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Effects of different feeding regimes applied during rearing of dairy calves: circulating adiponectin and insulin sensitivity in early life and around the first lactation

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Julia Kesser

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Referent: Prof. Dr. Dr. Helga Sauerwein

Korreferent: Prof. Dr. Karl-Heinz Südekum

Fachnahes Mitglied: Prof. Dr. Karl Schellander

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English abstract

Metabolic programming is defined as an early stimulus with long term effect. In dairy cows, feeding in the preweaning period has long term effects on later milk production. However, the influence on metabolic and endocrine changes is not fully clarified. The aim of this thesis was to investigate the influence of different feeding regimen on performance data and metabolic and endocrine variables in dairy calves and around the first lactation. Special attention was directed to insulin sensitivity and adiponectin, an adipokine with insulin sensitizing properties. However, its concentration and changes with age were unknown in calves. To characterize the effects of colostrum feeding and of different feeding intensities before weaning, samples from different trials were used; In Manuscript I, twenty dairy calves fed with colostrum and then restrictively with milk replacer (MR; 130 g/L, 6 L/d) for 110 d post natum (p.n.) were studied. In addition, calves receiving either colostrum (n = 7) or formula (n = 7) until d 4 p.n., and calves born either at term (n = 7) or preterm (n = 7) receiving their first colostrum only at 24 h p.n. were included. Blood and milk samples were taken and adiponectin was measured by an in-house developed ELISA and by Western blot to assess the different molecular weight forms. In Manuscripts II, the influence of different feeding regimen on performance including milk yield in the first lactation was investigated; in Manuscript III, the animal trial from manuscript II was extended to assess metabolic and endocrine variables. The experiment comprised dairy calves fed at 3 different regimen until d 25 p.n. (MR restricted, n = 20; MR ad libitum (ad lib), n = 17; whole milk (WM) ad lib, n = 20) and observed thereafter until d 110 p.n. The female calves (n = 28) were further pursued in their first pregnancy from 3 months ante partum until 10 weeks post partum. Blood samples were taken in regular intervals. During calfhood, glucose tolerance tests and insulin tolerance tests (only male calves) were done and liver biopsies were taken. Performance data like body weight, average daily gain, food and energy intake, milk yield were recorded and economic outcomes were calculated. The postpartal increase in adiponectin serum concentrations depended on colostrum intake suggesting a transfer of colostral adiponectin to the calves' circulation. The different feeding regimen yielded differences in the metabolic and endocrine variables as well as the performance data but these were not sustained at later age. Around the first lactation the metabolic and endocrine variables were not different, albeit the heifers from in the WM-ad lib group had numerically greater milk yields thus compensating the higher costs during the rearing period. The present thesis provides information about the circulating adiponectin concentrations in dairy calves and about the effects of different feeding regimen on performance and endocrine and metabolic variables in blood.

