calf has not been studied despite that it might exert some negative effects because of the potential increased glucose supply to the fetus (Bach, 2012). Indeed, an elevated maternal BW and plasma triglycerides have been related with elevated birth weight and adiposity in humans (Heerwagen et al., 2010). Maternal overnutrition during a premating period or early pregnancy resulted in increased porcine embryo and fetal mortality (Einarsson and Rojkittikhun, 1993). And, interestingly, similar to a situation of undernutrition, overfeeding leads to retardation in fetal growth in pigs (Cole, 1990) and adolescent sheep (Wallace et al., 2004). Also, Long et al. (2010) conducted a study with overfed ewes that gave birth to lambs that were leptin resistant. Adiposity in dairy cattle has been linked with reduced reproductive performance and poor health (Roche et al., 2009). Furthermore, overconditioned cows during late pregnancy have more risk to incur metritis, ketosis, milk fever, and cystic ovaries (Wu et al., 2006). Thus, it may be important to study the consequences of the currently recommended high-energy diets for the "close-up" period to evaluate its potential negative effects in future heifers.

1.1.2. Fetal and placental development

1.1.2.1. Development during early pregnancy

The bovine embryo enters the uterus 4 days after ovulation and fecundation. Then, maternal recognition starts, with the critical period being between days 15 and 18 after ovulation. It is surprising that current models (NRC, 2001) for dairy cattle ignore the needs during early pregnancy even knowing that poor nutritional environment is

quite common in livestock because of the precocious inseminations and competition between nutrient needs for milk production and fetal development (Reynolds and Caton, 2012). It is in the early stages of pregnancy, when nutrient requirements do seem to have an important influence on the offspring, when maximal placental growth, vascularization and fetal organogenesis take place (Redmer et al., 2004; Vonnahme, 2007).

The placenta plays a fundamental role for a proper fetal development, as it provides the fetal metabolic demands (Vonnahme, 2007). Thus, one of the earliest requirements during conceptus development is the establishment of a functional fetal/placental vascular system (Reynolds and Redmer, 1995). A large increase in transplacental exchange is essential to support the exponential increase of fetal growth during the last half of gestation (Prior and Laster, 1979), however, it depends directly on the proper growth of the uteroplacental vascular beds during the first half of pregnancy (Reynolds and Redmer, 1995).

In cattle and as shown in Figure 1.1. (adapted from Reynolds et al., 1990), as gestation advances, the fetal and placental weight increase exponentially but the absolute rate of increase is greater in fetal weight. A positive correlation exists between fetal and placental weights (Ferrel and Reynolds, 1992; Reynolds and Redmer, 1995). It seems that during early pregnancy, the placenta grows beyond its needs in order to be able to support the later fetus demand necessary for growth during late pregnancy (Reynolds and Remer, 1995). Indeed, uterine blood flow volume (FV) increases exponentially during pregnancy (Bollwein et al., 2002), meaning that even if the placental weight does not increase as fast as fetal weight, its capacity to supply nutrients does.

A meta-analysis has shown little influence on birth weight when maternal dietary supplements have been administrated to pregnant women (Harding, 2001). Bloomfield and Harding (1998) explained that there exists a "supply line" which links maternal diet at one end with fetal tissue uptake at the other. Inside the mentioned "supply line", there is maternal metabolism, endocrine milieu, uterine and umbilical blood FV and placental transfer and metabolism. For that reason, if the fetal "supply line" works properly, it allows a large margin of safety for fetal growth even if maternal nutrition changes. Likewise, a reduced uterine blood FV or reduced placental transfer capacity

may limit fetal nutrient supply without the need of a change in the maternal diet (Harding, 2001). Thus, the placenta has multiple roles. It influences not only fetal nutrition, via its capacity to transport nutrients from maternal to the fetal circulation, but also the metabolism of key nutrients such as fetal AA or glucose (Harding, 2001). Indeed, the placenta also has additional functions that have an influence in growth and development of the fetus such as production of hormones (Anthony, 1995), and thus, metabolic function of the offspring could be modified by the fetal environment without necessarily affecting birth weight (Dabelea et al., 2000). Godfrey and Barker, (2001) proposed that variations in the endocrine, nutrient and cardiovascular milieu in utero influence expression of the fetal genome, leading to development adaptations. These adaptations may be in the fetal or neonatal advantage of the offspring but then may predispose them to disease later in life.
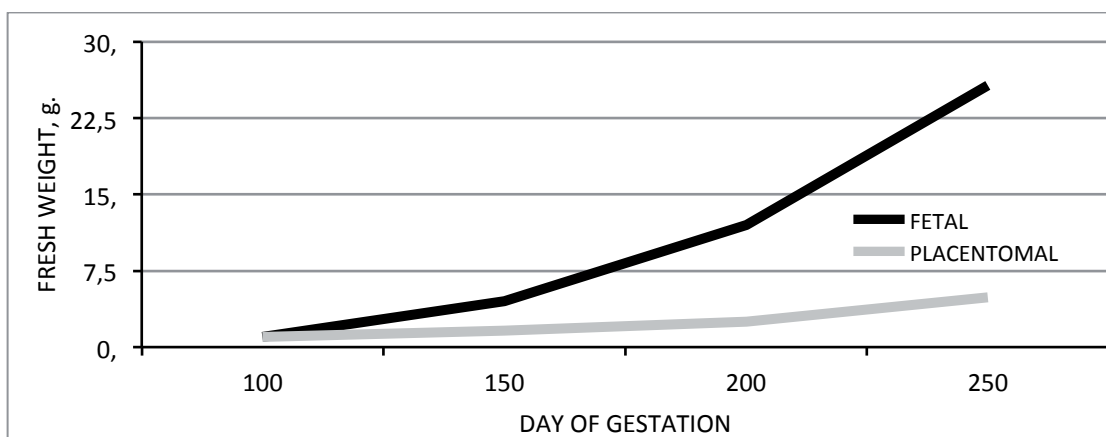
Figure 1.1. Regressions of fetal and placentomal weights on day of gestation in cows. Adapted from Reynolds et al. (1990).

Apart of the importance of a good establishment of the uteroplacental vascular bed, and of its proper functioning, retardation of fetal growth early in gestation should have more severe effects on subsequent neonatal development than retardation during latter in gestation even though retardation of bovine fetal growth in any time during pregnancy would have an influence in latter development of the offspring (Prior and Laster, 1979). These authors reported that in the bovine fetus, hyperplasia occurs throughout gestation, however the instantaneous growth rate due to hyperplasia

decreases near the end of gestation while growth due to hypertrophy continue to increase with increasing fetal age.

1.1.2.2. Uterine blood flow

The placenta transports respiratory gases, nutrients, and wastes between the maternal and the fetal systems. The placenta in the mammalian organism is situated between the maternal and the fetal compartment and it connects both through the uterine and umbilical vein and artery (Fig. 1.2.). Thus, to evaluate transplacental exchange, and estimate the nutrients that reach the fetal compartment, the maternal uterine blood FV must be determined.
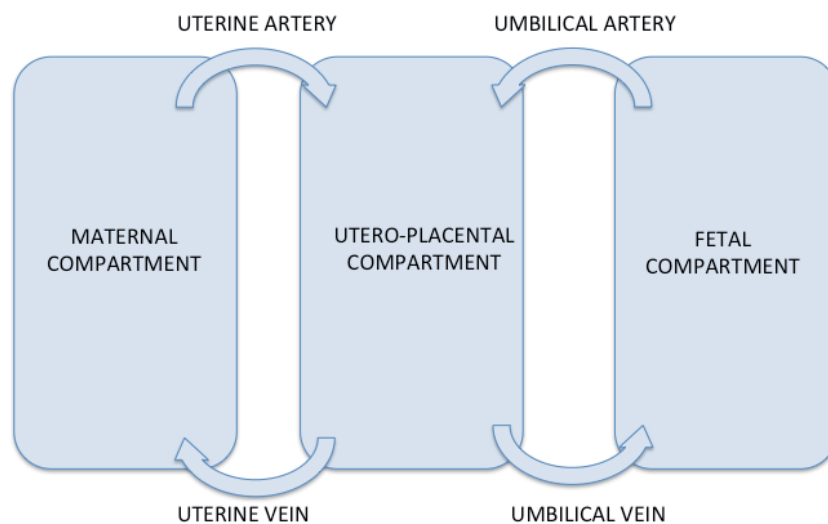
Figure 1.2. Schematic representation of the maternal utero-placental, and fetal compartment of a pregnant female.

Nutrient uptake by the gravid uterus in domestic livestock, including cows, is primarily depending on the uterine blood FV (Ford et al., 1984). Throughout pregnancy functional and structural changes in the utero-placental vascular system are necessary to satisfy fetal needs. The uterine artery adapts to these hemodynamic changes to ensure an adequate blood supply to the developing placenta. As pregnancy advances, and more markedly over the last half of the pregnancy, a progressive decrease in uterine arterial tone, and an increase of arterial diameter and blood FV is clearly appreciated (Ford, 1995). In fact, studies measuring resistance index (RI), which

represents the negative relationship between resistance and vascular perfusion of a tissue, reported a decrease of RI during the first 8 months of pregnancy and remained constant thereafter (Bollwein, 2002; Panarace et al., 2006). As mentioned above, blood FV supply to the uterus increases exponentially during pregnancy (Bollwein et al., 2002) and it seems that the increase is mainly due to the growth of the blood vessel diameter rather than to a rise of blood flow velocity.

In the past, measuring the uterine blood FV was expensive and complicated, and further, it required the utilization of invasive methodology such as the implantation of electromagnetic flow probes (Ferrell and Reynolds, 1992) or indwelling catheters. Transrectal Doppler ultrasonography has been described as a suitable and non-invasive technique for measuring uterine vessels hydrodynamics (Bollwein et al., 2002; Panarace et al., 2006) and during the last years, it has been increasingly used.

1.1.2.3. Doppler Ultrasonography

As mentioned before, Doppler technology is a non-invasive method suitable to measure uterine FV during pregnancy in livestock (Bollwein et al., 2002; Panarace et al., 2006). The Doppler effect is defined as the change in frequency of waves due to motion between source of wave and receiver (Maulik, 2005; Abramowicz and Sheriner, 2008). The change in frequency is known as Doppler frequency shift or Doppler shift (fd), being fd = ft − fr, where ft is the transmitted frequency and fr is the received frequency (Maulik, 2005). When artery blood FV is measured, the transducer is the stationary object and the red blood cells the moving reflectors that produce the echoes (Fig. 1.3.).

To obtain the blood FV of a certain vessel, a sample gate cursor should be placed on the image of the vessel of interest. In order to be able to measure FV, it is necessary to know the angle of isonation (α; Fig. 1.3.), and the cross sectional area of the vessel. In order to avoid error in calculating velocity, it is important to keep in mind while measuring FV that α should be lesser than 90º, and preferably between 30 and 60º (Maulik, 2005). Nevertheless, indices generally used in studies (as it is the case in Study 3 of the current thesis) are independent of α. Those indices are (Fig. 1.4.):

Pulsatility index (PI) = (peak systolic velocity (PSV) - end diastolic velocity (EDV))/mean velocity (MV); Resistance index (RI) = (PSV-EDV)/PSV and FV (ml/min) = Mean velocity (cm/s) x (π/4) x diameter^2 (cm^2) x 60 s.
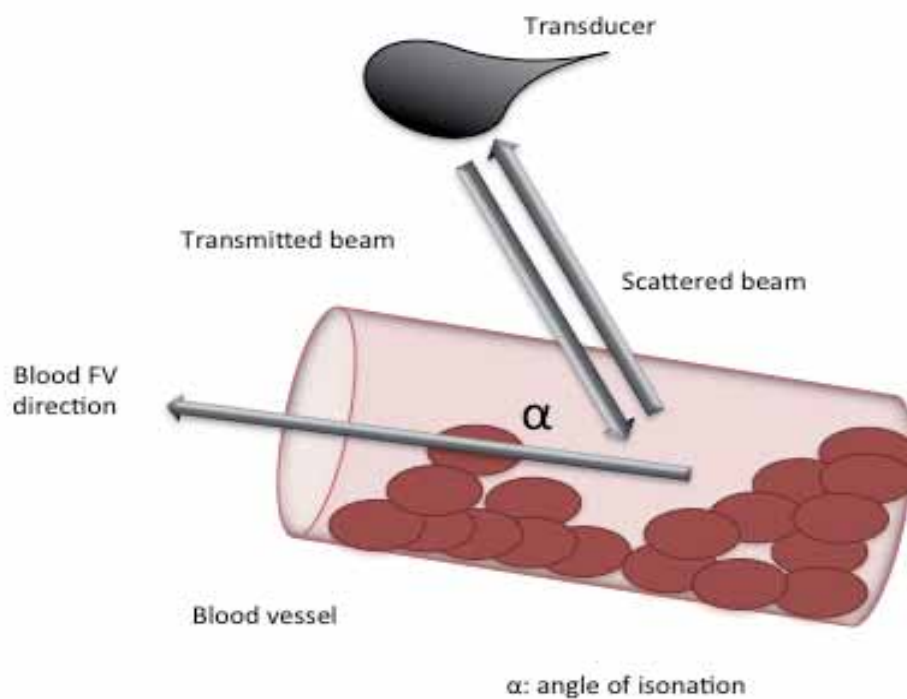
Figure 1.3. Doppler ultrasonography of a blood vessel. (Adapted from Maulik, 2005).
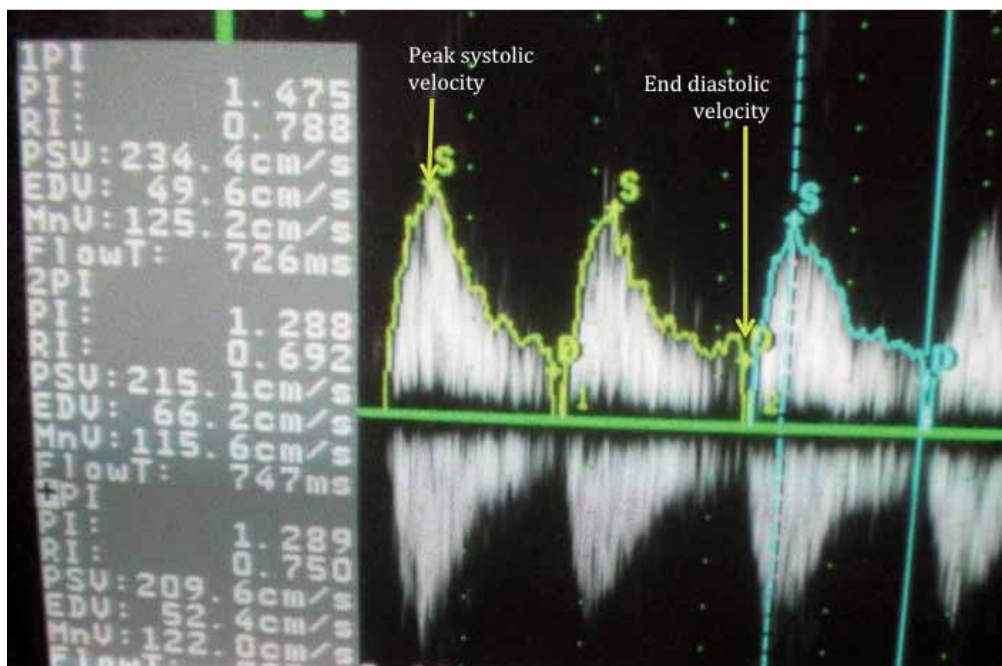
Figure 1.4. Doppler waveform of the uterine artery of a Holstein heifer.

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1.1.3. Provision of micronutrients

The supplementation of micronutrients may ameliorate the development of the fetus. Vitamins, minerals, and amino acids (AA) have been studied during pregnancy in animal and humans models. For example, vitamin A deficiency has been associated with reduced fetal survival (De Costello and Osrin, 2003). Vitamin B_6 seems to play an important role in the development of the central nervous system and its deficiency in rats leads to impaired physical and neuromotor development (Alton-Mackey and Walker, 1973). Also, a deficiency of folic acid has been associated with dysfunction in rapidly dividing cells (De Costello and Osrin, 2003) and vitamin E deficiency has been linked to malformations and fetal death (Basu and Dickerson, 1996). Also, minerals such as zinc, iron, copper, and magnesium have been linked with different fetal deficiencies (De Costello and Osrin, 2003). Maternal supplementation of calcium was found to reduce the offspring's blood pressure in childhood without affecting birth weight (Belizan et al., 1997).

Studies about supplementation of micronutrients in ruminants during pregnancy are not very numerous, however, some can be found in literature. In dairy cattle, AA are commonly in short availability. In the last years, feeding an adequate amount of each AA has been shown to allow reducing of the amount of protein included in the diets. Diets with an unbalanced AA profile cause, not only an excess of certain AA, but also a reduction of the absorption of other AA. Methionine has been identified as a limiting AA (Bach et al., 2000). Because of its participation in the regulation of translation and DNA methylation (Métayer et al., 2008), a methionine deficiency can lead to alterations in gene expression and metabolism of the offspring (Petrie et al., 2002). In fact, Sinclair et al. (2007), found that adult ewes that were offered a diet with restricted levels of methionine, B_{12} and folate had heavier and fatter offspring with altered immune system, insulin resistance, and had elevated blood pressure. In fact, methionine is a source of methyl groups, thus it should reduce the requirements for folic acid and vitamin B_{12} (Preynat et al., 2009). Indeed, a parenteral supplementation of vitamin B_{12} given to animals supplemented via its diet with folic acid and methionine, increased milk components yield (Girard and Matte, 1998). Furthermore, the supplementation of folic acid and/or vitamin B_{12} has been shown to increase milk production in dairy cows (Girard et al., 2005; Graulet et al., 2007; Preynat et al., 2009).

No available research in dairy cows about how methionine, vitamin B_{12} or acid folic can influence fetal programming was found. However, due to the increase in milk yield when supplementations of these nutrients were performed, it can be hypothesized that during lactation there may exist a limited supply of them, and thus, these micronutrients may also alter fetal development and gene expression of the offspring (Bach, 2012).

1.1.3.1. Arginine supplementation

It is important to make emphasis on arginine (Arg) because of its relationship with nitric oxide (NO). Arg is considered an essential AA for dairy cattle that participates in an array of body functions (Wu et al., 2009) such as lactation, growth, development, tissue remodeling, and response to several hormones and signaling molecules (Flynn et al., 2002). More important for fetal programming is the fact that Arg is an essential substrate for the synthesis of NO (Wu and Morris, 1998) and polyamines (Palmer et al., 1988; Wu et al., 2008). Nitric oxide is a vasoactive compound that participates in placental growth and angiogenesis and thus, it is necessary for increasing uterine and placental-fetal blood FV (Wu et al. 2008) and to secure the fulfillment of fetal nutritional needs (Bird et al., 2003). In addition polyamines regulate DNA replication and protein synthesis, and therefore, cell proliferation and differentiation (Flynn et al., 2002).

Some authors have proposed that manipulating the Arg-NO pathway can be a solution to prevent IUGR. Studies with livestock species have pointed that the administration of Arg increased protein accretion in ovine fetuses (De Boo et al., 2005), or the number of piglets born alive (Mateo et al., 2007). Furthermore, Chew et al. (1984) reported that Arg stimulated the synthesis of growth hormone (GH), prolactin (PRL) and insulin but in a transient way. Thus, more studies on Arg supplementation in dairy cattle during pregnancy could clarify in which amount and when Arg supplementation could be beneficial for fetal development and indirectly affect future offspring performance.

1.2. NEONATAL IMPRINTING

Some evidence for metabolic programming after birth in animal systems can be found in literature. Maybe, one of the most known is the familiar honeybee (*Apis mellifera*). The honeybee female embryos and newly born larvae are identical until they begin to be fed with either worker or royal jelly. Then signals are sent to the brain via the insulin signaling pathway. The results is that juvenile hormone levels are high in queen-destined larvae and low in worker destined larvae, contrary to what happen with DNA methylation levels that are higher in worker-destined larvae (Maleszka, 2008). As a consequence of that, anatomical and physiological differences are found between both castes.

Already in 1962, McCance conducted a study with rats where he demonstrated by adjusting litter size that the quantity of food consumed during early periods of postnatal life has long term consequences on growth. Interestingly, when the feeding limitations were applied later in life they had no consequences because the underfed rats had compensatory growth when they were re-fed at normal levels. In livestock species, there is little evidence of these mechanims. However, it has been observed recently that greater ADG during preweaning leads to greater milk yield during the first lactation (Bach and Ahedo, 2008; Bach, 2012; Soberon et al., 2012).

1.2.1. Effects of colostrum feeding

Colostrum is the first milk neonates receive after birth. Colostrum is rich in nutrients and biologically active compounds such as growth factors and hormones. The importance of feeding newborn calves an adequate volume of high quality colostrum is nowadays widely recognized as maternal IgG cannot traverse the placenta. To reduce the incidence of disease, a concentration of 10 mg of IgG/mL in the calf serum at 48 h after birth is commonly recommended (Donovan et al., 1986; Robison et al., 1988; Selim et al., 1995). And therefore, it is necessary to feed 4 L of colostrum immediately after birth. The relationship existing between plasma IgG concentration shortly after birth and future cow productivity was demonstrated by first time by DeNise et al. (1989). In the mentioned study, calves were allowed to suckle their dams for 24 h and thus, no quantification of colostrum consumption was made, however, regression

analysis between IgG concentration shortly after birth and milk production indicated that milk yields increased by 8.5 kg per IgG unit during the first lactation. Nevertheless, the authors suggested that these results were not only due to IgG directly but also to other factors present in colostrum. DeNise et al. (1989) hypothesized that these other factors could influence growth, protection against pathogens, metabolic performance and subsequent milk production. Faber et al. (2005), found reduced veterinary costs, greater growth rate, and greater milk when 4 L colostrum were offered instead of 2 L in Brown Swiss heifers.

Besides the colostrum IgG content, colostrum can influence postnatal intestinal development because of the presence of bioactive factors such as insulin-like growth factor (IGF)-I, IGF-II, growth hormone (GH), insulin, prolactin (PRL), and leptin (Odle et al., 1996; Blum, 2006). As first shown by Widdowson et al. (1976) in pigs, colostrum intakes lead to changes in morphology and function of the gastrointestinal tract (GIT) in neonates. More recently, various researchers had reported that the non-nutrient factors of colostrum modulate the GIT changing epithelial cell proliferation, migration, differentiation and apoptosis capacity, digestion, absorption and motility, and immune system development and function (Blum, 2006). Therefore, colostrum has also the potential to modify functions outside the GIT as metabolism and endocrine system, vascular tone and systemic growth (Blum, 2006). However, it is interesting to note that supplementation with a single growth-promoting factor such as lactoferrin or vitamin A, has no positive effects (Rufibach et al., 2006). Furthermore, these active factors present in colostrum do not seem to be absorbed in significant amounts early after birth to be able to induce the animal programing (Grütter and Blum, 1991; Donovan et al., 1997). Sparks et al. (2003) reported colostral IGF-I absorption into systemic circulation but at relative importance compared with endogenous sources. Also, calves fed colostrum had 6-fold greater IGF-I serum concentration than calves fed milk replacer during the first week of life (Hammon and Blum, 1997). Birth weight was not correlated with serum IGF-I (Sparks et al., 2003), thus birth weight does not influence the differences in serum IGF-I among neonates.

Referring to intermediary metabolism, there is a growing body of evidence that colostrum has a significant impact on the calf glucose status (Hammon et al., 2013). At birth, calves have a reduced glucose concentration, but after the first meal, it

increases depending on the amount and time point of colostrum feeding (Hammon and Blum, 1998). It seems that colostrum intake enhances glucose absorption by increasing intestinal surface (Hammon et al., 2013) and improves lactose digestion. Moreover, plasma insulin concentration was also reduced at birth and increased after first meal (Hammon and Blum, 1998). As previously said, there is no evidence that colostral insulin is absorbed in significant amounts in the GIT of neonatal calves (Grütter and Blum, 1991). However, calves fed colostrum had greater post-prandial insulin concentration than the ones that did not receive colostrum (Blum and Hammon, 1999), indicating that colostrum has an effect on intermediary metabolism.

1.2.2. Effect of liquid feeding sources: Whole milk vs. milk replacers

Liquid feeding options are mainly milk replacers (MR), saleable whole milk (WM), non-saleable whole milk (NSWM), and pasteurized NSWM (and also pasteurized WM, but this is fairly uncommon). Before 1956, only WM was offered to dairy calves and feeding rates were not established (Otterby and Linn, 1981). The first MR was developed around 1950 and its use increased progressively until nowadays. However, their prices have been rising lately, and have reached historic heights due mainly to the price of protein and dry whey, and thus other liquid feeding options have been evaluated. For example, in order to decrease MR costs, alternatives of non-milk proteins have been used, but calves fed non-milk protein sources have been reported to have reduced growth and performance compared with those fed high-quality MR or WM (Gardner et al., 1990; Quigley, 2002; Touchette et al., 2003) and it is commonly accepted that non-milk derived protein products are not suitable for very young calves (Drackley, 2008).

Milk replacer continues to be the most commonly used liquid feeding in dairy farms. However, other available sources such as WM or NSWM are also an option. NSWM correspond to transition milk or waste milk (medicated animals). All these options are suitable to rear dairy calves when used properly (Keys et al., 1980). WM is the natural liquid feeding for calves, and in humans it has been largely demonstrated that maternal milk is the best nutritional support for the neonate (Donnet-Hughes et al., 2000) and it also provides a greater protection against respiratory tract infections and diarrhea (Hanson et al., 2003). In dairy cattle, a more economical option can be

feeding NSWM (waste or transition milk). However, calves fed milk containing antibiotics have greater preweaning mortality (Losinger and Heinrichs, 1997), and also antibiotic resistances may appear. Little studies have been performed about the subject and some of them did not find differences in antibiotic resistance index when comparing calves fed milk with or without antibiotics (Wray et al., 1990), but others did report differences (Selim and Cullor, 1997).