German abstract

„Metabolische Programmierung" meint den Einfluss eines Reizes in der frühen Entwicklung und dessen langfristigen Wirkung auf den Stoffwechsel eines Individuums. Bei Milchkühen konnte nach einer intensiven Aufzucht in den ersten Lebenswochen eine gesteigerte Milchleistung beobachtet werden. Die Folgen auf den endokrinen und metabolischen Status sind jedoch nicht eindeutig geklärt. Ziel dieser Arbeit war es, den Einfluss unterschiedlicher Fütterungsmethoden in der Aufzucht von Kälbern auf den endokrinen und metabolischen Status in der ersten Laktation zu untersuchen. Ein besonderes Augenmerk wurde dabei auf die Insulinsensitivität und das Adipokin Adiponektin gelegt, dessen Blutkonzentration und zeitlicher Verlauf bislang in Kälbern nicht bekannt war. Um die Effekte von Kolostrumfütterung und verschiedener Fütterungsintensitäten zu untersuchen, wurden Proben bzw. Daten aus 3 verschieden Versuchen verwendet. Manuskript I beinhaltet die Ergebnisse von 20 Kälbern, die mit Kolostrum und danach mit Milchaustauscher (MR, 130 g/L, 6 L/Tag) bis zum Alter von 110 Tagen verfolgt wurden. Zudem gingen die Daten von Kälbern, die entweder Kolostrum (n = 7) oder MR (n = 7) erhielten und nur bis zum 4. Lebenstag beobachtet wurden, sowie von Kälbern, die entweder natürlicherweise geboren wurden (n = 7), oder vorzeitig per Kaiserschnitt auf die Welt kamen (n = 7), und nach 24 h zum ersten Mal gefüttert wurden, in das Manuskript ein. Es wurden jeweils in regelmäßigen Abständen Blut- und Milchproben genommen und Adiponektin mittels einem, im Institut entwickelten ELISA, sowie zur Charakterisierung der verschiedenen Molekulargewichtsformen im Western Blot nachgewiesen. In den Manuskripten II und III wurde der Einfluss verschiedener Fütterungsintensitäten auf die Wachstumsleistung, aber auch auf metabolische und endokrine Parameter bis hin zur ersten Abkalbung herum zu untersuchen. Dazu wurden Holstein-Kälber in den ersten 25 Lebenstagen aus 3 verschiedenen Fütterungskonzepten (MR restriktiv (n = 20; 130 g /L, 6 L/Tag); MR ad libitum (ad lib; n = 17, 160 g/L) und Vollmilch ad lib (n = 20)) bis zu ihrem 110. Lebenstag beobachtet. Die weiblichen Tiere aus diesem Versuch wurden weiter bis um ihre erste Abkalbung herum verfolgt. Während der Aufzuchtphase wurden zudem Glucose- und Insulin-Toleranztests durchgeführt sowie Leberbiopsien gewonnen und Daten zum Körpergewicht, Gewichtszunahme, Futter- und Energieaufnahme erhoben sowie später die Milchleistung notiert und auch die betriebswirtschaftlichen Ergebnisse berechnet.

Der postnatale Anstieg der Adiponektinblutkonzentration bei neugeborenen Kälbern erwies sich als abhängig von der Kolostrumaufnahme, was auf einen Transfer von kolostralem Adiponectin in den Blutkreislauf des Kalbes weist. Die getesteten, unterschiedlichen Fütterungsintensitäten ergaben lediglich während der differenzierten Fütterung Unterschiede zwischen den Gruppen in Hinblick auf die metabolischen und endokrinen Parameter. Auch um die erste Abkalbung herum gab es keine Unterschiede in den Blutparametern, obwohl Färsen, die als Kälber Vollmilch ad lib bekommen hatten, numerisch mehr Milch gaben. Die höheren Aufzuchtkosten mit Vollmilch konnten so kompensiert werden.

In der vorliegenden Arbeit wird die Adiponektinblutkonzentration bei Kälbern unter verschiedenen Bedingungen charakterisiert und der Einfluss verschiedener Fütterungsintensitäten auf die Wachstums- und spätere Laktationsleistung dokumentiert.

a.p.	ante partum
aa	amino acids
Acrp30	adipocyte complement-related protein of 30kDa
ADF$_{OM}$	acid detergent fiber
ADG	average daily weight gain
AdipoR1	adiponectin receptor 1
AdipoR2	adiponectin receptor 2
AF	allantoic fluid
AFC	age at first calving
ad lib	ad libitum
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
aNDF$_{OM}$	neutral detergent fiber
apM1	adipose most abundant gene transcript 1
APPL1	adapter protein containing phosphotyrosine binding domain & leucine zipper motif
AT	adipose tissue
AUC	area under the curve
BAT	brown adipose tissue
BHB	beta-hydroxybutyrat
BW	body weight
CA	crude ash
cDNA	complementary deoxyribonucleic acid
CF	crude fiber
CL	crude fat
COL	colostrum
CP	crude protein
CV	coefficient of variation
DIM	days in milk
DM	dry matter
DMI	dry matter intake
ECM	energy corrected milk

List of abbreviations

$ED_{50,1}$	insulin concentration to elicit a half-maximal effect
$ED_{50,2}$	increased insulin concentration to elicit half of the maximal effect
EDTA	ethylenediaminetetraacetic acid
EIF3K	eukaryotic translation initiation factor 3, subunit K
ELISA	enzyme-linked immunosorbent assay
FA	fatty acids
FFA	free fatty acids
FM	fresh matter
FOR	formula
GBP28	gelatin binding protein of 28kDa
GfE	German Society of Nutrition Physiology
GLUT	glucose transporter protein
GTT	intravenous glucose tolerance test
HMW	high molecular weight
HOMO-IR	homeostasis model assessment insulin resistance
HPCAL1	hippocalcin-like protein 1
IgG	immunoglobuline G
IOFC	income over feed costs
ISBGR	insulin stimulated blood glucose response
ITT	intravenous insulin tolerance test
kDa	kilo Dalton
LMW	low molecular weight
LOD	limit of detection
LRP10	low density lipoprotein receptor-related protein 10
MMW	middle molecular weight
MR	milk replacer
MW	molecular weight
NDF	neutral detergent fiber
NEFA	non-esterified fatty acids
NEL	netto energy lactation
P	phases
p.n.	post natum

p.p.	post partum
p38-MAPK	mitogen-activated protein kinase
PBS	phosphate-buffered saline
PC	pyruvate carboxylase
PEPCK-C	phosphoenolpyruvate carboxykinase, cytosolic
PPARα	peroxisome proliferator activated receptor α
PT	preterm
qPCR	quantitative polymerase chain reaction
QUICKI	quantitative insulin sensitivity check index
$\text{QUICKI}_{\text{Glycerol}}$	quantitative insulin sensitivity check index including glycerol
r	restrictive
R_{max1}	maximal biological effect
R_{max2}	decreased biological effect
RQUICKI	revised quantitative insulin sensitivity check index
$\text{RQUICKI}_{\text{BHB}}$	revised quantitative insulin sensitivity check index including beta-hydroxybutyrate
SCC	somatic cell count
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGLT	sodium-dependent glucose transporter
T	term
TBST	tris buffered saline containing 0.05 % tween 20
TMR	total mixed ration
TPP	total plasma protein
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten
VFA	volatile fatty acids
WAT	white adipose tissue
WM	whole milk

Meinen Eltern

Table of contents

1   Introduction:

In the last four decades, milk production per cow doubled in many countries (Oltenacu and Algers, 2005; Oltenacu and Broom, 2010). Improved feeding regimen and herd management, as well as improvements in the genetics of dairy cows strongly contributed to this increase (Oltenacu and Broom, 2010). However, negative side effects like metabolic problems, lameness, mastitis or reduced fertility and reduced production lifespan may occur (Oltenacu and Broom, 2010). Studies have shown that nutrition in early life can support the performance and health of adult individuals (Lucas, 1991). In dairy cows, nutrition in the first weeks of rearing may influence milk production in later life (Bach, 2012). For understanding the influence of nutritional stimuli in dairy calves on their later milk production sound knowledge about the physiology of dairy calves and cows and the underlying mechanisms of metabolic programming in the relevant target tissues and at the systemic level is required.