Not matter which type of WM, it contains hormones and growth factors mentioned for colostrum, but in lesser quantities. However, it may be possible that little quantities of these active nutrients may exert some benefits in the metabolism of the calf and may provoke an increase of its future milk production. Indeed, studies comparing growth and future performance on calves fed MR and WM are reported in Table 1.1. As it can be seen, contradictory results are found; some authors (Lynch et al., 1978; Jaster et al., 1990) did not find differences between feeding MR and WM. But, in some studies, greater average daily gain (ADG) during preweaning was found when calves were fed MR compared with calves fed WM (Hill et al., 2008) and in contrast, in others studies, calves fed WM had better performance and lesser incidence of disease than calves fed MR (Fisher, 1976; Godden et al., 2005; Gleeson and O'Brien, 2012) as WM improved growth and decreased morbidity of the animals compared with MR. Moreover, WM seems to give a greater protection against diseases such as it has been demonstrated in humans (Donnet-Hughes et al., 2000). WM-fed calves had lesser mortality and need of veterinary treatments (Losinger and Heinrichs, 1997; Godden et al., 2005) than calves fed MR. Such amelioration could be due to the presence of protective IgG, nonspecific immune factors (Godden et al., 2005) and other hormones such as growth factors that may play a function in the regulation of the immune system (Clark, 1997). Moreover, it seems that a beneficial effect exists when WM was offered compared with MR in future milk performance of the heifers (Bar-Peled et al., 1997; Shamay et al., 2005; Moallem et al., 2010). However, the number of animals of these studies was low, and no clear explanation of the potential underlying mode of action has been provided. Furthermore, in many studies, the amounts of milk provided differed between WM- and MR-fed calves.

Table 1.1. Summary of research studies evaluating different milking feeding sources in dairy calves performance, health and future productivity.

Study	Treatment[1]	n[2] and sex	Feeding times per day	ADG, g/day	P-value[3]	Milk yield, kg/300d	P-value[4]
Lynch et al., 1977	WM/MR;12%BW	4/4 ♂	2	1050 / 270	<0.01[a]		
	WM/MR; 8% BW	4/4 ♂	2	270/200	NS	--	--
	WM/MR; 8L/d	4/4 ♂	2	710/70	<0.01[a]		
Bar-Peled et al., 1997	Suckling vs. MR; (360g/d)	20/20♀	3 vs. 1	820/680[b]	<0.05	9,624/9,171	< 0.1
Shamay et al., 2005	WM (*ad libitum*)/MR (450 g/d)	10/10♀	2 vs. 1	882/591[c]	<0.05	12,104/11,558	<0.05[d]
Godden et al., 2005	WM/MR; (3.8 to 5.6L/d)	223/ 215 1/2♂+1/2♀	2	470/350[c]	< 0.001	--	--
Hill et al., 2008	WM/MR; (418g/d)	16/16 ♂	2	509/554[c]	<0.05	--	--
Moallem et al., 2010	WM/MR; (*ad libitum*)	8/8♀	2	807/733[c]	<0.01	10,170/8.820	<0.05[d]
Gleeson and O'Brien, 2012.	WM(4.5L/d)/MR (454g/d)	18/18♀	1	680/790[c]	<0.1	--	--

[1] WM= Whole milk; MR=Milk replacer .

[2] n= number of animals per treatment and sex. ♀: female; ♂:male.

[3] Effect of feeding source in ADG. NS: No significant.

[4] Effect of feeding source in milk yield.

[a] differences only during 7th week of life; [b] from birth to conception; [c] nursing period; [d] only treatments with additional protein after weaning are consider.

1.2.3. Effect of milk quantity

Calf management programs commonly consists of feeding MR at 10% of calf BW twice a day diluted at 12.5% DM to achieve a target growth rate of 500 g per day. During decades, feeding strategies focused on attempting an early weaning (Savage and McCay, 1942; Kertz et al., 1979; Baldwin et al., 2004) and improving calf starter intake

by reducing the amount of milk fed to calves. But calves fed low milk allowances have low growth rates (Flower and Weary, 2001) and high rates of mortality (NAHMS, 2007). In the last years, several studies have investigated the effect of increasing milk allowances (Diaz et al., 2001; Jasper and Weary, 2002; Bartlett et al., 2006; Terré et al., 2007). It seems that achieving greater growth rates at early stages in life (2-3 months) might be profitable, as increases in relative BW and wither height are more cost-efficient during the first 6 months of life (Kertz et al., 1998).

Increased ADG had been reported when milk was fed *ad libitum* (Jasper and Weary, 2002) or at increased rates (Diaz et al., 2001; Quigley et al., 2006). The critical point when high quantities of milk are offered to calves is the reduction of liquid feed during preweaning (Jasper and Weary, 2002; Shamay et al., 2005; Quigley et al., 2006; Terré et al., 2007) because it may negatively influence rumen development (Anderson et al., 1987b) and nutrient provision to the animal (if solid feed consumption does not rapidly compensate the lack of milk). Indeed, when high nutritional planes are used, during the weaning process, when MR was reduced to stimulate starter intake (Jasper and Weary, 2002; Brown et al., 2005; Terré et al., 2009) or during the week after weaning (Bar- Peled et al., 1997), ADG was markedly reduced. However, after weaning, equal starter intakes (Quigley et al., 2006) and similar ADG (Terré el al., 2009) have also been reported in enhanced-fed and conventional-fed calves. The ability to compensate or not the lack of liquid feeding is not clear, as different weaning methodologies have led to different results. It may be hypothesized that it is the combination of several factors (milk quality, starter quality and management) that can influence the final result. Another critical point is the risk of increasing diarrhea, as higher faecal scores have been found (Diaz et al., 2001; Raeth-Knight et al., 2009) in calves fed high amounts of milk. However, several studies have reported no differences in faecal scores in calves fed different nutritional planes (Jasper and Weary, 2002; Terré et al., 2009).

Moreover, there are several studies that showed a link between milk yield and plane of nutrition in early life. Calves that received large amounts of whole milk or were allowed to suckle from their dams had (Shamay et al., 2005; Drackley et al., 2007) or tended to have (Bar-Peled et al., 1997) a greater milk yield during their first lactation than restricted calves. However, other studies did not show this difference between planes of nutrition (Terré et al., 2009). Part of the improvement in milk yield can be

due to the increased of BW at calving, as these both parameters are positively correlated (Keown and Everett, 1986; Bach and Ahedo, 2008). Thus, it is important that when implementing enhanced feeding programs, weaning processes that allow maintaining the BW gain are in place.

Another benefit associated with feeding high amounts of MR is the amelioration of the immune response. Pollock et al. (1994) were the first studying whether calves receiving a high feeding level would react differently to an immune stimulator (keyhole limpet haemocyanin). In the same direction, Nonnecke et al. (2003) also hypothesized that an enhanced plane of nutrition may ameliorate the immune function because it increases plasma IGF-I concentrations (Bartlett, 2001; Smith et al., 2002), and IGF-I acts as a cell proliferation cofactor and differentiation factor on B cell development and it enhances the proliferative response of lymphocytes to mitogens (Clark, 1997).

The critical period for mammary development seems to occur between 90 and 300 kg of BW (Sejrsen et al., 1982; Capuco et al., 1995) and some authors have questioned enhanced feeding programs because it may impair the development of the mammary gland (Lammers et al., 1999; Silva et al., 2002). However, the majority of the studies reported that an increase of nutrient intake during the first weeks of life results in an increased of milk yield during the first lactation (Bach and Ahedo, 2008; Bach, 2012; Soberon et al., 2012). In fact, Brown et al. (2005) reported that increasing protein intake at the same time as increasing energy intake during the preweaning period increased mammary parenchyma mass without increasing intraparenchymal fat content. In the same direction, Meyer et al. (2006) reported almost 40% increase in mammary epithelial cell proliferation when calves were fed an enhanced feeding program compared with a conventional feeding program during the first 60 days of life. Thus, according to the previous studies reported herein, offering high amount of milk or MR to calves seems a good management practice. However, it is important to decide the frequency of the feedings. When calves are allowed to suckle they can have around 22 meals per day (Appleby et al., 2001). However, only two or three meals are usually offered in commercial farms and those will be followed by a long fasting period and this situation can lead to insulin resistance (Bach et al., 2013) that could potentially imprint the metabolism of the heifer. In fact, some studies have already reported insulin resistance in dairy calves fed high amounts of MR, which indicates a

non-efficient use of glucose (Hostettler-Allen et al., 1994; Hugi et al., 1997a; Bach et al., 2013).

1.2.4. Effect of weaning methods and calf management

A smooth transition from liquid to solid feeding is important to minimize weight loss and stress at weaning (Weary et al., 2009). Stress at weaning should be minimized because it may compromise the immune response of calves for at least 2 weeks after weaning (Hulbert et al., 2011). Studies evaluating the effect of weaning calves in groups or individually on performance (Terré et al., 2006), behavior (Keil and Langhans, 2001), and health (Terré et al., 2006) can be found in literature with different results. Bach et al. (2009) recommended moving calves into groups at 49 days of age and then starting the weaning process as it improves solid feed intake, growth performance and health condition. However, Terré et al. (2006) did not find differences in final BW or starter dry matter intake (DMI) between calves that were located individually or in groups throughout the entire rearing period, but the decrease of ADG during the week following weaning was more pronounced in calves that were in groups (mainly due to increased incidence of disease). Thus, when health levels are high and the environment for the calves is adequate, weaning in groups is recommended, but if producers are incapable of providing adequate conditions for the calves, then weaning individually may be a safer option.

Also, it is important to decide which criterion has to be followed to decide when a calf should be weaned. A Swedish study (Pettersson et al., 2001) reported that 46% of the herds used calf age as weaning criterion, and only 18% of the herds used concentrate consumption. The same study explained that the most common management practice to wean is to dilute the milk with successively increasing volumes of water (32%), followed by weaning calves abruptly (21%), or offering one MR meal per day for 5 days (21%). Early weaning (at 4 weeks instead of 6 weeks of age) is possible (Anderson et al., 1987a; Hopkins, 1997) but starter intake may be not enough to compensate the lack of MR intake at that stage (Quigley, 1996) and thus ADG losses may occur. To avoid that, Greenwood et al. (1997) suggested to wean when calves reach a dry feed intake of 1% of initial BW as it does not impair performance compared with calves weaned with a starter intake of 1.5% or 2% of initial BW. Actually, even though

removal of milk is the major stimulus for dry feed consumption (Appleman and Owen, 1975), the greater the intake of starter before weaning the greater the chances to sustain a desirable growth rate after weaning (Kertz et al., 1979). In the same direction, Davis and Drackley (1998) suggested that the minimum daily consumption of starter after weaning should be 0.8-0.9 kg as it would allow the calf to cover all the maintenance needs and to do a modest weight gain.

Long term effects of weaning methods are not very often reported, and little literature can be found about this subject. No differences in future milk production was found when calves were allowed to stay (and suckle or not) during 4 days with their dams compared with the ones that were separated at birth (Krohn et al., 1999), or reared individually or in groups (Mogensen et al., 1999). However, Arave et al. (1985) reported that calves housed individually and in isolation (without visual access to other calves) prior to weaning produced significantly more milk than calves housed in hutches or in groups of six. In the same study, behavioral measurements (vocalization, defecation, and urination during handling experiments) were greater in calves housed in groups and the authors suggested that these behaviors could indicate increased stress in group-housed calves. However, subsequent work (Arave et al., 1992) did not show an increase in milk production or changes in calf behavior. Last, Creel and Albright (1985) reported that calves reared in isolation appeared more stressed as indicated by greater standing behavior and increased serum cortisol concentrations. In view of the non-consistency of the results in how rearing and weaning strategies affect future milk performance, more studies will be necessary to elucidate which is the most suitable strategy.

Chapter 2

OBJECTIVES

The main objective of this thesis was to evaluate different feeding managements for pregnant cows and neonatal calves that could ameliorate future performance and productivity of heifers. The specific strategies considered were:

1. To compare intake, growth, immune response, and reproductive parameters in dairy calves fed either milk replacer or whole milk.

2. To compare intake and growth performance, and reproduction parameters, as well as glucose metabolism in dairy calves fed different allowances of milk replacer.

3. To evaluate the effects of Arg supplementation on uterine artery hemodynamics between 40 and 140 days of gestation in pregnant dairy heifers.

To achieve these objectives, three studies were conducted:

Study 1: Exploring potential changes in performance and immune response in dairy calves offered milk replacer or whole milk.

Study 2: Determining short- and medium-term changes in performance and metabolism of dairy calves offered different amounts of milk replacer.

Study 3: Evaluating the impact of Arg supplementation on placental vascularization in pregnant dairy heifers.

Chapter 3

POTENTIAL CHANGES IN PERFORMANCE AND IMMUNE RESPONSE IN DAIRY CALVES

OFFERED MILK REPLACER OR WHOLE MILK.

3.1. Introduction

One of the main objectives of dairy farms is to rear healthy female calves that will in turn become healthy and productive milking cows. It is progressively becoming clear that metabolism in mammals is influenced by their perinatal nutritional environment (Bartol et al., 2008; Bach, 2012). Liquid feeding options for calves are mainly milk replacers (MR), saleable whole milk (WM), non saleable WM, and pasteurized non saleable WM. Milk replacers have been used for many years now, but their prices have been rising lately, and have reached historic heights due mainly to the price rise of protein and dry whey (Jones and Heinrichs, 2003). In order to decrease MR costs, alternatives of non-milk proteins have been used. However, calves fed non-milk protein sources have been reported to have reduced growth and performance (Touchette et al., 2003) and it is commonly accepted that non-milk derived protein products are not suitable for very young calves (Drackley, 2008).

On the other hand, in humans, it is well established that breast milk is the best nutritional support for the neonate (Donnet-Hughes et al., 2000), and some studies report that breastfed-children are better protected against respiratory tract infections and diarrhea than those fed infant formulas (Hanson et al., 2003). Calves fed WM had better performance and lesser disease events than calves fed MR (Fisher, 1976; Godden et al., 2005), but Lynch et al. (1978) found no differences between feeding MR and WM.

Herein, we hypothesized that offering WM to neonate calves would improve growth performance and immune function. Thus, the objective of the present study was to compare intake, growth, immune response and reproductive performance in dairy calves fed either MR or WM.

3.2. Materials and methods

3.2.1. Animals and treatments

Seventy dairy female Holstein calves were weighed at birth (42.7 ± 0.57 kg), offered 4 L of colostrum within 2 h after birth and housed in individual hutches where they received 3 meals more of colostrum every 12 h. Calves (n = 35) were randomly assigned to either a conventional feeding program based on MR or the same feeding program using WM, in both cases milk was offered twice a day. Before every feeding,

raw milk from healthy cows was collected from the milking parlor and then immediately offered to calves. All milking cows were blood sampled to ensure that both the colostrum and the fresh milk that were used in the study were free from *Mycobacterium avium paratuberculosis*.

In both cases (MR or WM), the amount of milk DM offered to calves was 500 g/day from day 2, and the amount was increased progressively until reaching 750 g/day. All the calves were fed 750 g/day from day 15 to 56, and 375 g/day from 56 to 63 days of life. Milk replacer was offered at 15 % DM and WM had on average 12.47 ± 0.404 % of DM. Moreover, the MR contained 22.9 % CP, 20.1 % fat, and 40 % lactose, whereas the WM contained on average 28.8 ± 6.9 % CP, 23.2 ± 3.5 % fat, and 36.3 ± 1.1 % lactose. Animals were housed in individual pens until they reached 10 days of age, when they were moved in pens of groups of 5 calves. In total, 14 pens of 5 calves were used in this trial. Calves were grouped by pen according to treatment (i.e., 5 calves on WM in one pen; 5 calves on MR in another pen) and remained in groups until they reached 70 days of age when they were grouped again in pens of around 80 heifers, and animals from both treatments were commingled. Calves had free access to water and chopped straw throughout the study. All animals were weaned at 63 days of age and starter feed (Table 3.1.) and water were offered *ad libitum* throughout the study. All calves were fed twice a day.

Table 3.1. Chemical composition of whole milk, milk replacer, and starter feed (% of dry matter).

	Whole milk	Milk replacer	Starter feed
Crude protein	28.8 ± 6.9	22.9	21.8
Fat	23.2 ± 3.5	20.1	4.1
Lactose	36.3 ± 1.1	40.0	--
Neutral detergent fiber	--	--	16.4
Acid detergent fiber	--	--	8.0
Ash	--	7.5	4.4

3.2.2. Measurements and sample collection

Whole milk samples were collected monthly across the study. Daily milk and feed intakes were recorded from day 2 to 63 and veterinary treatments were recorded throughout the study. Animals were weighed weekly, and average daily gain (ADG) and gain to feed (GtoF) were then calculated. Also, daily crude protein (CP), fat and metabolizable energy (ME) intake were calculated. Reproduction parameters including age at first effective insemination (FEAI), and number of previous insemination were also recorded.

At days 14, 28, 42 and 56, 4 to 6 h post feeding, blood samples were collected to determine glucose and insulin concentrations. Determination of plasma glucose was performed using a glucose reagent (Beckman Coulter, Brea, California) and serum insulin using a bovine insulin ELISA kit (Mercodia, Uppsala, Sweden). Immune response was evaluated in blood samples collected at 35 days of age by measuring TNF-α after a lipopolysaccharide (LPS) in vitro challenge in the extracted blood. Also, at days 7, 21 and 35, 1-ml of a solution containing 0.5 mg of ovalbumin was injected to calves and blood samples were collected at days 7 and 56 of life to quantify antibody titers against ovalbumin before and after the ovalbumin challenge. For this analysis, an ELISA test was used.

3.2.3. Statistical analysis

Growth performance, blood, and immune test data were analyzed using a mixed-effects model with repeated measures that accounted for the fixed effects of treatment, day, and their 2-way interaction, and the random effect of calf. Body weight at day 0 entered the model as a covariate, and time as a repeated measure. Data from blood insulin and insulin to glucose ratio were previously log10-transformed to reach a normal distribution. The variance components structure yielded the smallest Schwarz's Bayesian criterion and thus it was used as the selected variance-covariance matrix for the analysis.

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3.3. Results and discussion

3.3.1. Growth and reproductive Performance

Calf performance between day 2 to weaning is presented in Table 3.2. Neither milk nor starter concentrate intake differed between WM and MR calves. The objective was to offer the same amount of DM from both milk sources (WM or MR) but it is clear that variations in WM could have occurred (Hill et al., 2008). Solid feed intake did not differ between treatments, and as a consequence no differences in total DM intake were found. However, as whole milk had a numerically greater concentration of CP and fat, calves fed WM had a greater CP (P < 0.05) and fat (P < 0.0001) intake. Metabolizable energy intake did not differ between both treatments (P = 0.41). Neither BW nor ADG differed between treatments and evolved similarly in both feeding plans (Fig. 3.1). Consequently gain to feed ratio did not differ between treatments. Because of the greater intake of fat and CP observed in WM-fed calves, growth performance differences were expected to occur. Similar to the present study, Lynch et al. (1978) and Jaster et al. (1990) did not report differences in performance when feeding MR or WM to dairy calves. In contrast, Hill et al. (2008) reported greater feed intake in calves fed MR compared with those fed WM. Consequently, an increase of 13% in preweaning ADG in calves fed MR than in calves fed WM was reported. Gleeson and O'Brien (2012) also found that ADG tended to be lesser in calves fed MR compared with calves fed WM. But remarkable differences (sex of the calves or number of feeding bouts) could have interfered in the results between these 2 trials.

It is important to note, that in the current study, animals did not have free access to the milk source that was offered. The recorded intakes for this study were, on average 592 ± 8.6 g/day, whereas when calves are offered WM *ad libitum*, intakes of 1,090 g/day (Moallem et al., 2010) or 995 g/day (Shamay et al., 2005) have been reached. In both of these mentioned studies, where WM was offered *ad libitum*, a beneficial effect compared with MR was found. Thus, it may be that in order to attain more noticeable differences between both milk sources calves may need to be fed high quantities of milk.

Table 3.2. Intake and growth performance of calves fed either whole milk (WM) or milk replacer (MR) during the preweaning period (from 2 to 63 days of age).

	Treatment[1]			P-value[2]		
	WM	**MR**	**SEM**	**T**	**A**	**TxA**
Intake, g of DM/day						
Milk	592	593	8.6	0.93	<0.0001	0.99
Starter feed	398	444	58.1	0.58	<0.0001	0.98
Total	990	1,037	57.5	0.92	<0.0001	0.58
CP[3]	281	236	11.9	0.01	<0.0001	0.09
Fat	165	142	3.5	<0.0001	<0.0001	0.70
ME[4], Mcal/day	2970	2897	58.5	0.41	<0.0001	0.79
Growth Performance						
ADG[5], g/day	620	625	13.9	0.78	<0.0001	0.17
GtoF[6]	0.60	0.58	0.034	0.63	<0.0001	0.27

[1] Type of milk offered: Whole milk (WM) or milk replacer (MR).

[2] T = effect of treatment; A = effect of age; T x A = interaction between the effect of treatment and age.

[3] Crude protein.

[4] Metabolizable energy.

[5] Average daily gain.

[6] Gain to feed ratio

No differences were found in reproductive performance between heifers that were fed WM or MR. The first effective insemination was, on average, at 490 ± 8.1 days of life. And the number of insemination needed to achieve conception did not differ between treatments either. Calves allowed to suckle their dams had a lower calving age than calves fed MR (Bar-Peled et al., 1997). Bar-Peled et al. (1997) explained that milk quality was the only difference that may contribute to improve performance, however, calves that were allowed to suckle consumed 14% more energy than the calves fed

MR. In the present study, this advantage in energy intake was not present, and thus, it may have hidden potential differences. Moreover, Moallem et al. (2010), also reported a numerically lower age at first calving of calves fed WM compared with MR, but as differences in intakes also have appeared in this study, reproductive performance could have been modified because of the higher intake rather than because of the differences in milk source.
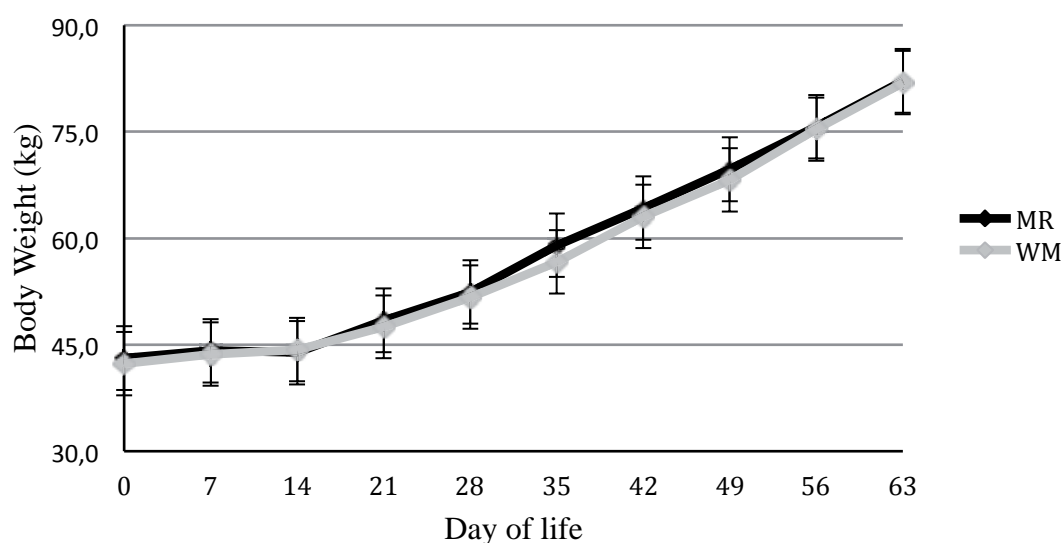
Figure 3.1. Evolution of body weight of calves fed either milk replacer (MR) or whole milk (WM).

3.3.2. Blood parameters

Overall glucose concentration did not differ between treatments and serum insulin concentration was lower (P < 0.001) in calves fed WM than in those receiving MR (Table 3.3). However, as shown in Figure 3.2, plasma glucose concentrations evolved differently during the study (P < 0.05) depending on the milk source. Plasma glucose concentration was constant throughout the study for MR-fed calves whereas WM-fed calves had increased plasma glucose concentrations at day 56 compared with those read at day 14. These results are surprising, as basal glucose concentration usually decreases with age (Wijayasingue et al., 1984; Quigley et al., 1991; Maccari et al., 2014). At day 14, concentrations were similar to those reported by Quigley et al. (1991) and typical of preweaned calves. However, glucose was expected to be lesser than the reported values and around 85 mg/dl at day 56, which would be the typical

values for mature ruminant animals (Wijayasingue et al., 1984; Quigley et al., 1991; Maccari et al., 2014).

Table 3.3. Blood metabolites, and immune responses to lipopolysaccharide in vitro challenge and ovalbumin injection of calves fed either whole milk (WM) or milk replacer (MR) during the preweaning period.