1.1   Dairy calves: changing from pseudo-monogastrics to ruminants

The physiology of digestion of newborn ruminants differs from adult ruminants. After birth the rumen of dairy calves is not yet fully developed (Warner et al., 1956). The reticulum, omasum and rumen are inactive and rudimentary (Heinrichs, 2005). Colostrum and milk (or milk replacer) are directly passed over the rumen via the esophageal groove into the abomasum (Fig. 1). In the first weeks of life the digestion of newborn ruminants is similar to non-ruminant monogastric animals (Leat, 1971). Therefore neonate dairy calves are referred to as pseudo-monogastrics or pre-ruminants. Carbohydrates are digested in the small intestine. The main energy source of non-ruminant dairy calves in their first weeks of life is milk or milk replacer. Only with the gradual increase of the intake of solid feed, which reaches the rumen directly, the rumen starts to develop physically and metabolically and a microbial population starts to develop in the rumen. The degradation of carbohydrates into volatile fatty acids, i.e. acetate, propionate, and butyrate, also supports the development of the rumen (Heinrichs, 2005). Additionally, the size of the rumen increases and there is an expansion of the papillae with growing age. In contrast, the size of the abomasum decreases (Warner et al., 1956). Depending on the development of the rumen and therefore on the quality and quantity of the solids eaten, calves can be weaned after 4 to 11 weeks post natum [p.n. (Anderson et al., 1987)].
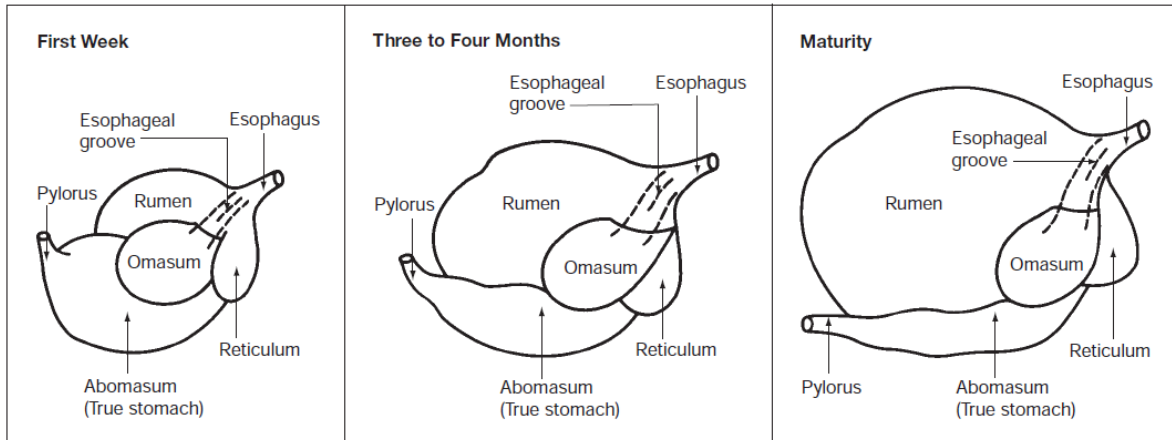
Figure 1: The development of the digestion system of the calf from the first week until maturity (modified from Heinrichs and Jones, 2003).

1.2 The physiology of dairy cows during the transition period

Throughout lactation dairy cows experience continued changes of energy supply and energy demand through changes in development of the offspring, milk production and feed intake. The difference between energy supply and energy demand is referred to as energy balance. In the transition period, defined as 3 weeks ante partum (a.p.) until 3 weeks post partum (p.p.), metabolic and endocrine changes occur to support the nutrient supply to the offspring including lactogenesis and galactopoiesis in the dam (Grummer, 1995), the energy requirements increase. Limitations concerning nutrition in the transition period may have long time effects on milk yield (Drackley, 1999). Furthermore, during this critical time dairy cows are prone to develop metabolic and infectious diseases, like ketosis, displaced abomasum or mastitis (Bell, 1995; Drackley, 1999). In addition to the increased energy demand, the feed intake of the dairy cow decreases due to the metabolic and endocrine changes around parturition (Allen et al., 2005). The energy balance turns into negative.

The most important nutrient for the developing mammary gland and the offspring is glucose (Bell and Baumann, 1997). Therefore ensuring an appropriate glucose supply for both, the offspring and the mammary gland, is a metabolic priority for the dam (Bell and Baumann, 1997). In consequence, gluconeogenesis in the liver is increased whereas glucose uptake in the muscle and adipose tissue is reduced due to a decrease in insulin sensitivity (Bell and Baumann, 1997). To

adapt to the increased energy demand, the dairy cow starts to mobilize body reserves, mainly from adipose tissue (Drackley, 1999).

1.3   Adipose tissue and its role in the dairy cow

Adipose tissue (AT) is a type of loose connective tissue composed of adipocytes (lipid filled cells) which are surrounded by a matrix of collagen fibers, blood vessels, fibroblasts and immune cells (Ashima and Flier, 2000). The number of adipocytes is determined in childhood and remains constant during adulthood (Spalding et al., 2008). The AT can be subdivided into brown AT (BAT) and white AT (WAT). The BAT plays an important role in the production of heat, especially in newborns. Brown adipocytes contain many small lipid droplets and a high amount of mitochondria (Saely et al., 2012). The amount of BAT decreases with age, whereas the size of WAT increases. Moreover, adipocytes of WAT have only one lipid droplet and less mitochondria. Beside its ability to store and to mobilize triglycerides, AT (BAT and WAT) metabolism is regulated trough endocrine, paracrine and autocrine signals (Mohamed-Ali et al., 1998). Adipokines, e.g. adiponectin, leptin, resistin, visfatin and apelin represent signal molecules of AT by which the AT is able to communicate with other organs such as brain, skeletal muscle, liver or gastrointestinal tract. Adipokines influence, among other things, glucose and lipid metabolism, insulin sensitivity and secretion, endothelial functions and blood pressure (Blüher, 2012).

In dairy cows the process of fat mobilization (lipolysis) occurs mainly during the transition period. Triglycerides which are stored in the AT are hydrolyzed into glycerol and free fatty acids (FA) and are released into the circulation as non-esterified FA (NEFA, McNamara, 1991). During late lactation and the beginning of the dry period when energy intake is increased and milk production decreases, the triglyceride storages in AT are refilled. This process is referred to as lipogenesis (McNamara, 1991).

1.3.1   Adiponectin

Adiponectin is a hormone that is produced by brown and white adipocytes and has important roles in regulating glucose and lipid metabolism (Berg et al., 2002; Waki et al., 2003; Iacobellis et al., 2013). It was discovered two decades ago by four research groups almost simultaneously

4

and therefore different names were used for it at that time: Acrp30 [adipocyte complement-related protein of 30 kDa (Scherer et al., 1995)], apM1 [adipose most abundant gene transcript 1 (Maeda et al., 1996)], adipoQ (Hu et al., 1996), and GBP28 [gelatin binding protein of 28 kDa (Nakano et al., 1996)].

The 30 kDa adiponectin monomer consists of 247 amino acids and is structured by a N-terminal sequence (17 amino acids (aa)), a variable species-specific domain (28 aa), a collagen-like domain (65 aa), and a C-terminal globular domain (137 aa) (Fig. 2, Scherer et al., 1995; Berg et al., 2002; Waki et al., 2003).

Figure 2: Structural domains of adiponectin (modified from Scherer et al., 1995; Waki et al., 2003).

In blood, several molecular weight (MW) forms of the full length adiponectin can be found: the low MW form (LMW), the middle MW form (MMW) and the high MW form (HMW) (Fig. 3, Berg et al., 2002; Waki et al., 2003). In addition, there is a form of adiponectin that is limited to the globular head, and that is biologically active (Fruebis et al., 2001).