	Treatment[1]			P-value[2]		
	WM	MR	SEM[3]	T	A	TxA
Glucose (mg/dl)	116.9	116.6	3.09	0.94	0.65	0.04
Insulin (µg/l)[3]	0.78	1.36	0.053	<0.0001	0.003	0.44
ItoG (10^{-4})[3,4]	0.07	0.12	0.535	<0.0001	0.0003	0.81
TNF-α[5] (ng/ml)	0.216	0.213	0.0487	0.98	-	-
Anti-Ovo[6]	1.85	1.80	0.108	0.68	-	-

[1] Type of milk offered: Whole milk (WM) or milk replacer (MR).

[2] T = effect of treatment; A = effect of age; T x A = interaction between the effect of treatment and age.

[3] Least squares means for insulin concentrations and the ratio insulin to glucose blood concentrations presented herein correspond to non-transformed data, and SEM and P-values correspond to the ANOVA analysis using log10-transformed data, respectively

[4] Insulin to Glucose ratio

[5] after a lipopolysaccharide *in vitro* challenge

[6] Antiovalbumin titers absorbance with 450 nm wavelength

One hypothesis for the high glucose levels found herein can be because calves were housed in groups when blood samples were taken. Therefore, when entering the pen to take the blood sample, five animals became stressed and triggered changes in the metabolism proper of an alarming situation (Temple et al., 2014). In addition, the time

spent in each pen was 5 times longer compared with blood sampling in individual hutches. The stress response is elicited by glucocorticoid hormones such as cortisol. These hormones have numerous effects throughout the body, including the mobilization of stored energy (Cherrington, 1999). Cortisol counters insulin by encouraging greater blood sugar concentrations and stimulating gluconeogenesis (Hoehn and Marieb, 2010). Thus, it could be that the glucose levels were increased because of the stress during blood collection. Nevertheless, comparison between treatment groups should still be valid.

Moreover, plasma glucose concentration was expected to decrease with age independently of the liquid feeding source (Wijayasingue et al., 1984). Interestingly, at day 56, WM calves tended to have a greater concentration of blood basal glucose concentrations than MR calves. This can be due to the greater fat intake of the WM-fed calves, as plasma glucose concentrations were increased in calves fed high-fat MR (Wijayasingue et al., 1984). Furthermore, Lee et al. (2009) found a greater serum triglyceride concentration in WM fed calves and they ascribed it to a greater milk fat content and also to an increased fat absorption in WM. Another possible explanation for the increased plasma glucose in WM-fed calves could be found in the presence of certain amino acids in whole milk that are not present in MR that could serve as glucose precursors (Young, 1977) or also due to the presence of some casein proteins. It is thought that some casein proteins induce diabetes (Clarke and Trivedi, 2014), and it has been demonstrated that A1 β-casein induced diabetes in non-obese mice (Elliot et al., 1997). Indeed, Cohick et al. (1986) already found a significant glucagon increase when casein was infused. However, this item still remains controversial as there are also studies reporting a non-influence of casein in diabetes (Hill and Boland, 2001). Anyhow, the presence or absence and the type of casein in MR and WM may be key to understand the differences in performance and immunology between different studies found in literature. The ratio of insulin to glucose (ItoG) in blood was lower ($P < 0.0001$) for WM than for MR calves (Table 3.3). At similar glucose intakes, an increase in ItoG ratio can be an indicator of insulin resistance (Hostettler-Allen et al., 1994). However, the values obtained in this study were collected between 4 to 6 h post feeding, and therefore they did not reflect early meal responses (which would more directly depend on the amount of glucose consumed).
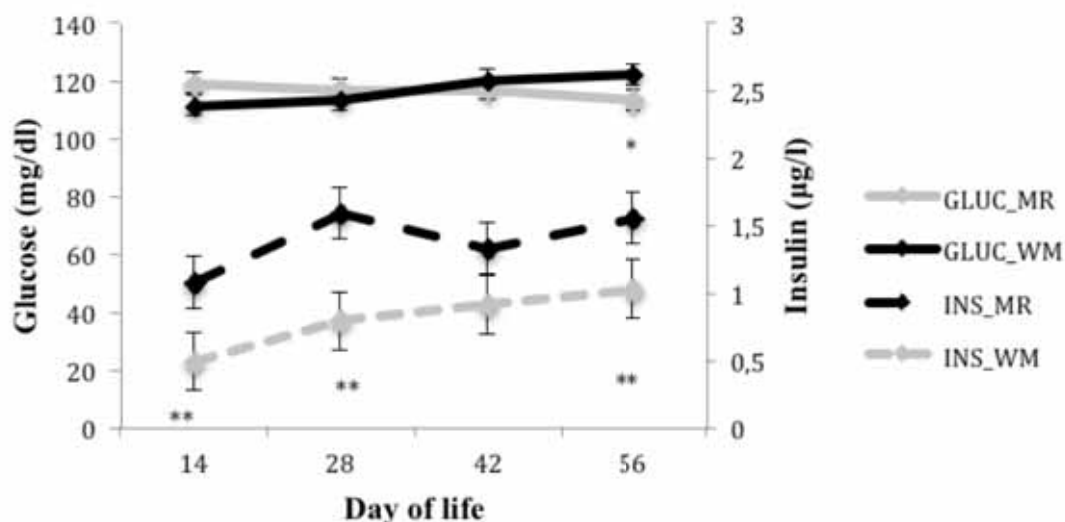
Figure 3.2. Evolution of plasma glucose (GLUC) and serum insulin (INS) concentration (mg/dl, and µg/l respectively) in calves fed milk replacer (MR) or whole milk (WM). * indicates differences between groups (* $P < 0.1$; ** $P < 0.05$).

In vivo challenge with ovalbumin was reported as a good indicator of β-cell activity (Foote et al., 2007). In the present study the immune response against ovalbumin did not differ between treatments and neither did the TNF-α concentrations after the LPS challenge. These results suggest that, contrary to our initial hypothesis, calves fed WM did not have an apparent immune benefit compared with calves fed MR. A better immunological response could have been expected in WM-fed calves because WM contains protective IgG, nonspecific immune factors (Godden et al., 2005) and other hormones such as growth factors that may play an important function in the regulation of the immune system (Clark, 1997). Although no differences in the immune parameters determined in the present study, WM calves tended ($P = 0.06$) to need less veterinary treatments than MR calves. Similarly, Godden et al. (2005) observed benefits of WM consumption in calves such as a reduction of mortality or a lesser risk of needing veterinary treatments. In contrast, Gleeson and O'Brien (2012) reported a greater incidence of pneumonia cases when calves were fed WM compared with MR but did no found differences in the number of cases of diarrhea. However, in the study conducted by Gleeson and O'Brien (2012) calves were only fed once per day and milk was offered at cold temperatures (10-15ºC) whereas in our study, calves were fed

twice a day and milk was offered at 38ºC. Whole milk contains others bioactive substances as for example lactoferrin. Lactoferrin is an antimicrobial glycoprotein present in the milk of most mammals (Masson and Heremans, 1971). Furthermore, supplementation of lactoferrin in neonatal calves improved faecal scores and reduced the number of days medicated (Robblee et al., 2003), thus its presence in WM also may help the immune response against diarrhea infections. The reason why those benefits were not apparent in the blood parameters measured (anti-ovalbumin and LPS challenge) in the current study could be because the techniques that were used were not the most appropriate ones or have not been performed in the right moment. As an example, Ballou (2012) measured several parameters of immune response in calves and most of the observed differences occurred during postweaning. Thus, in the present study, measures during postweaning could have been useful in order to detect any difference in immune response. Furthermore, more immunological challenges could have been performed. For example, measurements of plasma concentrations of haptoglobulin could have been interesting as haptoglobulin is not present in healthy calves but increased dramatically with inflammatory disorders even before the clinical signs appears (Gånheim et al., 2007; Cray et al., 2009).

3.4 Conclusions

Feeding WM to calves did not result in any differences in intake, BW, ADG, or GtoF in comparison with those calves fed MR. No differences in immune response were detected in blood parameters after anti-ovalbumin or an LPS challenge. However, calves fed WM tended to need less medication than calves receiving MR. It may be possible that the moment or the technique that was chosen to measure the immune response was not the most adequate. Further research is needed to achieve a better understanding of the relationship between milk source and immune function.

3.5 Acknowledgements

Chapter 4

SHORT- AND MEDIUM-TERM CHANGES IN PERFORMANCE AND METABOLISM OF

DAIRY CALVES OFFERED DIFFERENT AMOUNTS OF MILK REPLACER.

A fraction of this research has been submitted to:

Livestock Science (April 2015)

4.1. Introduction

Most recommendations for feeding replacement dairy heifers focus on rearing the animals in a cost-efficient manner, but tend to omit the potential long-term consequences that early calf nutrition may exert. In the last decade, though, several studies have evaluated the short-term effects of large milk allowances during the first weeks of life (Diaz et al., 2001; Bartlett et al., 2006; Terré et al., 2007). According to those studies, feeding large milk allowances could be beneficial as it improves preweaning ADG (Terré et al., 2009), and reduces the days at first calving (Bar-Peled et al., 1997; Moallem et al., 2010).

Thus, it would seem that dairy herds could benefit from offering high amounts of MR to calves. However, calves on high MR allowances have been reported to have increased blood insulin to glucose ratio (Hammon et al., 2002; Terré et al., 2009) and become insulin resistant at early ages of life (Hostettler-Allen et al., 1994; Hugi et al., 1997a; Bach et al., 2013). This problem could be aggravated if feeding frequency (FF) is low. Commercial dairy herds usually feed calves only twice a day. Thus, if high amounts of milk or MR are offered only twice a day, the metabolic capacity of the calf could be overwhelmed as their capability to metabolize large amounts of nutrients is limited (Hostettler-Allen et al., 1994). In fact, decreasing the FF for the same milk allowance has negative effects in glucose metabolism as hyperglycemia and glucosuria (Vicari et al., 2008) and also provokes a decrease of the efficiency of utilization of protein and energy (Van den Borne et al., 2006). Indeed, Kaufhold et al. (2000) found lower concentrations of plasma glucagon when calves were allowed to suckle several times compared with calves fed only twice a day with the same amount of milk replacer per day.

Therefore, it was hypothesized that feeding high amounts of MR during preweaning to calves would ameliorate growth and reproductive performance compared with calves fed conventionally, but a greater milk allowance could alter glucose metabolism, provoking an undesired insulin resistance that could be sustained long after weaning. Thus, the objective of this study was to assess the impact of high milk allowances offered twice a day on short- and medium-term growth performance and glucose metabolism in dairy calves.

4.2. Materials and methods

4.2.1. Treatments and measurements

All procedures for this experiment were approved and monitored by the Animal Care Committee of Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Barcelona, Spain). One hundred and twenty female Holstein calves born between April and June were weighed at birth (40.7 ± 4.94 kg), offered 6 L of good quality colostrum in two separate doses (a first meal of 4 L and a second of 2 L) and housed in individual pens. Calves were randomly assigned to one of the three following treatment groups (n=40): a conventional feeding program consisting of 4 L/day of MR (4L); another group that received 6 L/day of MR (6L); and a third group that received 8 L/day of MR (8L). The MR was diluted to a final DM content of 15 % and contained 22.8 % CP and 19.4 % fat (DM basis). Water was available *ad libitum* throughout the study. All animals were weaned at 63 days of age. Calves on the 4L group were offered 4 L/day of MR from days 2 to 57 and then received one meal per day of 2 L of MR from days 58 to 63. Animals in the 6L treatment were offered 4 L/day of MR from day 2 to 7, 6 L/day of MR from day 8 to 50, then 4 L/day of MR from day 51 to 57, and one meal per day of 2 L of MR from day 58 to 63. Lastly, calves in the 8L group were offered 4 L/day of MR from day 2 to 7, 6 L/day of MR from day 8 to 11, 8 L/day of MR from day 12 to 46, then 6 L/day of MR from day 47 to 50, 4 L/day of MR from day 51 to 57, and one meal per day of 2 L of MR from day 58 to 63. Calves in all treatment groups received MR twice a day at 5:30 and 15:30 in either 2 or 3 L bottles and had *ad libitum* access to the same starter feed (Table 4.1) from day 2 until one weak after weaning. At day 71, calves from the three treatments were comingled and moved in pens of groups of 10 calves. At day 180, animals were grouped again in pens of around 90 heifers.

Daily MR and feed intake were recorded from days 2 to 70. Calves were weighed at days 0, 35, 63 and 300. Veterinary treatments were recorded throughout the study. Reproductive parameters (age at first breeding, conception rate, and age at pregnancy) were recorded for all heifers in the study.

A glucose tolerance test (GTT) was performed at 42, 86 and 300 days of life in a total of 45 heifers (15 per group) after a 5-h fasting period. These 45 animals were chosen randomly within each group. Animals were weighed the day before the GTT, and the day of the test, a catheter was placed in the jugular vein of the animals and then each

subject was infused with 180 mg of glucose per kg of BW. Blood samples were collected at -5, 0, 4, 8, 12, 18, 25, 35, 35 and 60 min relative to glucose infusion. At each time point, blood was obtained using two different evacuated tubes, one containing sodium fluoride (to recover plasma for the subsequent analysis of glucose), and the other (with no additive) to obtain serum for subsequent measure of serum insulin concentrations. Determination of plasma glucose was performed using a system glucose reagent (Beckman Coulter, Brea, California) and serum insulin using a Bovine Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Table 4.1. Chemical composition of milk replacer and starter feed (g/kg of DM).

	Milk replacer	Starter feed
Crude protein	228	238
Ether extract	194	450
Neutral detergent fiber	--	181
Acid detergent fiber	--	106
Ash	77	64

4.2.2. Calculations and Statistical Analyses

Total dry matter, protein and fat intake, GtoF, and ADG were calculated based on recorded observations. Metabolizable energy (ME) was determined following NRC equations (2001). Data pertaining to GTT as plasma glucose and serum insulin basal concentrations, and their respective areas under the curve (AUC), maximum increase, clearance rate (CR), and time to baseline concentration as well as blood insulin to glucose ratio (ItoG) and insulin sensitivity (IS) were also calculated. Basal concentrations for plasma glucose and serum insulin represented average concentrations between -5 and 0 min before glucose infusion. Areas under the curve of the blood concentrations of glucose and insulin were calculated using the trapezoidal method after discounting the baseline concentrations for each metabolite. Clearance rate was calculated following Pires et al. (2007). Insulin sensitivity was

calculated following a simplified alternative model to the minimal model (Bergman, 1989) described by Christoffersen et al. (2009).

Data pertaining to feed intake, growth performance, age at first insemination, and age at conception were analyzed using a mixed-effects model that accounted for the fixed effects of treatment, day, and their 2-way interaction, and the random effect of calf. Body weight at day 0 was used as a covariate. Day entered the model as a repeated measure. The variance components structure yielded the smallest Schwarz's Bayesian criterion and thus it was used as the selected variance-covariance matrix for the analysis.

All parameters derived from the GTT were analyzed as previously described, including time of GTT as a repeated measure using a compound symmetry structure, as it yielded the smallest Schwarz's Bayesian criterion. Conception rate was analyzed using a logistic regression that accounted for the effect of treatment.

For all data, least squares means and standard errors are reported. Differences among treatment means were obtained using a Tukey's multiple comparison test. Tendency and significance were declared at $P < 0.1$ and $P < 0.05$ respectively.

4.3. Results

4.3.1. Growth and Reproductive Performance

Calf performance data from day 2 to weaning are presented in Table 4.2. No differences in veterinary treatments were found, and on average 28% of the animals were treated at least one time throughout the experiment. Due to the experimental design, MR intake was different ($P < 0.0001$) among groups. Calves that were fed a greater amount of MR (6L and 8L) consumed less ($P < 0.0001$) starter feed than 4L calves. In addition, starter intake evolved differently throughout the study (Fig. 4.1). Calves receiving 4 L of MR/day started to consume more solid feed earlier ($P < 0.0001$) than the calves in the other 2 groups, and consumed more ($P < 0.05$) solid feed than calves in the other 2 groups between day 29 and weaning (day 63). Indeed, starter feed intake was already greatest ($P < 0.05$) for 4L calves at day 29 and continued to be greatest until day 68 when similar starter feed intakes were observed among all treatments (Fig. 4.1). On average from day 0 to weaning, total DMI (solid plus liquid) was the greatest ($P < 0.05$) for 8L calves, and it evolved differently ($P < 0.0001$) among

treatments, being greater for 8L calves at the beginning of the study and for 4L calves towards weaning (Fig. 4.2). As a consequence of MR and starter feed intake, overall protein, fat and ME intakes were greatest in 8L calves (P < 0.05). However, the three variables also evolved differently during the study (Fig. 4.3 and 4.4). From day 0 to 35, average protein, fat and ME intakes were greater in 8L calves (P < 0.05) than for 6L and 4L calves (Fig. 4.3 and 4.4).

Calves fed 8 L/day of MR had a greater (P < 0.0001) ADG during the first 35 day of life and grew 268 g/day more than calves fed 4 L of MR /day, and 188 g/day more than calves fed 6 L of MR/day. Moreover, at day 35, 8L calves weighed 12 kg more (P < 0.05) than 4L calves and were about 7 kg heavier than 6L calves. At day 35, calves in the 6L treatment were also 5 kg heavier than 4L ones. Gain to feed ratio was similar for the three groups throughout the study.

Table 4.2. Intake, growth, and reproductive performance of calves until weaning as affected by milk allowance.

	Treatment[1]				P-Value[2]		
	4L	6L	8L	SE	T	A	T x A
Intake, kg/day (DM basis)							
Milk replacer	0.55[c]	0.72[b]	0.90[a]	0.004	< 0.0001	< 0.0001	< 0.0001
Starter feed	0.40[a]	0.23[b]	0.18[b]	0.021	< 0.0001	< 0.0001	< 0.0001
Total DM	0.95[b]	0.95[b]	1.01[a]	0.022	< 0.0001	< 0.0001	< 0.0001
Growth Performance							
BW[3], kg							
day 0	40.1	41.3	41.0	0.82	0.891	--	--
day 35	56.4[c]	60.8[b]	68.0[a]	1.54	< 0.0001		
day 63	74.9[b]	74.7[b]	79.0[a]	1.54	< 0.0001		
day 300	310.6[b]	319.4[a]	323.0[a]	2.48	< 0.0001		
ADG [4], g/day							
day 35,	484[c]	564[b]	752[a]	28.9	< 0.0001		
between 35 and 63 day	660[a]	495[b]	394[c]	28.9	< 0.0001		
between 63 and 300 day	1,045	1,056	1,065	43.5	0.749		
Gain:Feed ratio	0.58	0.54	0.60	0.025	0.2632	< 0.0001	0.7861
Reproductive Performance							
Age at breeding, day	416[a]	409[b]	410[b]	1.3	0.0024	--	--
Age at conception, day	433	425	428	4.6	0.468	--	--
Conception rate at first AI[5], %	66.7	62.2	57.9	8.06	0.74		

[1] Amount of milk replacer (4L = 4 L/day; 6L = 6 L/day; 8L = 8 L/day); [2] G = effect of treatment; A = effect of age of measurement; G x A = interaction between the effect of treatment and age of measurement; [3] Body weight; [4] ADG = Average daily gain; [5] AI = Artificial insemination; [a,b,c] Values within row with unequal superscripts differ at P <0.05.
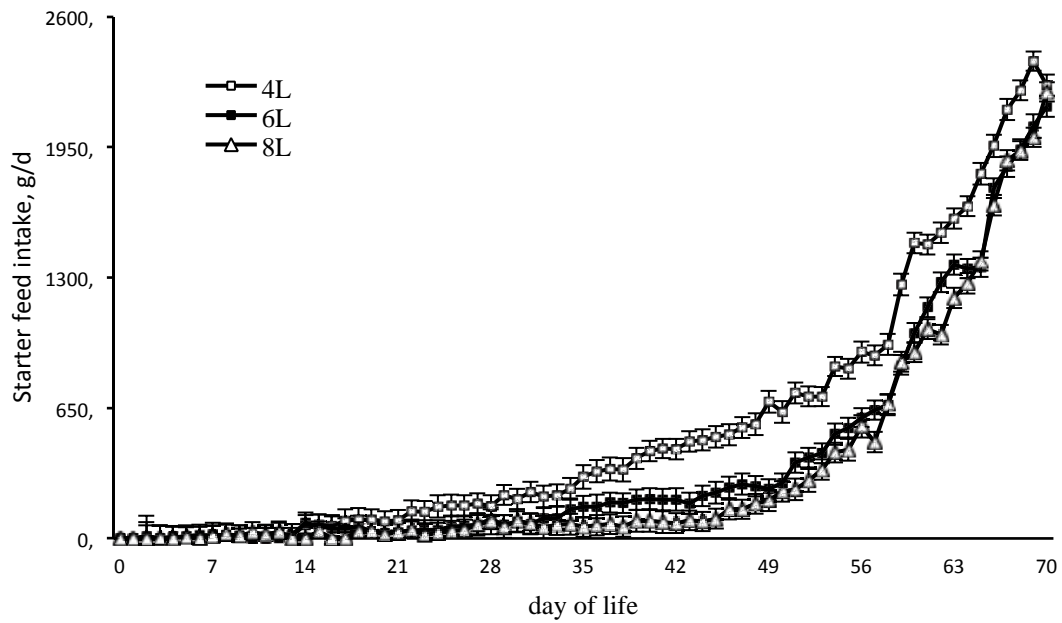
Figure 4.1. Starter feed intake (DM basis) of calves fed 4 (4L, □), 6 (6L, ■) or 8 (8L, △) liters of milk replacer per day. Weaning was at 63 days of age.
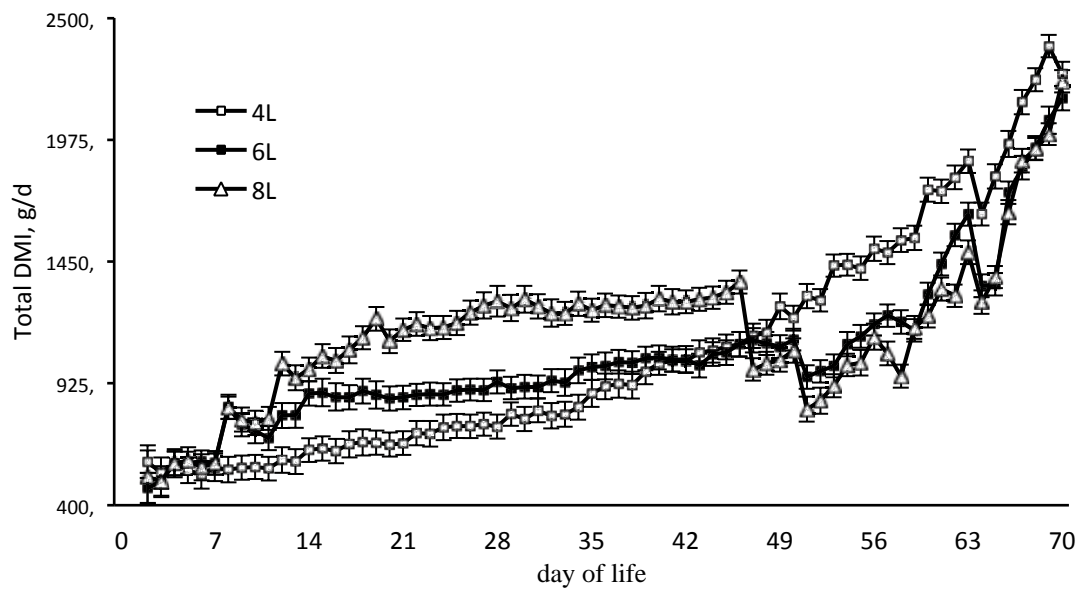
Figure 4.2. Total dry matter intake (DMI) of calves fed 4 (4L, □), 6 (6L, ■) or 8 (8L, △) liters of milk replacer per day. Weaning was at 63 days of age.

After weaning, the situation changed. During the beginning of the week following weaning, 4L calves consumed more solid feed (P < 0.05) than 6L and 8L calves (Fig.4 .1); but towards the end of that week, differences in intake among groups disappeared (Fig. 4.1). Nevertheless, 6L and 8L calves did not begin to consume progressively greater amounts of solid feed until day 57 of life (Fig. 4.1). Because of that, 4L calves had the greatest protein intake (P < 0.05) between days 35 to 70. However, ME intake was similar in 4L and 8L calves (P < 0.05), but fat intake continued to be the greatest in 8L calves (P < 0.05) during this period although differences between groups were less marked (Fig. 4.3, 4.4).

Body weight and ADG were also influenced by starter feed intake. Although 8L calves were 4-kg heavier (P < 0.0001) than 4L and 6L calves at day 63, at day 300, the weight advantage of calves was no longer present between 8L and 6L, but 8L calves were 12-kg heavier than 4L calves (P < 0.05). Average daily gain from days 35 to 63 was greatest (P < 0.05) for 4L calves, but no differences were found (P = 0.75) in ADG between days 63 to 300.
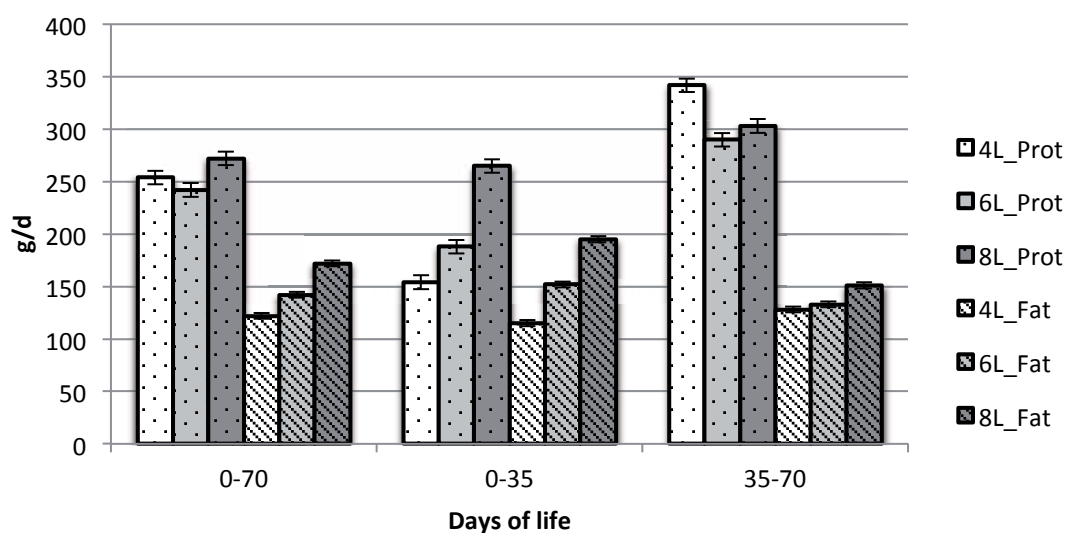
Figure 4.3. Protein and fat intake of calves fed 4 (4L,□), 6 (6L, ■) or 8 (8L, ■) liters of milk replacer per day.
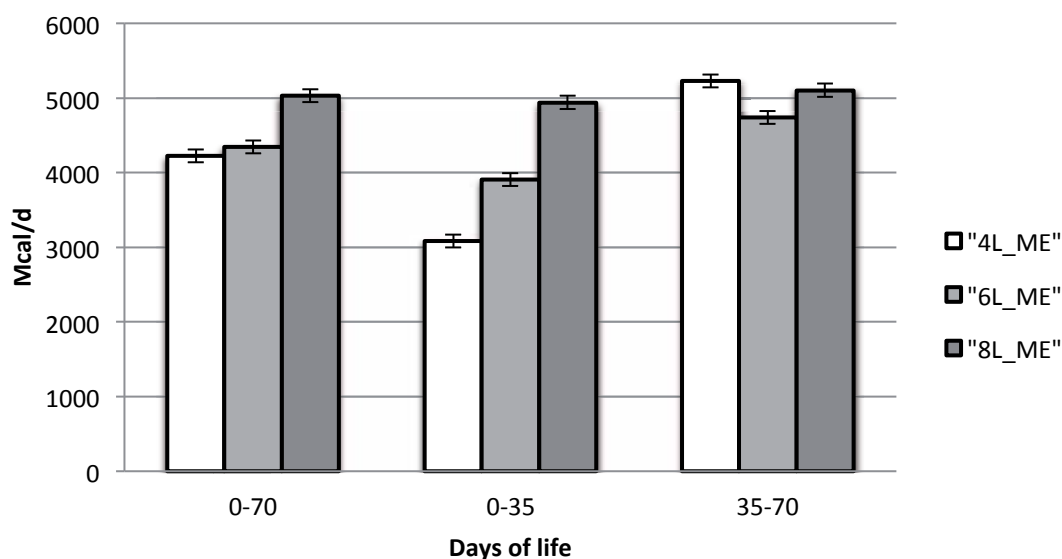
Figure 4.4. Metabolizable energy intake of calves fed 4 (4L,□), 6 (6L, ■) or 8 (8L, ■) liters of milk replacer per day.

Age at first breeding (Table 4.2) was greater (P < 0.05) for calves receiving 4 L of MR/day than for calves receiving 6 and 8 L of MR/day. However, conception rate at first breeding and age at conception did not reach significance.

4.3.2. Metabolic Response

Main results for GTT are presented in Table 4.3. Throughout the study, basal concentration of plasma glucose was greater (P < 0.05) in 8L than in 4L and 6L calves. Overall, basal plasma glucose concentration decreased with age (P < 0.0001). There was an interaction between treatment and age that led to differences (P < 0.05) at 42 day of age between 8L (120 ± 2.3 mg/dl) and 6L (104 ± 3.1, mg/dl) and 4L (97 ± 3.0 mg/dl) groups. Similarly, basal serum insulin concentration was also greater at 42 day of life (P < 0.0001) for 8L (51 ± 0.08 µU/ml) than for 6L (10 ± 0.08 µU/ml) and 4L (10 ± 0.08 µU/ml) calves. Plasma glucose AUC did not differ among treatments and increased (P < 0.0001) with age for all the groups. The maximum plasma glucose concentration was overall lesser (P < 0.05) in calves receiving 4 L than for calves receiving 8 L of MR/day. Overall, insulin release, measured as insulin area under the curve (IAUC), after a GTT did not differ among groups (P = 0.37) but it evolved differently among treatments (Figure 4.5). Insulin release after a GTT increased (P <

0.005) with age, being the increase more marked in 4L calves (Fig. 4.5). At day 42, 8L calves had a greater (P < 0.05) insulin serum concentration increase than calves in the other 2 treatment groups. The amount of MR offered had short- and medium-term effects on ItoG, with calves fed 4 and 6 L of MR/day having lower (P < 0.05) values (131.0 ± 16.60 and 159.3 ± 16.60 µU/mg, respectively) than calves fed 8 L of MR/day (215.6 ± 16.02 µU/mg) independently of age. Overall, glucose clearance rate (CRG) did not differ among treatments (P = 0.67), although it decreased (P < 0.0001) with age and insulin clearance rate (ICR) was greater (P < 0.005) in 8L calves. Overall insulin sensitivity tended (P = 0.07) to be lesser in calves fed 8 L of MR/day than in calves fed 6 or 4L of MR/day (1.38 ± 0.044; 1.74 ± 0.045; and 1.95 ± 0.045 ml/min x µU/ml per kg of BW, respectively). And at day 42, IS tended to be lower (P = 0.06) in 8L calves than in 4L or 6L (Figure 4.6).

After weaning, basal plasma glucose differences disappeared at days 86 (P = 0.36) and 300 (P = 0.73) of life, when the average basal plasma glucose concentrations for all the calves were 93 ± 1.8 and 80 ± 1.9 mg/dl at day 86 and 300, respectively. Similarly, differences in basal serum insulin also disappeared at day 86 (P = 0.65), but reappeared at day 300, when 8L calves had again greater (P < 0.05) basal serum insulin levels (51 ± 0.08 µU/ml) than 6L (16 ± 0.09 µU/ml) and 4L (19 ± 0.08 µU/ml) calves. At 300 days of life plasma glucose AUC was similar (P = 0.63) for all treatment groups, but heifers belonging to the 6L treatment had greater (P < 0.0001) IAUC at day 300 compared with 4L and 8L heifers (Fig. 4.5). Similar to day 42, at day 86, 8L calves had a greater (P < 0.05) insulin serum concentration increase following the GTT, however, at day 300, the maximum serum insulin increase after a GTT, was greater (P < 0.05) in 4L and 6L than in 8L calves. Insulin clearance rate was greatest (P < 0.05) in 8L calves at 300 days of age. Time to reach insulin basal serum concentrations was almost 20 min shorter in 8L than in 4L and 6L calves at day 300. Furthermore, after weaning, IS tended (P = 0.06) to become similar among treatment groups (Fig. 4.6).

Table 4.3. Blood glucose and insulin responses after a glucose tolerance test of calves as affected by milk allowance.

	Treatment[1]				P-value[2]		
	4L	6L	8L	SE	G	A	GxA
Basal glucose concentration, mg/dL	90[b]	91[b]	97[a]	1.8	0.0208	< 0.0001	0.0004
Basal insulin concentration, μU/mL	12[b]	11[b]	32[a]	1.1	< 0.0001	< 0.0001	0.0025
Glucose AUC[3], mg/dL x 60 min	1,874	1,921	1,795	106.3	0.7025	< 0.0001	0.7150
Insulin AUC, μU/mL x 60 min	3,319	3,886	2,939	242.6	0.3710	< 0.0001	0.0169
Maximum glucose increase, mg/dL	177[b]	183[a,b]	188[a]	2.7	0.0241	< 0.0001	0.6497
Maximum insulin increase, μU/mL	88	105	109	0.05	0.2746	0.3391	0.0107
Glucose clearance rate, %/min	7.4	8.0	7.2	0.67	0.6764	< 0.0001	0.0718
Insulin clearance rate, %/min	9.1[b]	9.2[b]	10.1[a]	0.68	0.0039	0.0396	0.9673
Time to glucose baseline, min	39	40	35	2.7	0.4094	< 0.0001	0.0259
Time to insulin baseline, min	34[a]	34[a]	24[b]	1.9	0.0004	0.0501	0.1050
Insulin:glucose ratio , μU/mg	131[b]	159[b]	216[a]	16.4	0.0023	< 0.0001	0.3572
Insulin sensitivity[4], mL/min x μU/mL per kg of BW	1.95[a]	1.74[a,b]	1.38[b]	0.045	0.0723	< 0.0001	0.0635

[1]Amount of milk replacer (4L= 4 L/day; 6L= 6 L/day; 8L = 8 L/day)

[2] G = effect of treatment; A = effect of age of measurement; G x A = interaction between the effect of treatment and age of measurement

[3] AUC: Area under the curve (discounting baseline concentration)

[4] Calculated following Christoffersen et al. (2009)

[a,b,c] Values within row with unequal superscripts differ at P <0.05
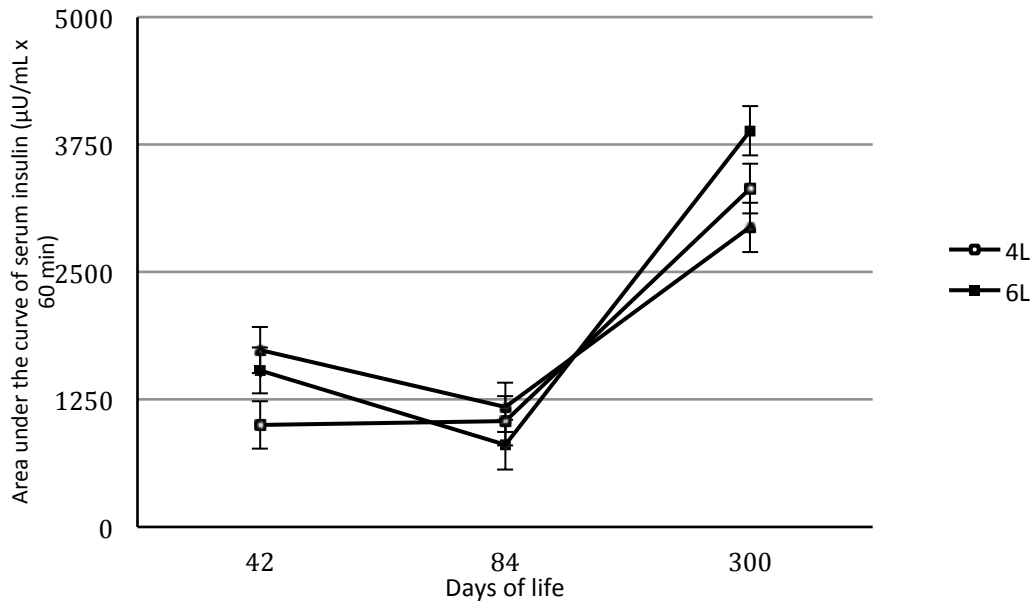
Figure 4.5. Area under the curve of serum insulin concentrations (AUC; µU/mL x 60 min) of calves fed 4 (4L,□), 6 (6L, ■) or 8 (8L, △) liters of milk replacer per day. Weaning was at 63 days of age.
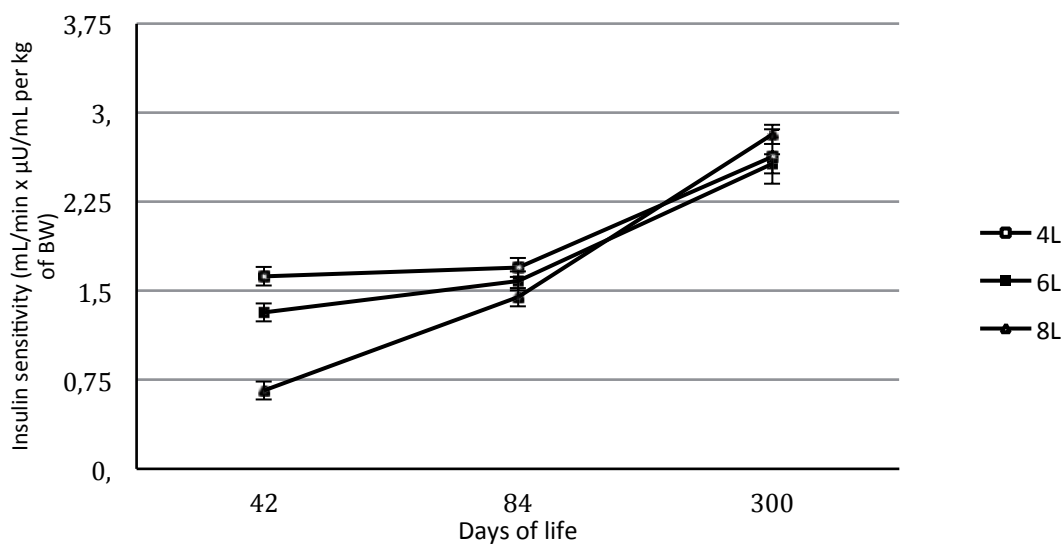
Figure 4.6. Insulin sensitivity (mL/min x µU/mL per kg of BW) of calves fed 4 (4L,□), 6 (6L, ■) or 8 (8L, △) liters of milk replacer per day. Weaning was at 63 days of age.

4.4. Discussion

4.4.1. Growth and Reproductive Performance

Calves allowed to consume more MR (6L and 8L) consumed less starter feed than 4L calves. This situation has been already reported (Jasper and Weary, 2002; Quigley et al., 2006; Terré et al., 2009). Due to the greater intake of MR, 8L calves had a greater ADG at day 35. Increased growth rates during the first weeks of life have been associated with increased milk yield in the first lactation in some studies (Bach and Ahedo, 2008; Bach, 2012; Soberon et al., 2012, 2013). However, as a result of the reduced total DMI that occurred in 6L and 8L calves when the weaning process begun, ADG at day 63 was the greatest for 4L calves (Fig. 4.2). The reduction in total DMI was due to the fact that 6L and 8L calves were not able to compensate the lack of MR allowance by increasing the amount of starter feed intake. Indeed, from day 47 until day 58, only a slight increase in starter feed intake occurred (Fig. 4.1) and it was only after MR was offered once daily (at day 58), when 6L and 8L calves quickly increased starter feed consumption. Similarly, protein, fat, and ME intakes were overall greater (P < 0.05) in 8L calves; but from day 35 onwards, a smaller increase in the protein intake was observed for calves in the 8L treatment, which resulted in a greater protein intake in 4L (P < 0.05) compared with 8L calves during that period. Also, ME intake was similar (P = 0. 28) for 4L and 8L calves. It has been previously reported that an increased crude protein concentration in diets with similar energy content stimulates growth of young dairy calves (Bartlett et al., 2006); therefore, greater ADG in 4L calves after day 35 observed herein could be partly explained by the protein to energy intake ratio.

A gradual decrease in milk allowance was chosen herein as weaning method as it has been reported to effectively ease the weaning process (Khan et al., 2007). However, it is important to note that the reduction of MR allowance towards weaning was performed differently as described by Khan et al. (2007). Khan et al. (2007) decreased the milk allowance by reducing the solid contents of the milk and thus, calves received always the same quantity of liters per meal but a lesser amount of DM. That may decrease the weaning stress as calves may find other aspect of feeding routine satisfying, as gut fill from liquid or sucking (Jasper et al., 2008). Indeed, Budzynska and Weary (2008) found a reduced stress in calves that were allowed to drink warm water

from the teat system the first three days after weaning. Moreover, weaning calves by gradually increasing the dilution of the milk has been proposed to be beneficial for calves, as it allows a slow adaptation by gradually increasing intake of starter feed (De Passillé, et al., 2004; Jasper et al., 2008). However, Nielsen et al. (2008) reported that calves weaned by diluting the MR with additional water consumed less starter feed than calves weaned by a progressive reduction of milk volume. Moreover, the rate of decrease of the amount of MR offered to calves was also different than the one used by Khan et al. (2007), who provided milk at 20% of BW and then gradually reduced during 4 days to 10% of BW for 16 days. Differences between these two weaning schemes may have caused the discrepancies in starter intake found herein and those reported by Khan et al. (2007).

Age at breeding was greater when less milk was offered to calves during the preweaning period. However, conception rate at first breeding and average age at pregnancy did not differ among treatments (Table 4.2). Results from previous studies are inconclusive and variable. For instance, Bar-Peled et al. (1997) reported that calves allowed to suckle their dam had a greater conception rate and calved 31 days earlier than calves fed 3 L of MR/day. Shamay et al. (2005) found that calves fed *ad libitum* reached puberty 23 days earlier than calves fed 0.45 kg/day of MR, but no differences in age at first calving were found. Davis-Rincker et al. (2006) observed numerical differences in breeding (P = 0.09) and calving (P = 0.11) ages in calves fed MR at 2.1% of BW, which were 15 days and 14 days younger at breeding and at calving, respectively, than in calves fed MR at 1.2 % of BW. Last, no differences in age at first breeding or calving were found by Terré et al. (2009) when comparing calves fed 4 or 7 L/day of MR. Even though differences in reproductive performance did not reach significance in all the studies, it seems that calves fed large milk or MR allowances reached puberty and pregnancy at earlier ages. A larger sample size may be necessary to clearly illustrate potential effects of MR allowances on reproductive performance.

4.4.2. Metabolic response

Results from GTT were performed at a moment when, in theory, should be unaffected by previous meal, as they were conducted after a 5-h fasting period and thus glucose and insulin concentrations should be at basal levels (Kamalu and Trenkle, 1978). As

expected, overall basal plasma glucose concentration was greater in 8L, as levels of blood glucose are related to feeding level (Hugi et al., 1997b). The greater amount of basal plasma glucose concentration at 42 days of age in the 8L group could be due to the greater consumption of lactose in 8L calves compared with 6L and 4L calves. Palmquist et al. (1992) reported that veal calves, fed high content of lactose (> 500 g of lactose /kg BW) had greater basal blood levels of glucose and insulin compared with calves fed a conventional diet. This hypothesis is further reinforced by the fact that after weaning, when no differences in lactose intake occurred among treatments, basal plasma glucose concentrations did not differ. However, in contrast, with the results from Hugi et al. (1997b), basal plasma glucose concentrations herein decreased with age. The differences between present results and those reported by Hugi et al. (1997b) could be related to weaning age, which was 23 days earlier herein.

During the GTT, all calves were able to control glycemia, calves had a greater plasma glucose AUC at 300 days than at 42 days of age. A decrease in glucose removal from blood with age has already been reported before (Colvin et al., 1967; Palmquist et al., 1992), meaning that the ability to clear glucose from the organism decreases with age. Serum insulin AUC after a GTT did not differ among treatments, but it evolved differently with age. At 42 days of life, 8L calves needed to release more insulin to control glycemia, but at 300 days of life, the situation was reversed. In all calves, insulin release increased with age, but the greatest increase was observed in 6L calves, which could be an indication of some degree of glucose intolerance (Palmquist et al., 1992). In contrast, 8L calves had a greater blood ItoG than that of 4L and 6L calves, independently of age. An increase in blood ItoG ratio could also indicate a certain degree of insulin resistance (Hostettler-Allen et al., 1994).

Insulin sensitivity tended to be lesser in calves fed 8 L of MR/day than in calves fed 4 or 6 L of MR/day at 42 days of age. But, as age increased, IS differences among groups disappeared. A lesser IS in 8L calves could have caused an increase in fat deposition (Palmquist et al., 1992), and could be partially responsible for the decreased growth rate found herein from days 35 to 63, as depositing fat has a low feed efficiency (Mader et al., 2009). Indeed, 4L calves may have had less fat deposition as their protein intake was greater after day 35 than that of 8L calves, and increased crude protein intake typically leads to decreased fat deposition (Bartlett et al., 2006).

In summary, feeding 4 L of MR twice daily, at 42 days of age, could be detrimental for the metabolic performance of calves because insulin release to control glycemia is greater than when feeding 3 or 2 L of MR twice daily. However, these differences disappeared at 84 days of life and the situation was actually reversed at 300 days of life, when 8L calves needed lesser amounts of insulin than 4L and 6L calves to control glycemia during a GTT. Furthermore, offering 8 L of MR/day elicited a decrease in IS while calves were consuming MR, but these effects were not sustained over time, and IS did not differ among calves 300 days of life.

4.5. Conclusions

Calves fed 3 or 4 L of MR twice daily had greater preweaning ADG and achieved a greater BW. However, during weaning, calves previously receiving 6 or 8 L of MR were not able to compensate the lack of nutrient supplied via MR, which indicates that the weaning scheme used herein for the enhanced feeding programs was not effective. Furthermore, when providing 4 L of MR twice daily glucose metabolism was altered at 42 days of age, as 8L calves needed to release more insulin to control glycemia after a GTT and had lesser values of IS were found. Nevertheless, the impaired glucose metabolism observed at day 42 for calves fed 4 L of MR twice daily did not persist after weaning.

4.6 Acknowledgements

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Chapter 5

IMPACT OF ARGININE SUPPLEMENTATION ON PLACENTAL VASCULARIZATION IN PREGNANT DAIRY HEIFERS.

5.1. Introduction

Nowadays, it is known that fetal organs and systems are sensitive to the environment of the uterus and that the influence of this environment can lead to long-term consequences (Barker, 2004), such as changes in performance and metabolic function of the offspring (Wu et al., 2006; Bach, 2012). These processes are usually called developmental programming. Thus, it is critical to provide the uterus with sufficient nutrients during pregnancy, and this can only be reached by a proper maternal nutrition that permits an optimal placental growth and development. However, according to Reynolds and Caton (2012), poor nutritional environment is quite common in livestock, mainly among other things, because of precocious inseminations and competition between nutrient needs for milk production and fetal development. Current nutritional models for dairy cattle ignore the needs during early pregnancy (NRC, 2001). This problem is aggravated in heifers when requirements for growth and development compete with pregnancy. In the early stage of pregnancy, when nutrient requirements do not seem to have an important influence on the future offspring, maximal placental growth, vascularization, and fetal organogenesis take place (Redmer et al., 2004; Vonnahme, 2007). Proper development of the placenta is essential for fetal growth because it supplies nutrients and oxygen to the calf (Reynolds, 1995). To solve this dilemma, the first thought is an accurate and adequate dietary nutrient (such as amino acid [AA]) that may target specific processes (Reynolds and Caton, 2012).

Arginine (Arg) is considered to be an essential AA for dairy cattle that participates in an array of body functions (Wu et al., 2009), such as lactation, growth, development, tissue remodeling, and response to several hormones and signaling molecules (Flynn et al., 2002). Arginine stimulates the synthesis of growth hormone (GH), prolactin (PRL), and insulin (Chew et al., 1984). Moreover, Arg is an essential substrate for nitric oxide (NO) synthesis (Wu and Morris, 1998) and polyamines (Palmer et al., 1988; Wu et al., 2008). Nitric oxide is a vasoactive compound, which participates in placental growth and angiogenesis, and thus, it is necessary for increasing uterine and placental–fetal blood flow (Wu et al., 2008) and to secure the fulfillment of fetal nutrient needs (Bird et al., 2003).

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In addition, differences due to site of administration of Arg should be studied. Few studies can be found describing this objective; however, the effect in some blood metabolites is not the same if the administration takes place via the jugular vein or via abomasal infusion. For example, Vicini et al. (1987) reported an increase of somatotropin and insulin when performing an injection of Arg into the jugular vein but not when the infusion was via the abomasum.

We hypothesized that supplying Arg to early pregnant heifers would lead to an increased uterine blood flow, which in turn would enhance placental and uterine development helping the calf to optimally develop in the uterus. Thus, the objectives of the study were to evaluate the effects of Arg supplementation on uterine artery hemodynamics between 40 and 140 days of gestation and investigate the different effects of Arg after a single peritoneal infusion (PrI) compared with a single jugular infusion (JI) in pregnant dairy heifers.

5.2. Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use of North Dakota State University (#A12043).

In experiment 1, Holstein heifers (n = 17) averaging 448 ± 73.9 kg of body weight (BW) and 552 ± 146.8 days of life were bred at Day 0 to the same bull by artificial insemination. At DAY 30, after checking that all heifers were pregnant using a transrectal ultrasonography, animals were moved to a tie stall facility, and at 41 ds of gestation, heifers were surgically fitted with a catheter in the peritoneal cavity. These catheters were later used to conduct infusions of treatments throughout the study. After surgery, nine heifers that were randomly selected received a daily intra-PrI of 40 mg of Arg/kg of BW (ARG), whereas the remaining eight heifers received a daily intra-PrI of saline solution at equivalent volumes as ARG heifers relative to BW (CTRL). Arginine was 100% L-Arg (Evonik industries, Hanau, Germany). The dose of Arg was based on the National Research Council (2001) model. Using this guideline, we calculated the Arg supply of the diet would be about 48 g of metabolizable L-Arg/day. We aimed to provide a 40% supplementation over the regular Arg intake of a heifer and that corresponded to a daily dose 40 mg of Arg/kg of BW. Arginine was dissolved in 1 L of distilled water, and 35 mL of 2N HCl was added to achieve a final pH of 7.

Infusions of either CTRL or ARG took place every 12 hours until heifers reached 146 ds of pregnancy. The BW of each heifer was determined every 3 weeks, and the quantity of Arg or saline solutions to be infused into each heifer was adjusted accordingly.