Globular head form | Monomer | Low molecular weight form LMW, Trimer | Middle molecular weight form MMW, Hexamer | High molecular weight form HMW

~ 16 kDa   ~30 kDa   ~ 90 kDa   ~ 180 kDa   S-S   S-S   >300 kDa
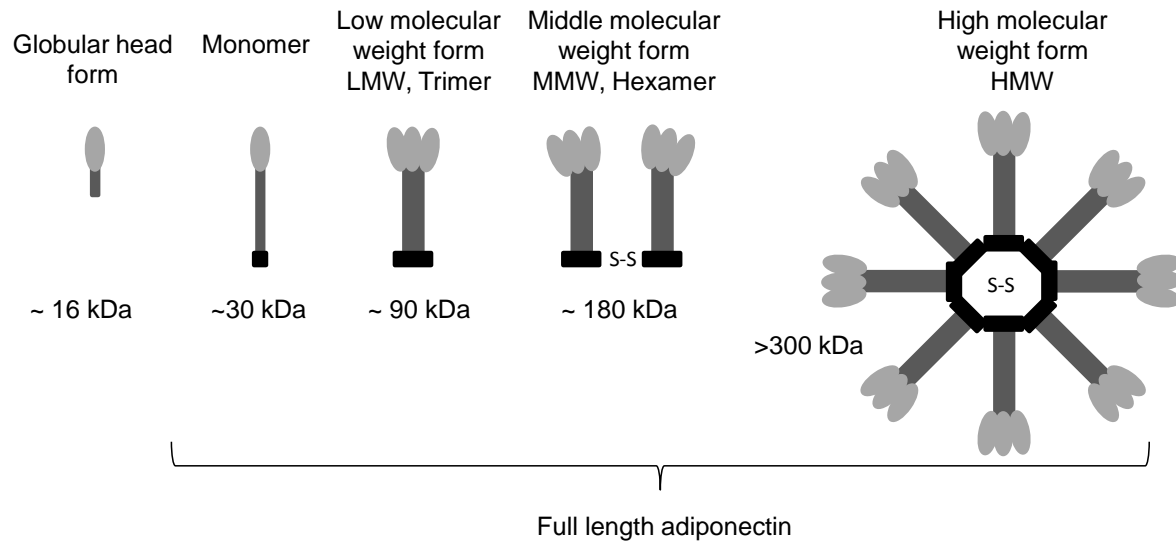
Full length adiponectin

Figure 3: Different molecular weight forms of adiponectin (modified from Fruebis et al., 2001 and Berg et al., 2002).