Animals had free access to water and were fed *ad libitum* exactly the same total mixed ration (corn silage: 60%, distillers' dried grains: 30%, and grass hay: 10%) that met NRC (2001) requirements. The ration was sampled once a week and analyzed for dry matter (DM), crude protein, neutral detergent fiber, nonfiber carbohydrates, and ether extract. Feed intake was recorded daily for each heifer. Three times a week, all the animals were walked for about 10 minutes.

At day 41, 62, 83, 104, 125, and 146 of gestation, all heifers were weighed, a blood sample was collected by venipuncture of the jugular vein, and the uterine artery blood hemodynamics was determined. Blood samples were analyzed to determine free AA concentration, NO, glucose, insulin growth factor 1 (IGF-I), progesterone (P4), GH, and PRL. Hemodynamics of the uterine artery was studied in both ipsilateral and contralateral arteries to the conceptus and was transrectally determined using a color Doppler ultrasonography (Aloka SSD-3500; Aloka America, Wallingford, CT, USA) with a convex probe (UST-995-7.5). Data collection was similar to that previously published (Camacho et al., 2014). Each examination lasted for approximately 30 minutes. After identification of the uterine arteries (Bollwein et al., 2000), at least three blood flow waveforms were recorded. Last, calves born to both CTRL and ARG heifers were weighed at birth.

Our initial hypothesis for this study was that providing Arg through the peritoneum would mimic more closely Arg bioavailability to the animal when provided through the diet, as it would be done under practical conditions (if Arg supplementation was deemed useful). Thus, in experiment 2, two 146-day pregnant Holstein heifers averaging 423.4 ± 18.3 kg of BW were placed in a tied-up stall where they had free access to water and were fed *ad libitum* the same ration described previously. Both heifers had participated in experiment 1 and were part of the CTRL group. Additionally to the peritoneal catheter already placed for experiment 1, another catheter was placed in their jugular vein. Those catheters were later used as described previously, for the infusion of a solution of 40 mg of Arg/kg of BW. In both animals, at time 0, the PrI of Arg took place followed by a JI after 24 hours. Blood samples were taken at time

0, 15, 30, 60, 120, 360, and 720 minutes after the infusions. Blood samples were then centrifuged to obtain plasma for later determination of AA, NO, and glucose concentrations.

5.2.1. Hormone and metabolite analyses

Blood P4 was analyzed following a previously described method (Galbreath et al., 2008). Briefly, a 50-mL sample of maternal serum was analyzed in duplicate. Progesterone concentrations were measured by chemiluminescence immunoassay using the IMMULITE 1000 (Siemens, Los Angeles, CA, USA), where lesser, medium, and greater progesterone pools were assayed in triplicate (1.6 ± 0.08, 2.6 ± 0.07, and 13.2 ± 0.40 ng/mL, mean ± standard error of the mean for lesser, medium, and greater pools, respectively). The intra-assay and inter-assay coefficients of variation were 4.4% and 6.7 %, respectively. Serum concentrations of GH were determined in the samples using the RIA procedures described by Hoefler and Hallford (1987). The double anti-body RIA used rabbit anti-oGH-3 (AFP0802210) and oGH-I-5 (AFP12855B) provided by the National Hormone and Peptide Program (Torrance, CA, USA). All samples were run in a single assay, and the coefficient of variation was 6.1 %. The concentration of IGF-I in serum was quantified by RIA according to the procedures of Berrie et al. (1995). All samples were run in a single assay, and the coefficient of variation was 15.3%. Serum PRL (Spoon and Hallford, 1989) concentrations were determined in duplicate by double antibody RIA using primary antisera (anti-oPRL-2, AFPC35810691R) and purified standard and iodination (oPRL-I-3, AFP10789B) preparations supplied by the National Hormone and Peptide Program. All samples were run in a single assay, and the coefficient of variation was 8.2 %. Total serum nitrites were determined using the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA). Briefly, following the manufacturer's recommendations, serum samples were deproteinized and quantified after the reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrite standard curve (0–100 mM), with a sensitivity of 0.6 mM and an intra-assay coefficient of variation of 7.3 %.

Maternal and fetal serum glucose concentrations were determined using a colometric assay, following Lekatz et al. (2010). Briefly, 5 mL of sample, standard and control serum (Accutrol; Sigma, St. Louis, MO, USA) was added to 96-well plates with the

addition of 250 mL of infinity glucose reagent (Thermo Electron Corporation, Pittsburgh, PA, USA). Plates were incubated at 37ºC for 15 minutes, and absorbance was measured at 340 nm (SpectraMax 340; Fullerton, CA, USA). Intra-assay and interassay coefficients of variations were 2.4 % and 4.8 %, respectively.

Serum AA profiles were determined using an ultra performance liquid chromatograph (UPLC). Two hundred fifty microliters of serum was deproteinized with 250 mL of 10 % sulfosalicylic acid to which 250-mM norvaline was added as an internal standard. This mixture was vortexed and centrifuged for 5 minutes at 16,000 g at 4ºC. Twenty microliters of supernatant was added to 60 mL of borate buffer and sodium hydroxide solution as well as 20 mL of MassTrac Amino Acid Analysis derivatazing reagent. The samples were then capped, mixed, and heated in a digestion block at 55ºC for 10 minutes. Samples were then injected into the UPLC. This method uses the MassTrac Amino Acid Analysis system for the full profile of AA in physiological fluids. Derivatization chemistry for physiological samples is a precolumn method and is based on a derivatizing reagent, 6-aminoquinolyl-N-hydrox-ysuccinimidyl carbamate, which converts both primary and secondary AA to stable chromophores for UPLC detection.

5.2.2. Calculations and statistical analyses

Once the ultrasound image was obtained, the angle of insonation and diameter were determined and measurements from three recorded waveforms, including heart rate (HR), uterine blood flow volume (FV), pulsatility index (PI), and resistance index (RI) were calculated. Pulsatility index was calculated as (peak systolic velocity [PSV] - end diastolic velocity)/mean velocity; RI was determined as (PSV - end diastolic velocity)/PSV; and FV (mL/min) was estimated as mean velocity $(cm/s) x (\pi/4)$ diameter^2 (cm^2) x 60 seconds. The values of the three waveforms were then averaged.

Doppler ultrasonography from day 41 was considered as the baseline. The remaining Doppler determinations (from 62 to 146 day of pregnancy) were corrected with the baseline value obtained on day 41. As no clear differences were observed between both uterine sides until day 146 and as our interest was to study whether total FV to the uterus increased with Arg, a mean from both sides was calculated for each heifer at each sampling point.

The experimental unit herein was the heifer. The number of heifers needed to be involved in the study was determined by a power analysis with an alpha = 0.05 and a power of 0.8, assuming a variance for the main outcome variable (uterine blood FV) based on previous reports (Reynolds et al., 1985). All measurements (intake, blood metabolites, and artery hemodynamics) were analyzed using a mixed- effect model that accounted for the fixed effects of treatment, day of gestation, and their interaction, with age and BW at 41 days of gestation as covariates, plus the random effect of heifers within treatment. Time entered the model as a repeated measure. As PRL values were not normally distributed, data were analyzed after a ln-transformation. The threshold for significance was set at $P < 0.05$.

In experiment 2, to ensure adequate sample size, a power analysis was also conducted as described previously assuming a variance for clearance rate (CR) and half-life on the basis of previous reports (Chew et al., 1984). Measurements for all the metabolites included basal concentration (C_b), peak concentration, time to peak, area under the curve (AUC), CR, half-life ($t_{1/2}$), and time to reach the basal level (tb). Actual values were used for C_b, peak concentration, time to peak, t_b, and AUC and were analyzed using a mixed-effect model (PROC MIXED; SAS Institute Inc., Cary, NC, USA) that accounted for the fixed effects of place of infusion, time since infusion, and their interaction, plus the random effect of heifers. Bleeding time entered the model as a repeated measure. Furthermore, values for CR and $t_{1/2}$ were calculated from monoexponential curves (Booth and McDonalds, 1988) describing blood metabolite concentrations for the elimination phase (thus from concentration pick to C_b), following the model:

$$C_t = A \cdot \exp^{(-\alpha t)}$$

Where C_t was the metabolite concentration at time t, A is the basal concentration and α was the regression coefficient.

The elimination $t_{1/2}$ was defined as follows:

$$t_{1/2} = \ln(2)/\beta = 0.693/\beta,$$

being β the slope of the elimination phase.

5.3. Results

5.3.1. Performance and blood dynamics

There were no differences in total BW gain (125 ± 13.4 kg) or DM intake (23 ± 0.5 kg/day) of heifers between treatments. Similarly, there were no differences in calf BW at birth (38 ± 1.8 kg) between groups.

Uterine blood FV did not differ between treatments, but it increased ($P < 0.001$) with pregnancy day and evolved differently between treatments (Fig. 5.1). For CTRL heifers, uterine blood FV increased only 17 ml/min from day 62 to day 83 of pregnancy, whereas the increase between day 125 and 146 of pregnancy was 415 ml/min. For ARG heifers, uterine blood FV increased 64 ml/min from day 62 to day 83 of pregnancy but only 234 ml/min between day 125 and 146, which resulted in a greater ($P < 0.001$) uterine blood FV of CTRL heifers at 146 day of pregnancy than that of ARG heifers (Fig. 5.1).

Diameter of the uterine artery did not differ between treatments, but, it increased ($P < 0.001$) with pregnancy day, starting at 0.25 ± 0.012 cm on day 41 and reaching 0.43 ± 0.012 cm on day 146 of pregnancy. Pulsatility index and RI of the uterine artery decreased throughout pregnancy but did not differ between treatments (Fig. 5.2a and 5.2b).

Heart rate was lesser ($P < 0.05$) in ARG (74 ± 1.4 beats/min) than in CTRL heifers (81 ± 1.5 beats/min).
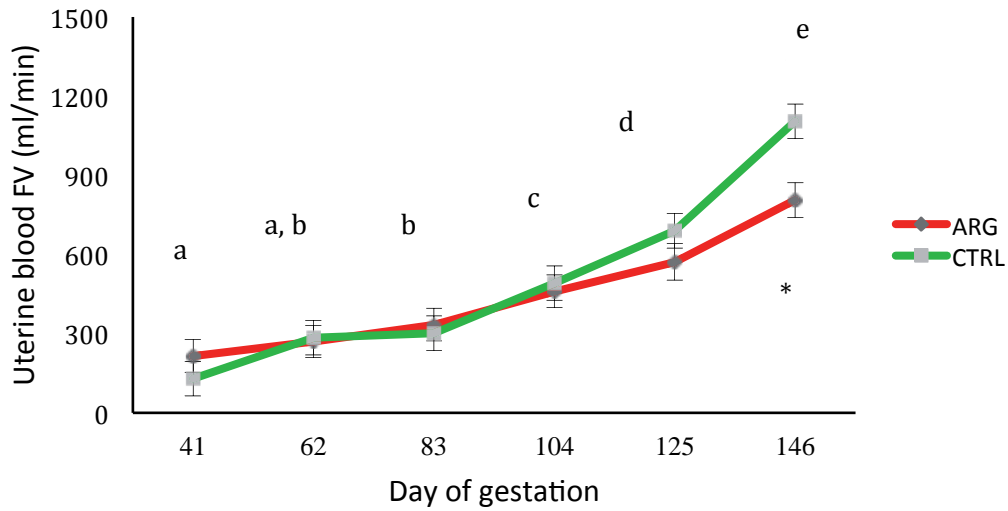
Figure 5.1. Blood flow volume (FV) in the uterine artery of heifers as affected by arginine supplementation. Values are means ± S.E. Different letters (a, b, c, d, e) indicate significant differences (P < 0.05) between sampling points. Values with * differ (P < 0.05) between treatments within sampling points.
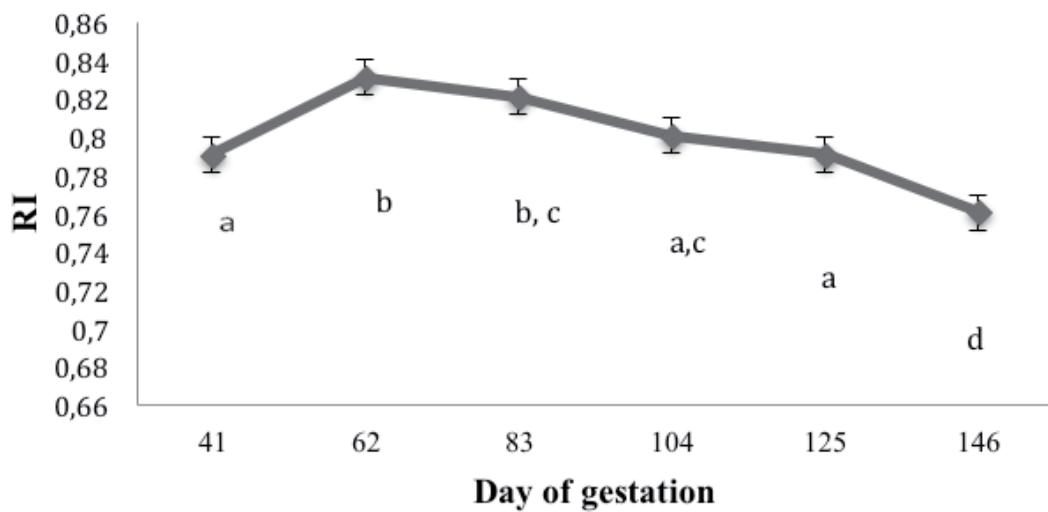
Figure 5.2a. Resistance index (RI) in the uterine artery of heifers as affected by arginine supplementation. Values are means ± S.E. of both treatments. Different letters (a, b, c) indicate differences (P < 0.05) between sampling points.
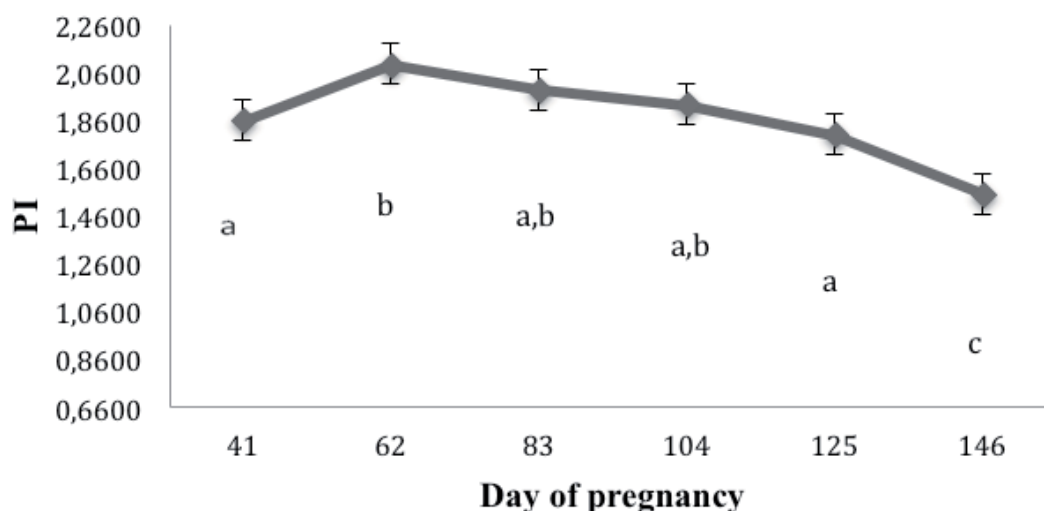
Figure 5.2b. Pulsatility index (PI) in the uterine artery of heifers as affected by arginine supplementation. Values are means ± S.E. of both treatments. Different letters (a, b, c) indicate differences (P < 0.05) between sampling points.

5.3.2. Blood metabolites

Plasma concentrations of Arg tended (P < 0.09) to be greater in ARG than in CTRL heifers, but carnitine, valine, isoleucine, leucine, phenylalanine and tryptophan were less (P < 0.05) in ARG than in CTRL heifers (Table 5.1). The proportion of essential amino acids (EAAs) in plasma was greater (P < 0.05) in CTRL (73 ± 2.4 %) than in ARG (65 ± 2.3 %) heifers (Table 5.1).

No differences between treatments were found in plasma PRL (P = 0.43), IGF (P = 0.97), glucose (P = 0.45) or GH (P = 0.39) concentrations. However, plasma GH concentrations decreased (P < 0.05) throughout the study (Table 5.2). Plasma P4 concentration was greater (P < 0.05) in ARG than in CTRL heifers (Fig. 5.3).
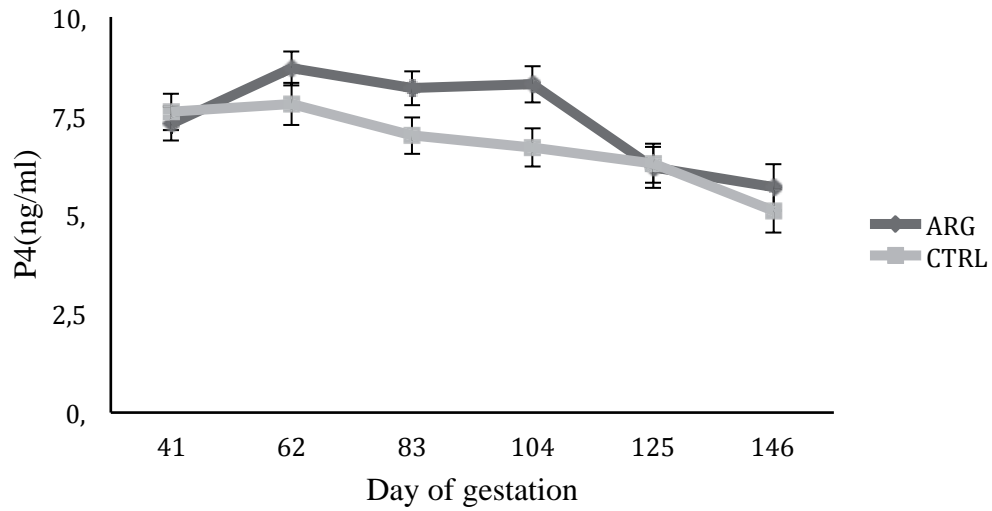
Figure 5.3. Blood progesterone (P4) concentration in heifers receiving arginine infusion (ARG) or not (CTRL). Values are means ± S.E. of both treatments. Values with * differ (P < 0.05) between treatments within sampling points.

Table 5.1. Average plasma amino acid concentrations concentration throughout the study in heifers as affected by arginine supplementation (ARG) or saline (CTRL) infusion. Data are given as means ± S.E.

	Treatment[a]		P-value
AA, μmol/L	**CTRL**	**ARG**	
Ala	183.6 ± 10.60	177.5 ± 10.11	0.5084
β-Ala	4.2 ± 0.25	3.83 ± 0.26	0.1938
Arg	155.9 ± 6.92	173.3 ± 6.65	0.0890
Asn	42.0 ± 2.37	43.0 ± 2.28	0.5988
Asp	10.8 ± 0.42	9.9 ± 0.41	0.1173
Carn	18.1 ± 0.82	14.2 ± 0.78	0.0044
Cit	70.1 ± 3.95	65.8 ± 3.77	0.2033
Cyst	3.5 ± 0.19	3.7 ± 0.18	0.2661
Gln	218.6 ± 10.1	227.6 ± 9.68	0.5080
Glu	61.1 ± 3.27	57.9 ± 3.12	0.7197
Gly	244.6 ± 7.36	254.3 ± 7.13	0.8979
His	71.5 ± 2.80	66.3 ± 2.70	0.3116
Ile	129.4 ± 3.61	116.7 ± 3.50	0.024
Leu	244.8 ± 8.57	217.1 ± 8.30	0.0340
Lys	81.1 ± 4.96	79.4 ± 4.78	0.2812
Met	28.3 ± 1.25	27.5 ± 1.20	0.2527
Orn	66.8 ± 4.12	68.2 ± 3.93	0.9037
Phe	73.1 ± 2.36	65.5 ± 2.27	0.0340
Pro	90.3 ± 5.58	95.7 ± 5.33	0.8650
Ser	85.9 ± 3.11	83.8 ± 3.00	0.0885
Tau	47.1 ± 3.62	49.3 ± 3.47	0.7413
Thr	86.1 ± 4.05	83.6 ± 3.91	0.5169
Trp	44.9 ± 1.54	37.9 ± 1.49	0.0050
Tyr	54.0 ± 2.66	49.1 ± 2.56	0.1484
Val	335.5 ± 9.13	300.3 ± 8.83	0.014
Total EAA[b]	1,251.2 ± 32.70	1,164.6 ± 31.66	0.076
EAA[c, %]	73 ± 2.4	65 ± 2.3	0.0240

[a] Peritoneal infusion of 40 mg of Arg/kg of BW (ARG) or the saline equivalent volume (CTRL).

[b] EAA. Essential amino acids.

[c] Proportion of EAA with respect total plasma AA.

Table 5.2. Average concentration of selected blood metabolites in heifers receiving arginine infusion (ARG) or not (CTRL).

	Treatment[a]			P-Value[b]		
	CTRL	ARG	SEM	TRT	D	TRT X D
IGF, ng/ ml	125.9	125.5	10.47	0.9708	0.5010	0.2501
P4, ng/ ml	6.8	7.4	0.29	0.0406	< 0.0001	0.2428
GH, ng/ ml	13.3	14.0	0.73	0.3891	0.0014	0.9821
PRL[c], ng/ ml	11.4	14.3	0.27	0.4354	0.9242	0.2773
NO, µmol	7.6	7.4	1.54	0.8864	0.2818	0.4043
GLUC, mg/dl	2.14	1.92	0.293	0.4532	0.8007	0.4447

[a] Peritoneal infusion of 40 mg of Arg/kg of BW (ARG) or the saline equivalent volume (CTRL).

[b] TRT = effect of treatment; D = effect of pregnancy day; TRT x D = interaction between the effect of treatment and pregnancy day.

[c] Least squares means for PRL blood concentrations presented herein correspond to non-transformed data, whereas SEM and P-values correspond to the ANOVA analysis using ln-transformed data, respectively.

5.3.3. Experiment 2

Hemodynamics of plasma concentration of Arg after a single peritoneal or jugular infusion is reported in Table 5.3. In experiment 2, overall plasma Arg concentration did not vary depending on the site of infusion (PrI or JI), however, it did vary with time after infusion ($P < 0.05$) and it was affected by an interaction between site of infusion and time ($P < 0.001$; Figure 5.4.). As expected C_b (117 ± 30.5 µmol/L) did not vary between treatments. Time to plasma Arg peak concentration was 15 min for JI and 60 min for PrI. Peak concentration was greater ($P < 0.001$) for JI (265 ± 30.5 µmol/L) than for PrI (210 ± 30.5 µmol/L) but in contrast, AUC was not different ($P > 0.1$) between

both infusion sites (71,219 ± 15,136 and 72,751 ± 15,136 μmol.min/L for JI and PrI, respectively). This can be due to a faster decrease in plasma Arg concentration with JI, since t_b occurred after 360 min after PrI and only after 120 min after the JI. After reaching plasma Arg peak concentration, $t_{1/2}$ was shorter (P < 0.05) in JI (15 ± 1.2 min) than in PrI (47 ± 1.2 min.), and the opposite occurred for CR (P < 0.05), being 4.6 ± 0.09 %/min for JI and 1.5 ± 0.09 %/min for PrI.

Table 5.3. Arginine hemodynamics after a jugular (JI) or a peritoneal (PrI) infusion of 40 mg of arginine per kg of body weight.

	Infusion Site		
	JI[a]	PrI[b]	P-value
Plasma conc[c] (μmol/L)	138 ± 25.7	133 ± 25.7	0.8133
C_b[d] (μmol/L)	108 ± 30.5	126 ± 30.5	0.5558
Time to peak[e] (min.)	15	60	< 0.0001
Peak conc[f] (μmol/L)	265 ± 30.5	210 ± 30.5	<0.05
AUC[g] (μmol.min/L)	71,219 ± 15,136	72,751 ± 15,136	0.8753
tb[h] (min.)	120	360	< 0.0001
t1/2[i] (min)	15 ± 1.2	47 ± 1.2	<0.05
CR[j] (%)	4.6 ± 0.09	1.5 ± 0.09	<0.05

[a] Jugular infusion; [b] peritoneal infusion; [c] plasma arginine concentration; [d] basal plasma arginine concentration; [e] time from the infusion until concentration peak; [f] peak concentration; [g] area under the curve; [h] time to reach basal level; [i] half-life; [j] clearance rate.

Plasma glucose concentrations were lower ($P < 0.0001$) in JI (73.4 ± 0.77 mg/dl) than in PrI (81.8 ± 0.77 mg/dl) and they evolved differently ($P < 0.0001$) between infusion sites (Fig. 5.5). The PrI of Arg led, after 120 min, to a 1.94-fold increase ($P < 0.0001$) in plasma glucose levels (compared with basal levels) reaching 135.8 ± 4.35 mg/dl. This increase was not observed with the JI of Arg, as basal levels of glucose were maintained steady during the whole recording time. The AUC of plasma NO tended to be greater ($P < 0.06$) when infusion took place via PrI (5,676.5 ± 1,789.44 µmol.min/L) compared with JI (3,849.9 ± 1789.44 µmol.min/L).
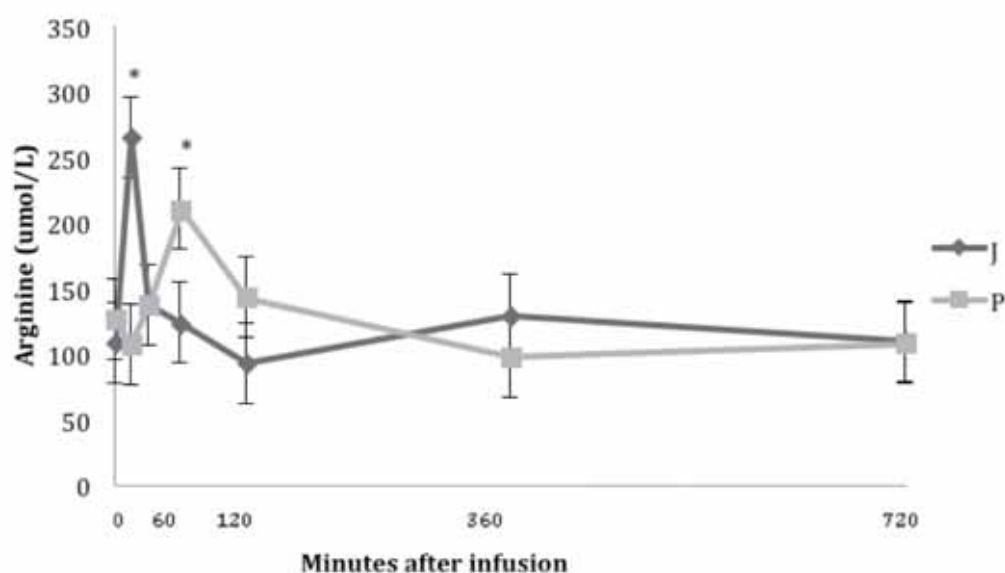
Figure 5.4. Plasma arginine levels after a jugular (J) or peritoneal (P) infusion of arginine. Mean ± S.E. Asterisks denote differences ($P < 0.05$) between infusion site within sampling points.
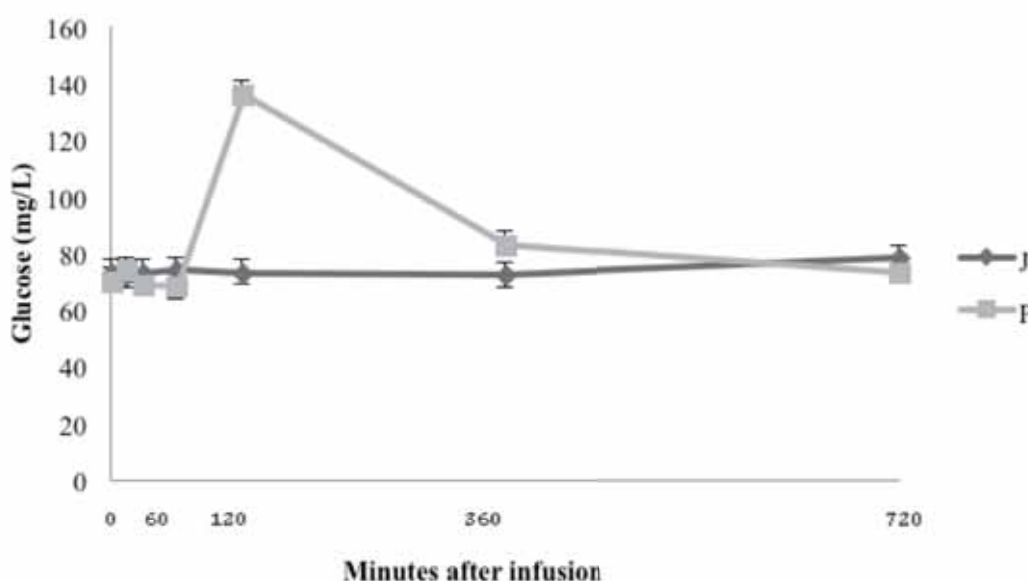
Figure 5.5. Plasma glucose levels after an arginine jugular (J) or peritoneal (P) infusion. Mean±S.E. Asterisk denotes differences (P < 0.05) between infusion site within sampling points.

5.4. Discussion

5.4.1. Performance and blood dynamics

Transrectal Doppler ultrasonography has been described as a suitable and non-invasive technique for measuring uterine vessels hydrodynamics (Bollwein et al., 2002; Panarace et al., 2006) and during the last years, it has been increasingly used (Herzog and Bollwein, 2007). There were no differences in total BW gain (125 ± 13.4 kg) or DM intake (23 ± 0.5 kg/day) between treatments. It has been previously reported that uterine blood FV seems to exponentially increase during pregnancy (Bollwein et al., 2002; Panarace et al., 2006). In the current study, uterine blood FV also increased (P < 0.001) with pregnancy day but it evolved differently between treatments (Fig. 5.1). Uterine blood FV of CTRL heifers progressively increased with days of pregnancy to a greater (P < 0.001) than in ARG heifers (Fig. 5.1). This response was unexpected as our hypothesis was that Arg supplementation might supply additional substrate for NO synthesis and that this would in turn increase uterine blood flow. It is now widely demonstrate that Arg is a common substrate for NO (Flynn et al., 2002). Furthermore, studies have shown that NO is a key regulator for angiogenesis and placental and fetal

growth (Reynolds and Redmer, 2001). Hefler et al. (2001) reported that the absence of NO synthesis in rats resulted in intra-uterine growth retardation, and in humans, intra-uterine growth retardation is also associated with low body NO synthesis (Hata et al., 1998). Mateo et al. (2007) reported an increase in fetus survival by supplementing of 1 % L-Arg between days 30 and 114 of gestation in gilts. Also, an intravenous administration of L-Arg (3 x 155 µmol/kg BW per day) prevented fetal growth restriction in underfed ewes (Lassala et al., 2010).

Diameter of the uterine artery did not differ between treatments, but it increased ($P < 0.001$) with pregnancy day, whereas PI and RI decreased throughout pregnancy but did not differ between treatments (Fig. 5.2). Both parameters PI and RI are commonly used in obstetrics; however, it is believed that PI provides more hemodynamic information than RI because in its calculation it includes the entire cardiac cycle (it enters the equation as a denominator). The decrease of resistance, showed by both PI and RI parameters, has been previously reported (Bollwein et al., 2002; Panarace et al., 2006) and has been associated with a progressive vascular bed development (Panarace et al., 2006). An accuracy of 1 mm in the measure of a small diameter (0.2 cm) can give errors of -75 and + 125 % in the calculation of blood flow volume (Herzog and Bollwein, 2007). During this study the diameters measured ranged from 0.25 to 0.43 cm, thus a small error in the measurement of the diameter could have hidden any potential effects of Arg.

Heart rate was lesser ($P < 0.05$) in ARG (74 ± 1.4 beats/min) than in CTRL heifers (81 ± 1.5 beats/min). An increased HR during pregnancy has been reported by Rezakhani et al. (2004) although they did not exactly specify at which days of pregnancy the rise of HR occurred. No studies reporting influences of maternal HR during pregnancy have been found, thus the consequences for the decrease of HR cannot be known. However, according to physical rules, it seems reasonable to assume that if the uterine artery has a bigger diameter, less beats per minute, thus a lesser HR, would be needed to ensure the same blood flow.

Our initial hypothesis was that Arg supplementation would increase placenta blood flow, and that might in turn also affect calf BW; however, no differences in calf BW at birth (38 ± 1.8 kg) were detected between groups, despite the fact, uterine blood flow was actually greater in CTRL than in ARG heifers at 146 day of pregnancy.

5.4.2. Blood metabolites

Uterine blood FV was negatively affected by L-Arg supplementation at day 146 of pregnancy. However, as mentioned above, errors measuring very small diameters can occur (Herzog and Bollwein, 2007) and then differences can be missed. For that reason, the measurement of NO, which could confirm potential changes in vasodilation, was performed. It could be that our Arg supplementation was too low to produce an increase of NO (no differences between treatments were found, P = 0.89) and thus none of the successive reactions as hormone secretion, vasodilation, etc. did take place. Indeed, in the literature we found transient increases in blood hormones in dairy cows after an infusion of 0.1 g/kg BW (Chew et al., 1984) that is 2.5 times more than the quantity infused in this study. However, Fligger et al. (1997) supplemented pre-ruminant calves with 0.5 g/kg BW of Arg and did not find changes in plasma GH concentrations. It is important to note, though, that in the present study, Arg supplementation took place during a more prolonged period of time and it was supplied via intra-peritoneal infusions which, as shown in Experiment 2, the absorption is slower than when directly infusion into the blood stream.

Plasma concentrations of Arg tended (P < 0.09) to be greater in ARG than in CTRL heifers, but the concentration of plasma was reduced. Reductions in plasma EAA concentrations could be attributed to either an increased uptake of EAA into protein (Bergen, 1979) or to a dietary protein deficit (Bergen, 1979). However, in the present study, all animals were fed exactly the same ration (balanced to meet the nutritional needs of the heifers) and no difference in feed intake was found, thus it is unlikely that a dietary protein deficit could have caused the differences in plasma AA. Other studies about protein metabolism after an L-Arg infusion have reported an increase in fetal protein accretion by decreasing protein turnover, and breakdown (De Boo et al., 2005). Also, L-Arg supplementation has been reported to ameliorate protein anabolism in rats (Cui et al., 1999)

Contrary to the data reported by Wu et al. (2009) when administering L-Arg i.v., plasma PRL, IGF, glucose, and GH concentrations were unaffected by treatments. De Boo et al. (2005) did not find differences in maternal hormone concentrations after an L-Arg infusion. However, other studies have shown that L-Arg stimulates GH secretion

(Merimee al., 1967; Koli et al., 2004; De Boo et al., 2008), decreases plasma levels of glucose, and increases plasma insulin concentration. Furthermore, contrary to the present study, an L-Arg infusion (0.1 g/kg BW per day) led to a transient increase in plasma concentration of PRL, GH, and insulin (Chew et al., 1984) in pregnant dairy cows. However, substantial differences as supplementation rate, physiological status of the animals, or supplementation methodology, can be found between the present study and the ones reported above. The current study is the only one administering Arg via peritoneum. De Boo et al. (2005) supplemented L-Arg in ewes fed under their nutritional recommendations, and infused intravenously 12.2 µmol of L-Arg per min during 180 min; while Chew et al. (1984) infused intravenously 0.1 g of Arg per kg of BW daily about 7 day prior calving. Thus, different procedures in the studies can lead to different results. A peritoneal infusion was chosen herein as a method to slowly supply Arg to the animal in a manner that would mimic Arg availability when providing Arg though the diet.

Contrary to the results found by Li et al. (2010) who reported a decrease in P4 when L-Arg was supplemented to gilts between day 0 and 25 of gestation, plasma P4 concentration herein was greater ($P < 0.05$) in ARG than in CTRL heifers. The differences between that study and ours could be due to different target species, supplementation time (day 41 of pregnancy herein whereas in Li et al. (2010) was initiated at the beginning of gestation), or the amount of Arg that was supplemented to the animals. Progesterone maintains endometrial functions as early embryonic development, implantation, placentation and fetal development (Bazer et al., 1994);thus, an increase of P4 concentration may have positives effects in fetal development. However, as depicted in Figure 5.3, differences between CTRL and ARG animals occurred at ds 83 and 104, and it should be further studied whether this relatively short window of time would be sufficient to elicit differences in fetal development.

5.4.3. Experiment 2

Overall plasma Arg concentration did not vary depending on the infusion site, however, substantial differences could be found in Arg hemodynamics. As expected C_b did not vary between treatments, thus the initial status of the animals was the same. However, it could be concluded that Arg availability was faster when infusion was performed via JI than when conducted as a PrI as the peak of Arg concentration took place 45 min sooner (Fig 5.4). Also, with JI, the peak was greater than in PrI but the AUC did not differ between the 2 infusion sites due to the faster disappearance of plasma Arg with JI; thus it could be concluded that total Arg bioavailability of Arg did not differ between infusion sites. Plasma glucose concentration was greater with PrI, and after 120 min, a 1.94-fold increase in plasma glucose occurred. This increase was not observed with the JI of Arg, as basal levels of glucose were maintained steady during the whole recording time. No explanation of this response has been found in literature. Nitric oxide did not differ between infusion sites; however, AUC of NO tended to be greater when infusion took place via PrI compared with JI. Chew et al. (1984) reported a transitory effect of intravenous Arg infusion on different hormones with a rapid but short peak of hormone concentrations. As PrI resulted in a slower clearance rate, less Arg may have been needed to activate the pathways to release glucose and NO and this could explain the AUC for these two metabolites in PrI than in JI.

5.5. Conclusions

In conclusion, contrary to our hypothesis, a peritoneal infusion of 40 mg/kg BW of Arg did not increase blood flow to the uterus but did change other parameters that could potentially influence placental and fetal growth such as heart rate, maternal concentration of plasma AA or P4 production. More research is needed to determine the optimal Arg supplementation rate and timing for dairy cows. As demonstrated in experiment 2, site of infusion (blood vs. peritoneum) affects the metabolic responses to Arg supplementation.

Chapter 6

GENERAL DISCUSSION

All the experiments conducted in this thesis aimed at studying different factors that may imprint the future performance of calves and heifers. Although each study had different and specific objectives, the results of all of them offer valuable information about short- and/or long-term consequences of different feeding management strategies.

As explained in the introduction, several studies have linked the neonatal management of heifers with their later performance. Thus, Studies 1 and 2 aimed at establishing neonatal feeding methodologies that could ameliorate the future performance and productivity of the female calves. For that reason, a comparison between liquid feeding sources and offered quantities have been compared in each study.

Recently, González-Recio et al. (2012) have demonstrated that the coexistence of pregnancy with high milk yields impaired the future milk yield performance of the new gestated calf. Thus, if on the one side we focus our efforts in rearing healthy and productive dairy heifers that can exhibit all their genetic merit but, on the other side we do not take any action to diminish the potential negative impact that the coexistence of pregnancy and milk production has, the outcome might be lower than expected. For instance, actions that permit a greater supply of nutrients to the fetus and mammary gland without increasing dramatically feeding costs should be studied. For that reason, the third study aimed at elucidating if arginine (Arg) supplementation may lead to uterine artery vasodilation, which would permit the fetus to obtain more nutrients from the dam.

It is the sum of both actions, and of course, good management practices that would permit future calves show the maximum of their genetic merit.

6.1. NEONATAL MANAGEMENT

The two studies with calves were carried out with the objective to compare the effect of different rearing strategies that may be used in dairy calves. In Study 1, the effect of feeding whole milk (WM) or milk replacer (MR) was evaluated because on the one hand, WM is an alternative that allows producers to dodge feed cost increases, and on the second hand because of studies (Bar-Peled et al., 1997; Godden et al., 2005; Shamay et al., 2005; Hill et al., 2008; Moallem et al., 2010; Gleeson and O'Brien, 2012) which have reported contradictory results in performances when comparing both

mentioned milk sources. As reported in Chapter 3, we did not find marked differences in performance between the 2 milk types when milk source was offered at the same DM amount. However, differences in glucose and insulin levels were observed. Studies reporting significant differences between WM and MR (Bar-Peled et al., 1997; Shamay et al., 2005) have concluded that milk allowances offered to calves is a major source of variation. Thus, a study in which different amounts of liquid feeding was offered to calves was carried out (Study 2). In addition, as differences in glucose and insulin appeared in Study 1, glucose metabolism was analyzed in depth in Study 2. Whole milk is not a constant feeding source, and thus feeding MR would be more consistent to study glucose metabolism than when feeding WM. Then, it was planned to use MR in Study 2 to analyze the effects on glucose metabolism and performance when offering three different amounts of liquid feed to dairy calves during rearing.

6.1.1. Performance

6.1.1.1.Growth Performance

The purpose of this thesis was to define strategies that may imprint the animals at early stages of life in order to improve their future performance and welfare. Several studies (Shamay et al., 2005; Bach and Ahedo, 2008; Soberon et al., 2012; Soberon and Van Amburgh, 2013) have linked greater nutrient intake, or growth in early ds of life with an increase in milk yield during first lactation.

As shown in Study 1, neither liquid and solid intake nor ADG have shown differences between WM- and MR-fed calves when fed at the same rate (750 g of DM/day). Whereas, in accordance with others studies (Bar-Peled et al., 1997; Diaz et al., 2001; Jasper and Weary, 2002; Terré et al., 2009), feeding high amounts of MR to dairy calves improved preweaning ADG. Obviously, MR intake was greater when greater amounts were offered to calves; however, total DMI was not always greater when more MR was offered as shown in Fig. 6.1.a. This was due directly to differences in the evolution of starter intake (Fig. 6.1.b). Analyzing the methodology used in each study, two main differences can be reported. First, the calf starter presentation, and second the weaning methodology.
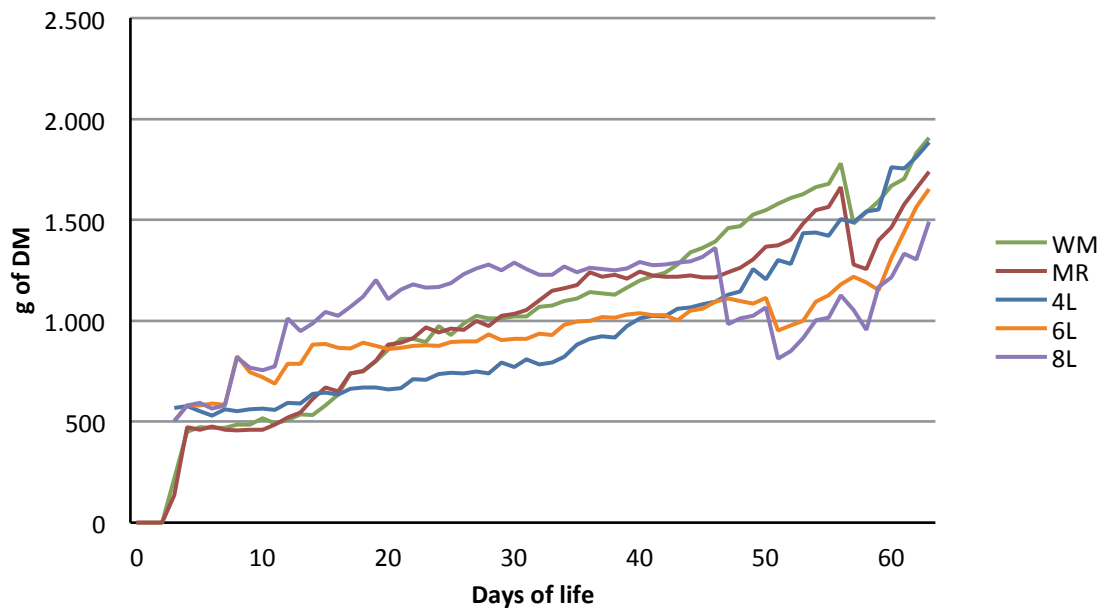
Figure 6.1.a. Total dry matter intake (DMI) of calves from Study 1 fed 750 g/day of whole milk (WM), 750 g/day of milk replacer type 1 (MR), and calves from Study 2 fed 600 (4L), 900 (6L) and 1200 g/day (8L) of milk replacer type 2.
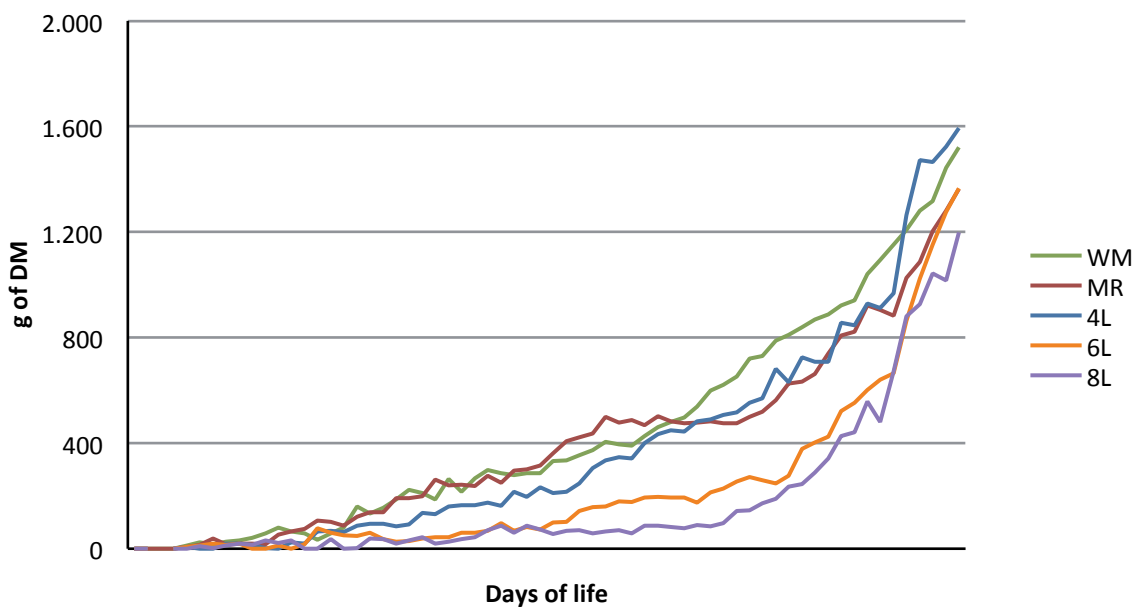
Figure 6.1.b. Starter intake of calves from study 1: fed 750 g/day of whole milk (WM), 750 g/day of milk replacer type 1(MR), and calves from study 2: fed 600 (4L), 900 (6L) and 1200 g/day (8L) of milk replacer type 2.
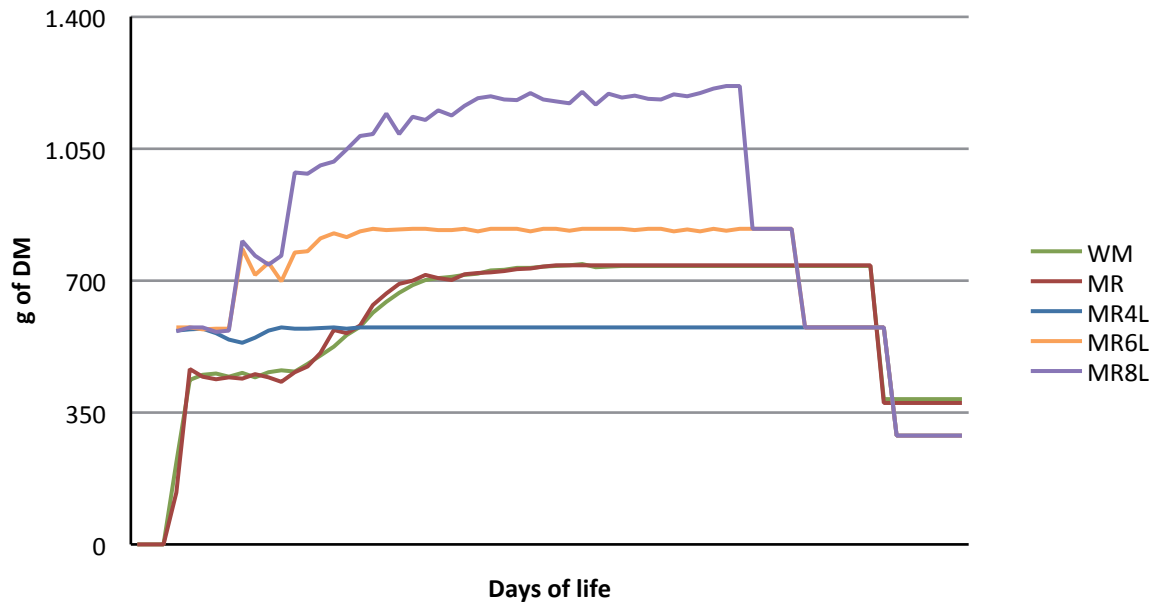
Figure 6.1.c. Milk intake of calves from study 1: fed 750 g/day of whole milk (WM), 750 g/day of milk replacer type 1 (MR), and calves from study 2 fed 600 (4L), 900 (6L) and 1200 g/day (8L) of milk replacer type 2.
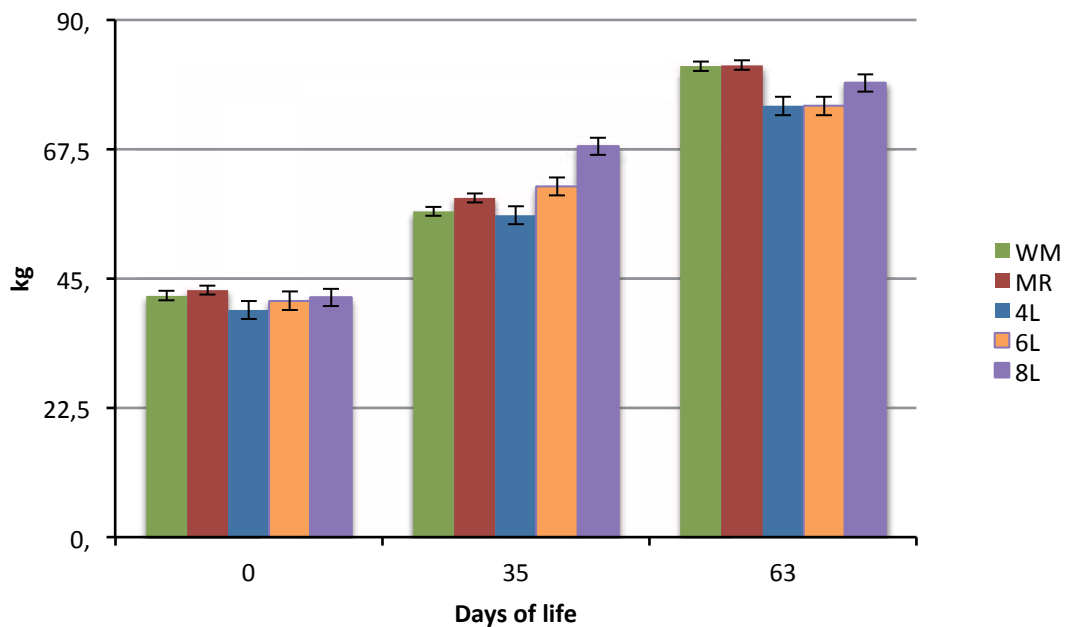
Figure 6.2. Body weight of calves from Study 1 fed 750 g/day of whole milk (WM), 750 g/day of milk replacer type 1(MR), and calves from Study 2 fed 600 (4L), 900 (6L) and 1200 g/day (8L) of milk replacer type 2.

As mentioned, calf starter presentation differed in both studies. In Study 1, starter was presented as pellet whereas in Study 2, the starter was presented as a multi-particle. The overall starter intake was 421 ± 58.2 g/day in Study 1 and 433 ± 26.5 in Study 2. Hence, there are not important starter intake differences. Other authors have reported lesser intakes when starter was offered as pellet compared with a texturized starter (Franklin et al., 2003) or multi-particle starter (Bach et al., 2007), although these authors reported similar intakes until preweaning. However, comparing these two studies, it can be easily observed that calves belonging to Study 1 (fed pellet), had a faster increase in starter intake (Fig. 6.1.b). This greater starter intake was maintained until around day 40 of life, when group 4L reached similar intakes levels. But it is only around day 60, when the rest of the calves (from groups 6L and 8L; Study 2) reached similar starter intake levels.

The second main difference between studies is the weaning methodology. All animals were weaned at 63 days of age, however in Study 1, and for group 4L of Study 2, the methodology used was to reduce liquid feeding a 50 % during one week whereas in Study 2, the groups 6L and 8L a more smoothly method was used. As the liquid feeding was removed progressively (Fig. 6.1.c). It can be observed (Fig. 6.1.a) that before day 47, when a large proportion of the total DMI depends on the liquid intake, the calves fed a greater amount of MR (8L) had the greatest total DMI. However, from day 47, when weaning process for group 8L started, their DMI dropped. As an example, their intake at day 51 was above 500 g lesser than at day 46. This observation seems to indicate that the weaning methodology was not adequate. Weaning methodologies will be discussed later in the section 6.1.2. of the present chapter.

Comparing the results of growth performance at day 35 is interesting. ADG in Study 1, were 717 and 701 g/day for MR and WM, respectively; and in Study 2, were 484, 564 and 752 g/day for calves in 4L, 6L and 8L, respectively. These results are in accordance with the total DMI reported above. The changes in ADG are reflected in BW variations throughout the study (Fig. 6.2). As expected 8L calves grew faster during the first 35 day of life but then all the gained advantage disappeared and became equal to that of calves consuming lesser amounts of MR; basically due to the fact that starter intake seems to be insufficient to compensate the withdrawal of the MR. Also, 6L calves

suffered a slump in weight gain during the last part of the experiment while calves in Study 1 (WM and MR), and 4L calves, to which an abrupt weaning was performed, seemed to be able to compensate the lack of MR with an increase of starter intake. Further, the overall GtoF ratio from day 0 to weaning was similar between studies ranging from 0.54, for 6L calves to 0.60 for 8L, and WM calves. But, in Study 1, GtoF ratio was almost constant from birth to weaning; whereas in Study 2, an important decrease in GtoF ratio was especially found in 8L group. The decrease in GtoF ratio was probably due to a greater MR intake during the first period, which is more digestible than starter (Davis and Drackley, 1998).

Veterinary treatments were recorded in both studies. In Study 1, 34 % of the calves received veterinary treatment compared to 28 % in Study 2. However, WM-fed calves needed less veterinary treatments than MR-fed calves (see Chapter 4). In contrast with results reported in other studies (Smith et al., 2002; Nonnecke et al., 2003), in Study 2, no differences were found between treatments, thus no immunological advantages were observed when feeding more MR to calves.

6.1.1.2. Reproductive performance

Some studies (Bar-Peled et al., 1997; Moallem et al., 2010) have reported a decrease in conception age in heifers fed greater milk quantities during the preweaning period. Both authors used 2 different milk sources (WM and MR), and in both cases calves fed WM had greater energy intakes. Therefore, it is difficult to elucidate whether this shortage in age at conception is due to milk quality or to the increased energy intake. In none of our studies, statistical differences were reached in conception age. In Study 1, where milk source was different but intake was similar, even no numerical differences were found. However, the milk amount fed to calves seemed to have an effect on age at first conception. Figure 6.3 shows the correlation between total milk intake during preweaning and age at conception. Even if the correlation is low ($R^2 <$ 0.3), the Figure shows that heifers eating more MR during preweaning had lesser age at conception. Also, a greater variability in conception age can be appreciated when heifers ate less than 40 kg of milk in a DM basis. Despite the fact that more data are necessary to reach a solid conclusion, according to the results presented herein, it

seems that efficiency in reproduction at first conception might be more imprinted by the amount of energy intake during preweaning than by the milk source utilized.
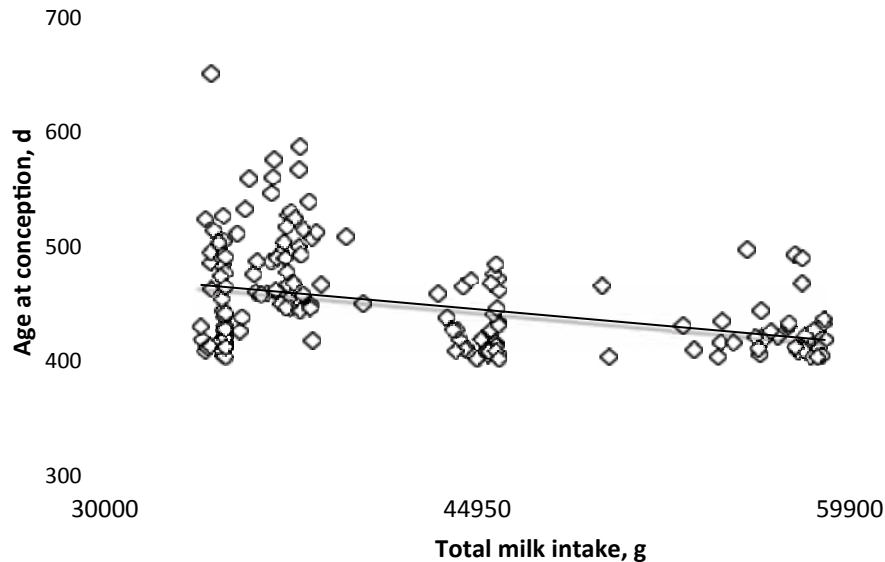
Figure 6.3. Correlation between total dry matter milk intake (g) during preweaning and age at conception (day) of heifers.

Meta-analysis of current reproductive data

A meta-analysis was performed using data from available calf studies. Many of the latest studies evaluating the impact of nutrient intake during rearing have shown a positive effect of enhanced feeding programs in reproductive performance parameters as age at first breeding (AFB), and age at first calving (AFC). However, the majority of them did not reach significance because of the low number of animals per treatment. Thus, integrating the available data might help to understand the effects of early feeding in reproductive performance.

We have found 10 studies reporting information about age at first calving in calves fed with different levels of preweaning nutrients (Table 6.1). Even if each study has differences in their methods, they present available data of number of calves per treatment, preweaning intake, preweaning ADG, and reproductive performance (AFC). Metabolizable energy (ME) intake was calculated according to NRC (2001).

The model was built to compare the effect of ME intake in ADG and AFC. Even though ADG ($P < 0.0001$) and ME ($P < 0.05$) were greater in enhanced-fed animals, the meta-

analysis resulted in similar AFC for calves reared under a conventional regime and calves reared under an enhanced regime (P = 0.15). Nevertheless, conventionally-fed calves were 11 days older at first calving. The integration of the actual available data did not allow to conclude whether neonatal intake directly influences AFC even if it seems that an enhanced feeding program may shorten AFC.

Table 6.1. Studies reporting information about age at first calving in calves fed with different levels of preweaning nutrients.

Study	N	Source of nutrients	ME above control calves, Mcal/day	ADG of control, kg	ADG of treatment, kg	Age at first calving of control, day	Age at first calving of treatment, day
Bar-Peled et al., 1997	20	WM/MR	0.29	0.56	0.85	700[a]	669[a]
Shamay et al., 2005	20	WM/MR	0.27	0.59	0.88	700[c]	683[c]
Drackley et al., 2007 (Exp.1)	10	MR	0.41	0.52	0.75	775[c]	808[c]
Drackley et al., 2007 (Exp.2)	14	MR	0.36	0.56	0.71	732[c]	741[c]
Raeth-Knight et al., 2009	26	MR	0.54	0.56	0.79	745[a]	717[a]
Terré et al., 2009[1]	30	MR	0.10	0.80	0.90	728[c]	720[c]
Morrison et al., 2010[1]	38	MR	0.16	0.34	0.50	727	730
Moallem et al., 2010	8	WM/MR	0.07	0.73	0.80	751[c]	705[c]
Davis-Rincker et al., 2011	40	MR	0.20	0.44	0.64	715[c]	701[c]
Soberon et al., 2012	400	MR	0.450	0.32	0.70	687[c]	691[c]
Kiezebrink et al., 2015	76	WM	0.53	0.62	0.78	759[c]	741[c]

[a]$P < 0.05$; [b]$P < 0.1$; [c]$P > 0.1$; 1 Data from age at first calving presented herein are age at pregnancy reported by the authors plus 280 days.

6.1.2. Weaning methodologies

As mentioned in the literature review, a smooth transition from liquid to solid feeding is important to minimize weight loss and stress at weaning (Weary et al., 2009). Weaning acquires even more importance when calves are fed high milk quantities, as a high milk intake before weaning can depress solid feed intake (Jasper and Weary 2002; Terré et al., 2009; Hill et al., 2010). Restricted milk feeding encourages solid feed consumption, which is essential for metabolic and physical rumen development (Baldwin et al., 2004). Weaning methods will influence growth rate during the preweaning period (Khan et al., 2011). In Study 2, a gradual weaning was chosen for calves as some studies (Roth et al., 2008; Weary et al., 2008) have reported that abrupt weaning in calves fed *ad libitum* results in a greater depression in growth. Khan et al. (2007) performed a study comparing a conventional feeding method (10 % BW) and an enhanced feeding method (20 % BW) following a step-down milk feeding regime. Calves in the step-down regime ate more solid feed before and after weaning and maintained their growth advantage over calves conventionally-fed after weaning. However, substantial differences exist between the study from Khan et al. (2007) and Study 2 presented in this thesis. First, WM instead of MR was offered to calves, but there is no evidence in the literature that shows that it can influence the weaning process. Second, Khan et al. (2007), fed the calves according to their individual BW and thus MR was corrected each 5 days; whereas, the amount of MR offered in Study 2 was the same for all calves on the same group. Adapting the MR quantity to the BW of each calf allows a better adaptation to their rumen capacity. Also, a very important difference resides in the fact that Khan et al. (2007), fed step-down calves (calves that were weaned progressively) several times per day in a way that they never provided more than 2 L in the same feeding, and feedings were evenly spaced. This would facilitate a better metabolic performance as it allows calves to express their natural behavior (De Passillé, 2001). Up to 22 suckling bouts daily can occur when calves are allowed to suckle from artificial teats freely (Appleby et al., 2001). This also allowed starting directly with around 9 L of WM per day, which is not possible when feeding only two times a day, as it was the case in Study 2. Moreover, weaning method itself also varied from the one performed in Study 2. First, complete weaning was at day 50 in the study of Khan et al. (2007), and 13 days later in ours. This should have given our

calves an advantage, as calves weaned at older ages are more able to compensate the lack of milk through starter intake (Khan et al., 2011). Also, in Study 2, the reduction in milk was performed by decreasing milk allowance, and Khan et al. (2007) decreased milk intake by offering a more diluted milk and thus, calves received always the same volume per meal. That may decrease the weaning stress as calves may find others aspects of feeding routine satisfying, as gut fill from liquid or suckling (Jasper et al., 2008).

By viewing the results in Study 2, the weaning methodology was not adequate, as calves lost the gained BW advantage earlier in life. However, the step-down methodology proposed by Khan et al. (2007) would be very difficult to apply in commercial conditions because of the limited number of operators and resources. In commercial farms, where Studies 1 and 2 were carried out, an increase in feeding bouts or an individualized feeding would be difficult to set. Therefore, feeding strategies facing real situations should be studied to properly weaned calves under an enhanced milk-feeding regime, which permit the maintenance of the BW advantage acquired during the suckling period.

6.1.3. Comparison of average daily gain prediction by the NRC and the one observed in our studies

The National Research Council (NRC, 2001) recommends to feed young calves milk at rates of 8-10 % of BW in order to encourage the consumption of dry feed at an early age to stimulate development of a functional rumen. The NRC (2001) reports several equations to predict nutrient requirements of young calves. As the actual tendency is to recommend a greater plane of nutrition for dairy calves, it would be interesting to check whether data from the two studies presented in the current thesis fit well into the NRC (2001) predicting equations. Data from both calves studies were used to compare the observed ADG during the preweaning period with that predicted based on the ME requirement equations from the NRC (2001).

The NRC (2001) equations are:

$$ME_i \text{ (ingested)} = (MRintake \times ME_{MR}) + (starter\ intake \times ME_s), \quad \textbf{[1a]}$$

$$ME_i \text{ (ingested)} = (WMintake \times ME_{WM}) + (starter\ intake \times ME_s), \quad \textbf{[1b]}$$

where ME_{MR}, ME_{WM}, and ME_s were considered for Study 1: 4.86 Mcal/kg DM of MR, 5.06 Mcal/kg DM of WM, and 3.37 Mcal/kg DM of starter. And for Study 2: 4.98 Mcal/kg DM of MR and 3.26 Mcal/kg DM of starter.

$$NE_m \text{ (maintenance)} = 0.086 \, BW^{0.75}, \quad \textbf{[2]}$$

where BW is body weight in kg.

$$MEm = NEm/\text{efficiency of use of MEm}, \quad \textbf{[3]}$$

where efficiency of the use of ME for maintenance was computed as weighted average of efficiencies, with values of 0.86 for MR and 0.75 for starter.

$$NE_g \text{ (gain)} = 0.84 \times BW^{0.355} \times ADG^{1.2} \times 0.69, \quad \textbf{[4]}$$

where ADG is the average daily gain in kg/day.

$$ME_g = NE_g/\text{efficiency of use of } ME_g, \quad \textbf{[5]}$$

where efficiency was computed as weighed average of efficiencies of MR (0.69) and starter (0.57).

Therefore,

$$\textbf{ADG} = e^{(\ln[((MEi - MEm) \times (\text{efficiency of MEg})) / (0.84 \times BW0.355 \times 0.69)]/1.2)} \quad \textbf{[6]}$$

Predicted individual ADG was calculated using the NRC (2001) equation reported above [6] and then, a linear mixed-effects model similar to the model used in Studies 1 and 2 was performed. For both studies, the least square means for the predicted ADG were lesser than the values observed (Table 6.2.). The ADG under prediction may be due to several causes, however, observing Figure 6.4 it seems clear that, after day 28, the under-prediction is almost constant. Then, it may be logical to think that it is due to differences in the fixed factors of the equation, especially those pertaining to solid feed. Furthermore, the linear regression presented in Figure 6.5 shows that ADG observed is more than 2-fold the ADG predicted by NRC. The different factors of the ADG equation can be evaluated to find an explanation of the under prediction. First,

the ME ingested calculated according to NRC (2001) may be too low. ME_{MR}, ME_{WM} and ME_s were calculated from the gross energy (GE) values from data on composition and heat of combustion (NRC, 2001). For MR and WM: GE (Mcal/kg) = 0.057 CP% + 0.092 fat% + 0.0395 lactose%. The digestible energy (DE) was calculated as 0.97*GE, and finally ME as 0.96*DE. Similarly, for starter, ME was calculated from NRC equations; GE (Mcal/kg) = 0.057 CP% + 0.094 ether extract (EE)% + 0.0415 carbohydrate%. DE values were calculated as the sum of the products of digestible CP, EE, and carbohydrates multiplied by their heats of combustion (NRC, 2001), and finally ME = (1.01 x DE - 0.45) + 0.0046 (EE - 3). Although data from the starter and WM are slightly lower than the one reported in NRC, it can be concluded that the ME obtained are in accordance with NRC (2001), therefore, ME_i calculation does not seem to be the cause of the obtained under-prediction.

Table 6.2. Comparison of observed average daily gain of calves with the predicted average daily gain values from NRC (2001) metabolizable energy (ME) equation of the calves under the 5 treatments reported in Studies 1 and 2.

	TREATMENT[a]								
Study 2				**Study 3**					
WM		**MR**		**MR_4L**		**MR_6L**		**MR_8L**	
Obs[b]	Pred[c]	Obs[b]	Pred[c]	Obs[b]	Pred[c]	Obs[b]	Pred[c]	Obs[b]	Pred[c]
625	427	620	399	515	427	550	424	678	448

[a]WM: whole milk; MR: milk replacer, MR_4L: milk replacer 4 L / day, MR_6L: milk replacer 6 L / day, MR_8L: milk replacer 8 L / day.

[b] Values observed in both studies.

[c] Values predicted; ADG = exp((ln[(((ME_I − ME_M) x (efficiency of MEg)) / (0.84 x $BW^{0.355}$ x 0.69)]/1.2); ME_i metabolizable energy intake, ME_m metabolizable energy for maintenance, BW body weight.

Another factor that may cause the under prediction is the ME of maintenance (MEm) that may be greater than expected due to an underprediction of the efficiency of use of ME for maintenance regarding milk sources and starter. Similarly, the efficiency of use of ME of the milk sources and starter for gain could have been underestimated. For instance, Davis and Drackley (1998) reported a range of 0.72 to 0.75 for the efficiency of use of ME of MR whereas NRC considers only 0.69.

A.
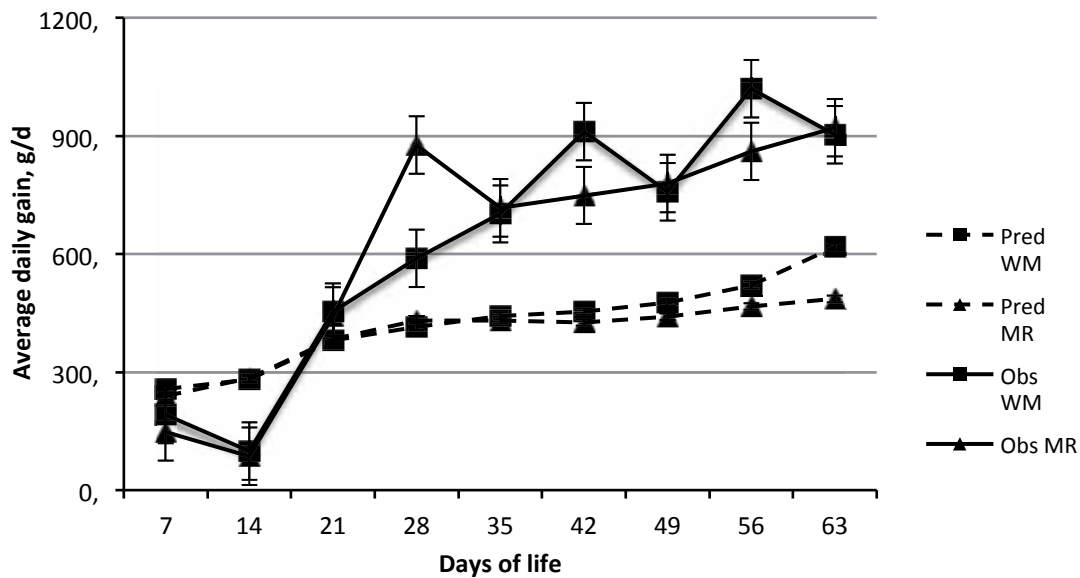
B.
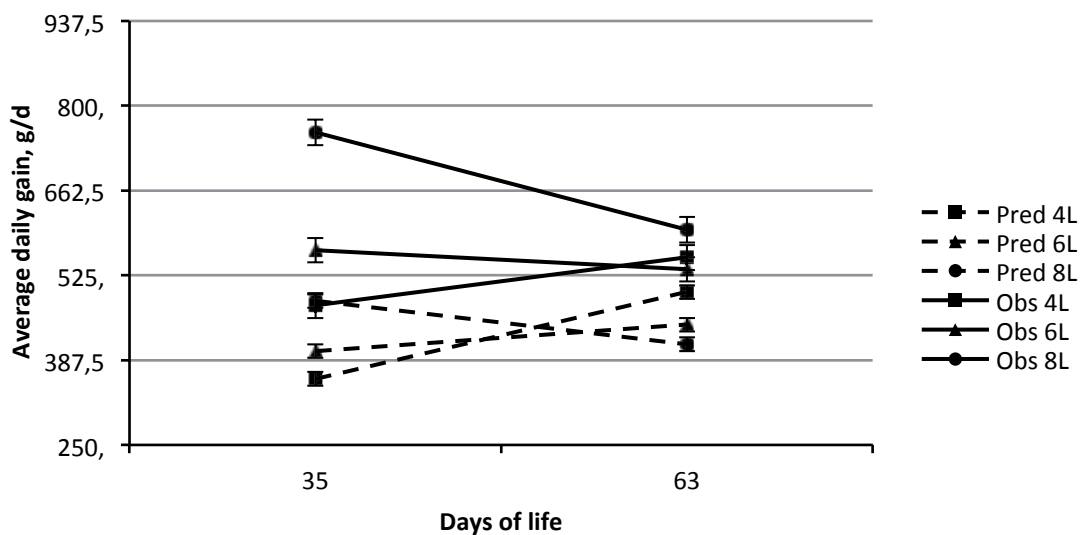
Figure 6.4. Comparison of observed and predicted average daily gain, using the equation based on ME requirement (NRC, 2001), values during the preweaning period of calves under Study 1 (A) and under Study 2 (B).

The chart shows a scatter plot with the equation y = 2,1721x - 264,16 and R² = 0,43242. The x-axis is labeled "Average daily gain predicted by NRC equations" (0, 375, 750) and the y-axis is labeled "Average daily gain observed" (0, 500, 1000, 1500, 2000).
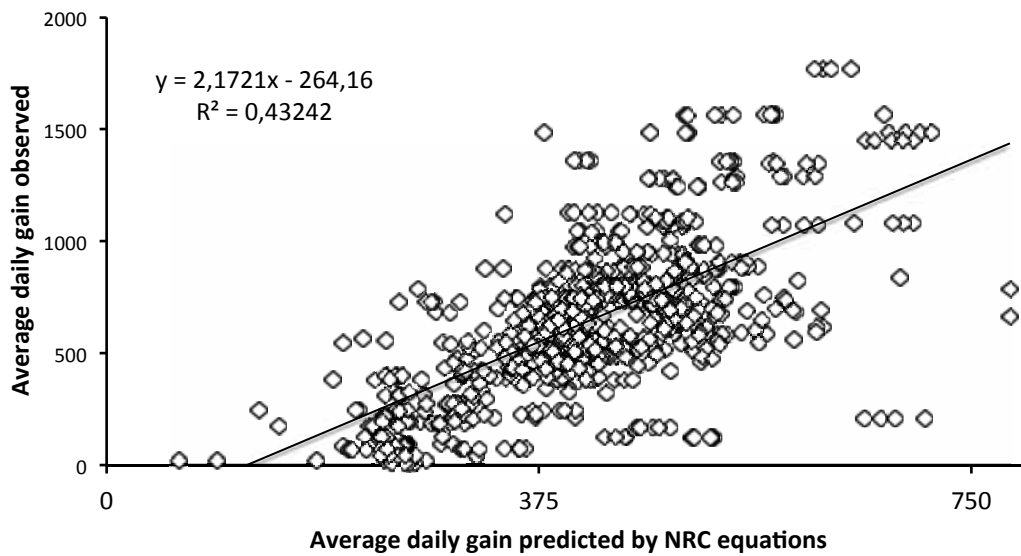
Figure 6.5. Linear regression between observed and predicted average daily gain of calves under Studies 1 and 2.

In the same direction, the NRC (2001) has estimated the biological value of the milk in 0.80, it seems to be reasonable (Blome et al., 2003), even when greater amounts of MR are offered to calves (Diaz et al., 2001; Bartlett et al., 2006). Therefore, 0.80 is used for the predictions in the present studies. However, the biological values of calf starter were based on NRC from 1978 (NRC, 2001), and apart of the fact that biological values for proteins in ruminants might vary considerably (Lofgreen et al., 1951), the studies in which NRC (1978) has obtained the values are from several years ago, and thus, it is logical to think that feed industries have improved their manufacturing process and therefore that the biological protein values of calf starter may have changed.

Summarizing, it seems that the values used by NRC (2001) results in a considerable underprediction of the observed performance in the present thesis. The predicted ADG would increase substantially if an efficiency of use of ME for gain of calf starter greater than 0.57 were used. Therefore, it may be useful to update these values to the actual circumstances of calves feeding.

6.1.4. Glucose and Insulin

It is relevant to discuss glucose and insulin blood concentrations and the ratio between both of them, because it can clarify how the metabolism responds to the different feeding methods. Glucose and insulin blood concentrations were analyzed in both calves studies. However, substantial differences in sampling methodology exist. First, in Study 1, plasma glucose and insulin were measured on days 14, 28, 42 and 56 from 4 to 6 h post feeding, whereas in Study 2, three glucose tolerance tests (GTT) at days 42, 86 and 300 were performed after a 5 h fasting period. Data from the GGT have already been discussed in Chapter 4, thus, only basal values in day 42 from both studies would be discussed in the present chapter, data of day 42 from glucose, insulin and their ratio is presented in table 6.3.

No differences were found in basal glucose concentration when comparing calves fed WM or MR at the same amount (750 g/day of DM) although the evolution over the study was different. As expected, plasma glucose concentration was greater in calves fed with greater amount of MR (Study 2). However, when all the data from the studies are compared, plasma glucose concentrations are surprisingly greater in calves from Study 1 (WM and MR at 750 g/day) than from calves fed with 1200 g/day of MR. As discussed in Chapter 3, the high glucose concentration can be due to stress, as calves in Study 1 were in groups and in Study 2 were housed in individual hutches.

In the same direction, blood insulin concentrations and ItoG ratio were similar in calves fed 750 g/day of MR from Study 1 than for calves fed 1200 g/day (Study 2). As a major lactose intake would be expected with increased milk allowances, the logical result would be to have also a linear increase in plasma glucose concentration when calves are fed more MR (Palmquist et al., 1992). Also, a linear increase in ItoG ratio should have occurred, as more insulin would be necessary to keep blood glucose levels steady. The differences found herein may be explained by the differences in sampling hours and methodology (as discussed before). Another possible source of variation could be the differences existing in the composition of the MR and the starter feed.

Table 6.3. Plasma glucose (mg/dL) and serum insulin (µg/L) concentrations and their ratio (ItoG; 10^{-4}) of calves fed whole milk (WM) and different milk replacement quantities (MR) from the studies reported above.

	Feeding Program					P-value[1]		
	WM(750 g/day)	MR(600 g/day)	MR(750 g/day)	MR(900 g/day)	MR(1200 g/day)	T	A	TxA
Glucose	$117^a \pm 3.1$	$90^c \pm 1.8$	$117^a \pm 3.1$	$91^c \pm 1.9$	$97^b \pm 1.8$	< 0.05	< 0.05	<0.05
Insulin[2]	$0.78^b \pm 0.053$	$0.46^c \pm 0.04$	$1.36^a \pm 0.053$	$0.42^c \pm 0.04$	$1.2^a \pm 0.04$	<0.0001	< 0.05	<0.05
ItoG[2]	$0.07^b \pm 0.535$	$0.05^b \pm 0.05$	$0.12^a \pm 0.535$	$0.04^b \pm 0.06$	$0.12^a \pm 0.05$	< 0.05	<0.0001	<0.05

[1]T=effect of feeding program; A = effect of age of measurement; TxA = interaction between the feeding program and age of measurement.

[2]SE =Least square means for insulin concentration and the ratio insulin to glucose presented herein correspond to non-transformed data, and SE and P-values correspond to the ANOVA analysis using ln-transformed data, respectively.

In both studies, blood samples were taken after at least 4 h of fasting to avoid differences due to feed intake. High plasma glucose concentration seems to be sustained several hours after feeding (Todd et al., 2000; Stanley et al., 2002) whereas blood insulin concentrations decrease 2 h after feeding (Kaufhold et al., 2000; Stanley et al., 2002; Herrli-Gygi et al., 2006). Thus, it is supposed that values obtained in both studies correspond to basal values. It can be hypothesized that the main source of variation could be the MR and the starter. As mentioned above, differences in intake exist between both studies; however, they do not seem to be enough to explain the important differences in blood basal values of glucose and insulin. Calves from Study 1 had an intake of 0.59 kg and 0.44 of DM per day of MR and starter respectively, and calves from group 4L on Study 2 had similar intakes, 0.55 kg and 0.40 of DM per day of MR and starter respectively. However, ItoG ratio of the calves belonging to Study 1 was more than two-fold greater than the value of the calves from Study 2. It is difficult to compare data from different studies found in literature as in each case feeding source would be different. Nevertheless, from the data of these two studies we can conclude that not important differences in glucose and insulin appeared between WM and MR and that MR at greater allowances increased ItoG rate but all the animals were able to control glycemia.

6.1.5. Economical analyses comparing rearing strategies

As mentioned before, calves are the future of any dairy farm, and thus raising dairy heifers in the most profitable way must be a priority. Rearing heifers represents an important future investment, as the cost of a heifer is the second largest after feeding costs in a dairy farm (Cady and Smith, 1996). It is interesting to perform an economical analysis of different situations that may take place in a dairy farm for heifer rearing. This will allow taking better decisions during the preweaning period. According to Tozer and Heinrichs (2001), the cost of growing a heifer includes mainly feed, labor, reproduction, health, and housing. As the source and amount of milk fed to calves affects calf growth (Diaz et al., 2001; Jaster and Weary, 2002; Quigley et al., 2006), health (Pollock et al., 1994; Nonnecke et al., 2003), reproductive performance (Bar-Peled et al., 1997; Davis-Rincker et al., 2006) and future milk yield (Bar-Peled et al, 1997; Shamay et al., 2005; Moallem et al., 2010; Soberon et al., 2012), there is an

economical impact on dairy production related to the calves feeding management of a farm. As presented in Chapters 3 and 4, any decision taken during the preweaning period may have consequences later during the life of the heifer. For example, calves under enhanced feeding programs may have a lesser age at first calving (Bar-Peled et al., 1997; Davis-Rincker et al., 2006), and a greater age at first calving increases total rearing cost (Tozer, 2000), as heifers have greater maintenance requirements (Kertz et al., 1998).

In order to measure the above-mentioned impact, it is necessary to identify the cost and income variations related to the source and amount of milk fed.

COST

- Price of each type of milk (€/kg DM): It is assumed that the associated costs for each type of milk: labor, water, etc., sum the same final value, thus only price of the milk source needs to be taken into account.
  - Milk replacer = $COST_{MR}$
  - Whole milk = $COST_{WM}$
- Sickness treatment (€/treated calf) = $COST_{SICKNESS}$
- Starter feed price (€/kg DM) = $COST_{STARTER}$

INCOME

- Milk price (€/kg DM) = $COST_{WM}$

In the present thesis data from calves in 5 different scenarios are reported. In all the cases calves are fed twice a day. The different scenarios are:

Study 1:

Feeding calves with milk replacer 6L/day and 12.5 % DM   **(a)**

 6L/day during 55 days

 3 L/day during 7 days

Feeding calves with whole milk 6L/day and an average DM of 12.5 %   **(b)**

 6L/day during 55 days

 3 L/day during 7 days

Study 2:

Feeding calves with milk replacer 4L/day and 15 % DM   **(c)**

      4L/day during 56 days

      2L/day during 6 days

Feeding calves with milk replacer 6L/day and 15 % DM   **(d)**

      4L/day during 6 days

      6L/day during 43 days

      4L/day during 7 days

      2L/day during 6 days

Feeding calves with milk replacer 8L/day and 15 % DM **(e**)

      4L/day during 6 days

      6L/day during 4 days

      8L/day during 35 days

      6 L/day during 4 days

      4L/day during 7 days

      2 L/day during 6 days

Parameters as housing and labor are assumed to be equal for the 5 situations presented here, and thus it will not be further studied. Also, no differences in calf mortality have been reported in the studies, thus it is also considered the same for all the cases. Some parameters, as starter intake (STi), medicaments, or expected future milk yield need to be associated to each group of calves. Starter intake (STi) from day 0 until day 63 is defined from now on: STa, STb, STc, STd, STe depending on the situation described above. Veterinary treatments are required for 37 % of calves if fed MR and 24 % of calves if fed WM (Study 1). Due to the big differences in intake data from both studies, and to better analyze the economical impact that type of liquid feeding source and amount may have, both studies will be analyzed separately.

6.1.5.1. Economical impact of feeding different liquid sources

Study 1 is used to evaluate which milk source is more interesting from an economical point of view. As there are not intake or growth performance significant differences, then there are only two parameters to compare: milk source and medicaments costs.

a) Comparable preweaning costs MR per calf =

$$43.875*COST_{MR} + 0.37*COST_{SICKNESS} + STa*COST_{STARTER} \quad [1]$$

b) Comparable preweaning costs WM per calf =

$$43.875*COST_{WM} + 0.24*COST_{SICKNESS} + STb*COST_{STARTER} \quad [2]$$

where 43.875 represents, in kg, the total amount of provided MR or WM in DM during the whole preweaning period. The values 0.37 and 0.24 represent the percentage of sickness events in the MR treatment and WM treatment respectively. Results from Study 1 show no differences on starter consumption. Then,

$$STa*COST_{STARTER} = STb*COST_{STARTER}.$$

When [1]/[2] = 1, there would be no special interest in either of the milk sources. That leads to the following relation

$$(COST_{WM}-COST_{MR}) / COST_{SICKNESS} = 0.00296$$

Therefore, values for $(COST_{WM}-COST_{MR}) / COST_{SICKNESS}$ above 0.00296 results in a higher profitability when using MR. Values below 0.00296 result in a higher profitability when using WM to feed the calves. As example, an estimation with current market prices shows that the use of MR is more profitable than WM.

$COST_{WM}$ = 2.56 €/kg DM

$COST_{MR}$ = 2.30 €/kg DM

$COST_{SICKNESS}$ = 15 €/treated calf

(2.56 – 2.30)/15 = 0.017 and then > 0.00296.

And therefore, in this case MR is more interested economically than WM. In addition, if there are no huge price variations in $COST_{MR}$, $COST_{WM}$ and $COST_{SICKNESS}$, then the usage of MR will always be more interesting economically than WM.

In summary, if happens that WM price is lower than MR price; it is more interesting the usage of WM. However, if WM is more expensive than MR, the better option depends on the costs of treatment for sick animals.

6.1.5.2. Economical impact of feeding different allowances

When analyzing Study 2, we try to find the most profitable amount of milk to feed during the preweaning period. In order to compare the three different management systems under Study 2, it has been developed a benefit index formula called comparison index (I). The highest result from the formula leads to the most interesting management system. The benefit index formula is built by the following parameters: MR intake (MRI), MR price, starter intake (ST), starter price, expected milk yield (Y) according to ADG and future whole milk price.

To calculate the values of the comparison index (I). It is required taking in account:

- the current market prices for whole milk, milk replacer and starter:

  $COST_{WM}$ = 2.56 €/kg DM

  $COST_{MR}$ = 2.30 €/kg DM

  $COST_{STARTER}$ = 0.26 €/kg

- The starter consumption during the whole preweaning period.

  STc = 25.2 kg

  STd = 14.49 kg

  STe = 11.34 kg

- The total MR consumption during the whole period (DM):

  $MRIc$ = 35. 4 kg

  $MRId$ = 48.3 kg

  $MRIe$ = 58.8 kg.

- The expected extra future milk yield (Y; extra kg in 1st lactation) that can be estimated in +1125 kg per extra 500 g in ADG during preweaning according to Bach (2012).

Future milk yield (Yc, Yd, Ye) needs to be multiplied by the price of the whole milk 24 months after the calf is born. Such value is of high uncertainty due to the high volatility in the dairy sector, uncertainty on market behavior due to the end of quotas system in March 2015, eventual changes in agricultural trade politics and eventual

sensationalism incidents or news within the next 24 months. Milk futures markets may be of interest to get data for such estimation, even when it is not possible to get a milk price value for a so far moment.

To calculate Y, it is necessary to know the ADG values for each group from day 0 to weaning:

ADGc=554 g/day

ADGd= 535 g/day

ADGe=599 g/day

Then, considering the group with the lowest ADG as the reference group, the extra future milk yield during first lactation (Y) are expected to be:

Yc = 42.75 kg

Yd = 0 kg

Ye = 144 kg

A polynomial relationship of second degree that relates total MR allowance (ALLOWANCE$_{MR}$) with Y during first lactation is obtained. Extra milk yield values are exclusively valid for a domain of R {4, 8} which covers the studied amounts interval from 4 to 8 L at 15 % DM.

$$Y = 23.344*(ALLOWANCE_{MR})^2 - 254.81*ALLOWANCE_{MR} + 688.5 \quad \textbf{[3]}$$

$R^2 = 1$ as we have a limited dataset of 3 values.

A 2nd degree polynomial relationship between MR allowance (ALLOWANCE$_{MR}$) and starter consumption (ST) can also be obtained:

$$ST = 0.945*(ALLOWANCE_{MR})^2 - 14.805*ALLOWANCE_{MR} + 69.3 \quad \textbf{[4]}$$

with a $R^2 = 1$ for a domain interval of R {4, 8}

Lastly, in order to automatize the formula in terms of ALLOWANCE$_{MR}$, a linear relationship between ALLOWANCE$_{MR}$ and MRI is obtained.

$$MRI = 5.85*ALLOWANCE_{MR} + 12.4 \text{ with a } R^2 = 0.997 \quad \textbf{[5]}$$

Now, it is possible to compare the three groups in economical terms with a comparison index (I):

$$I = [3]*v - [4]* COST_{STARTER} - [5]*COST_{MR} \qquad \textbf{[6]}$$

where "v" is the estimated net income whole milk price in 24 months. Prior settle Class III milk futures at CME (2015) group for February 2017 is 4 % higher than today's price (checked on March 29th 2015 at 19:00).

It leads to:

$$v = 1.04 * COST_{WM} - 1.44 \text{ €/kg DM} ,$$

where 1.44 € refers to the cost of producing 1 kg of DM milk. 1.44 € are split in 1.28 € of TMR plus 0.16 €/kg related to other variable costs (higher water intake, higher milking costs, etc.).

Hence, v = 1.22 €/kg DM for WM with 12.5 % of solids content.

When analyzing the formula [6] for I with the current market situation, the following results are found (Fig. 6.6.a):

      4L: Ic = -36.72

      6L: Id = -113

      8L: Ie = 36.62

It can be concluded that the increase on milk yield due to a higher amount of MR during the preweaning period plus the lowest starter intake are sufficient to overcome the increase on MR costs in the 8L treatment, but not in 6L treatment. By analyzing the results curve, a treatment of daily MR fed of 7.15L is as interesting as 4L treatment in financial terms when only considering milk yield in the first lactation. Treatments above daily MR of 7.15L are more interesting than 4L and 6L treatments. Being 8L the most interesting due to the extra income from higher milk yield in the first lactation. Furthermore, it must be taken into consideration that there are other possible benefits when increasing MR that have not been considered here because they were not statistically significant in our studies, as example: earlier conception date and higher milk yield in 2nd and later lactations when applicable. Nevertheless, according to Soberon and Van Amburgh (2013) due to standard deviation on milk yield in first

lactation, a sample size of around 128 heifers per treatment is necessary to obtained reliable data to estimate future milk yield.

In addition, there is a big drop of ADG for 6L and 8L groups due to the manner in which weaning has been carried out. Thus, it may be also important to compare those groups taking into account ADG values at day 35, to evaluate what would have happened if the advantage of BW gain during the first months had been maintained.

ADG35c = 484 g/day

ADG35d = 564 g/day

ADG35e = 752 g/day

Following the same approach, the new values for extra milk yield in first lactation (Y') due to a greater ADG are

Y'c= 0 kg

Y'd= 180 kg

Y'e = 603 kg

It results in a well-defined linear relationship between $ALLOWANCE_{MR}$ and Y'

$$Y' = 150.75*ALLOWANCE_{MR} - 643.5 \quad [7]$$

with $R^2 = 0.95$.

At this point, it is possible to get a new comparison index(I'):

$$I' = [7]*v - [4]*COST_{STARTER} - [5]*COST_{MR} \quad [8]$$

The new index is presented in Figure 6.6.b. As it can be observed in Figure 6.6.b, a new situation appears when ADG at day 35 were taken into account. That leads to the hypothesis that if BW gain would have been maintained until weaning, the increase on milk yield due to a higher amount of MR during the preweaning period would have been sufficient to overcome the increase on MR costs to any treatment above 4L up to 8L. It has to be kept in mind that, the new comparison index (I') is slightly overestimated for calves fed 6 and 8 L of MR, as if BW were maintained, a greater starter intake should be expected.
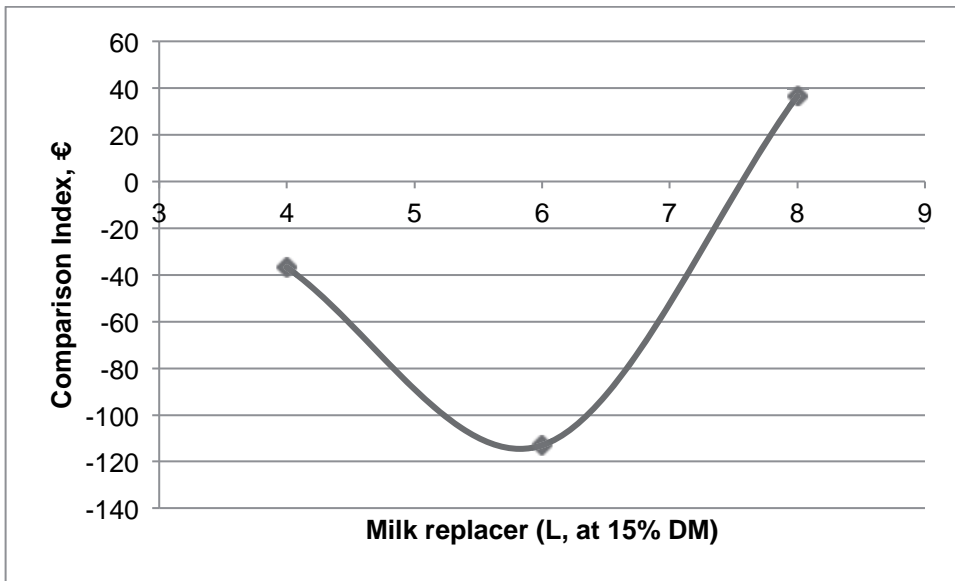
Figure 6.6.a. Comparison index I (€) for different milk allowances (L) taking into account ADG until day 63.
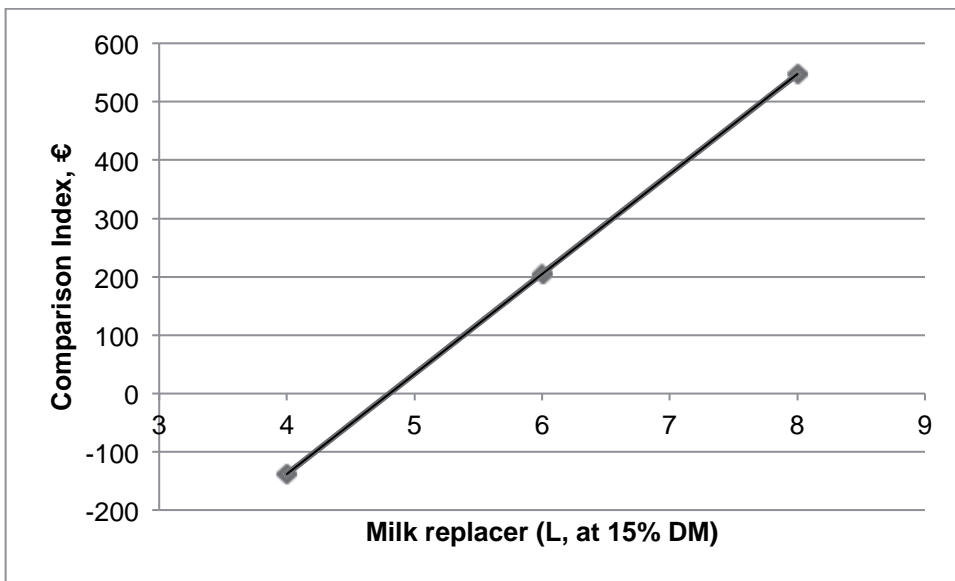
Figure 6.6.b. Comparison index I' (€) for different milk allowances (L) taking into account ADG until day 35.

6.2. NOURISHING AND MANAGING THE DAM

As coincidence with lactation and pregnancy often occurs in dairy cattle, and thus methods to mitigate the competition for available nutrients should be studied in order to ameliorate lactation yield and fetal development. The Arg Study tries to elucidate whether fetal nutrient supply can be ameliorated through a micronutrient supplementation via maternal nutrition. As mentioned above, pregnancy requirements are only taken into account in current nutritional models at the end of the pregnancy, however, during the beginning of the pregnancy, pregnant heifers have to have sufficient nutrients to achieve growth and pregnancy requirements. A possible way to solve this dilemma would consist of giving dietary nutrients that target specific processes (Reynolds and Caton, 2012), such as, for example, AA.

In the Study 3 of the present thesis, Arg was supplemented to pregnant heifers at the beginning of the pregnancy because it is nowadays well known that it is a common subtract of NO (Flynn et al., 2002). As already reported in Study 3, contrary to our hypothesis, Arg supplementation did not lead to an increase of uterine artery FV. The possible causes of this have already been discussed in Chapter 5. As a summary of those causes: first, small errors in diameter determination with the Doppler can lead to errors of -75 % and +125 % in the calculation of FV (Herzog and Bollwein, 2007). However, the amount of Arg infused also failed to increase blood NO, and thus, a second cause could be that the amount of Arg was not sufficient to increase NO and none of the successive reactions (vasodilatation, hormones secretion, etc.) occurred.

Nevertheless, according to our knowledge, no studies where Arg was supplemented via peritoneum have ever been performed in cattle. NRC (2001) recommends 48 g of metabolizable Arg/day. In Study 3, 40 mg of Arg/kg BW were supplemented, as it is a 40 % over the regular Arg of a heifer intake. That dose is lesser than the one used in other reports but, as demonstrated in Study 3 (Experiment 2), supplementation via peritoneum and jugular lead to complete different metabolic responses. It would be interesting to perform further studies regarding NO secretion after Arg peritoneal infusion, in order to elucidate which supplementation rate could be effective in the vasodilation of the uterine artery.

Apart from the growing challenge, during their pregnancy, heifers also have to face mammary gland development. In Study 3, milk yields from the studied heifers were

recorded. As only data from 15 animals were available, differences in milk yield were not significant (P = 0.64). However, heifers receiving Arg supplementation produced 293 kg more milk than control heifers in a 305-day lactation. However, due to the poor sample size a conclusion cannot be made. However, it seems that Arg supplementation could have ameliorated mammary gland development. According to our knowledge, no study has been performed in dairy heifers evaluating mammary gland development with Arg supplementation. Nevertheless, it can be hypothesized that a major blood flow caused by Arg supplementation during mammary gland development could have ameliorated the efficiency of nutrient transport to mammary gland (Reynolds and Redmer, 1995) and then, ameliorate future milk synthesis of the supplemented heifers. It would be interesting to further study the action of Arg supplementation on mammary gland development.

In parallel, as reported in Chapter 1, other micronutrients could also have been supplemented to prevent pregnancy deficiencies that may negatively imprint the fetus. In particular, an increased interest in methionine, folic acid and/or vitamin B_{12} has lately raised. Lactation increases demand for methylated compounds (Girard et al., 2005), and as reported in the introduction, an increase in milk yield has been observed when cows are supplemented with the folic acid and/or vitamin B_{12} (Girard et al., 2005; Graulet el al., 2007; Preynat et al., 2009). A lack of folic acid and/or vitamin B_{12} during pregnancy resulted in metabolic changes (Sinclair et al. 2007). Thus, the amount of those micronutrients may alter fetal development and gene expression of the offspring. Studies in such direction have still not been conducted in dairy cows, however, supplementation with methionine, folic acid and/or vitamin B_{12} could lead to interesting results.

Besides nutrition, management practices could also be studied. For example, the potential benefits from expanding or shortening calving interval (CI) could be investigated. Due to the highest milk yield at the beginning of the lactation, a shorter CI seems to be economically optimal (Dijkhuizen et al., 1985; Sørensen and Østergaard, 2003; Groenendaal et al., 2004; Steeneveld and Hogeveen, 2012). However, the economic effects observed were not very important, and varied between 0 and 2 euros per extra day open (Groenendaal et al., 2004, Inchaisri et al., 2011). However, none of these studies take into consideration a possible detrimental effect about the

coincidence of fetal development and lactation. González-Recio et al. (2012) found that females born from mothers that were in their first lactation while pregnant produced 52 kg less milk, lived 16 day less and were less metabolic efficient than females born to heifers. Thus, as the economic effect observed when CI was prolonged were not very high (Inchaisri et al., 2011), it may be beneficial to delay breeding for some days during the of the lactating cows. No economical studies that take into account all these factors have been found, and therefore, it is difficult to determine under which circumstances each management practice should be selected.

Chapter 7

CONCLUSIONS

The results obtained in this thesis allow us to conclude that:

1. Feeding WM to dairy calves does not result in differences in intake, BW, ADG or GtoF compared with calves fed MR.

2. Feeding calves following an enhanced-growth feeding program (3 or 4 L of MR twice daily) improves ADG and allows to obtain greater BW before weaning.

3. Calves that under an enhanced-growth feeding program are not able to compensate the lack of nutrient supply via MR during the weaning process experience an important decrease in ADG postweaning.

4. Feeding WM does not improve immune response measured in blood parameters (antiovalbumin and LPS challenge) but calves fed WM tend to need less medication than calves receiving MR.

5. Age at first breeding is greater in calves fed 4 L/day of MR than in calves following an enhanced-growth feeding program but it is not influenced by liquid source (WM or MR).

6. Glucose concentration does not differ in calves fed WM or MR, but calves fed MR have greater insulin levels.

7. Calves fed under an enhanced-growth feeding program have increased glucose and insulin levels and an impaired IS at day 42 of life, but those differences disappear with age.

8. A daily peritoneal infusion of 40 mg/kg BW of Arg from day 40 until day 140 of pregnancy does not increase blood flow to the uterus or affects plasma PRL, IGF, glucose, and GH concentrations, but increases plasma P4 concentrations.

9. A daily peritoneal infusion of 40 mg/kg BW of Arg from day 40 until day 140 of pregnancy causes the decrease of plasma EAA that can be due to an increased fetal protein accretion.

Chapter 8

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