1.3.2 Adiponectin and its receptors

Several receptors and transcription factors mediate the adiponectin signal (Fig. 4). Adiponectin acts through its two receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) (Yamauchi et al., 2003). AdipoR1 can be found in skeletal muscle and liver whereas AdipoR2 predominantly acts in hepatocytes (Yamauchi et al., 2003). AdipoR1 binds to all molecular forms of adiponectin, whereas AdipoR2 has a low affinity to the globular form of adiponectin and prefers the full length form of the protein.
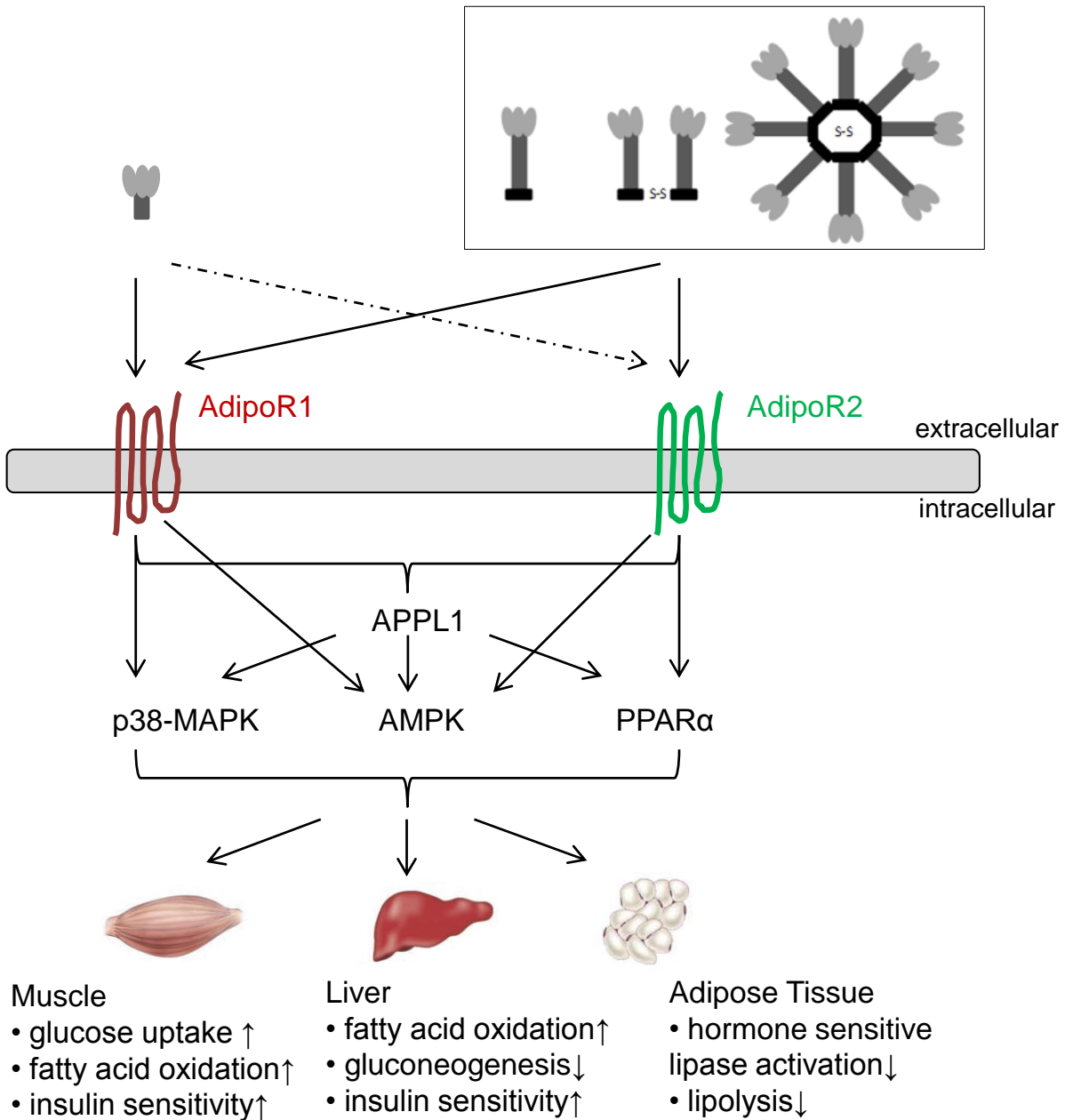
Figure 4: Signaling pathway of adiponectin and its receptors adiponectin receptor 1 and 2 (AdipoR1 and AdipoR2). Adiponectin activates the signaling molecules AMPK (adenosine monophosphate-activated protein kinase), PPARα (peroxisome pro-liferator activated receptor α), and p38-MAPK (mitogen-activated protein kinase). The protein APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif) acts as a link between the adiponectin receptors and the signaling molecules. In muscles, the glucose uptake, fatty acid oxidation and insulin sensitivity are stimulated by adiponectin. In liver, gluconeogenesis is decreased and the fatty acid oxidation as well as insulin sensitivity is increased. In adipose tissue the hormone sensitive lipase activation and lipolysis are inhibited (modified from Chandran et al., 2003 and Kadowaki et al., 2006).

1.3.3   Adiponectin and insulin sensitivity

Adiponectin is known for its insulin sensitizing effects (Fruebis et al., 2001; Yamauchi et al., 2001; Berg et al., 2002). In the liver, insulin sensitivity is increased while adiponectin decreases gluconeogenesis and the NEFA influx but increases the NEFA oxidation (Yamauchi et al., 2002). The hepatic glucose output and triglyceride synthesis are reduced. In muscle cells, adiponectin increases glucose uptake, FA oxidation and lactate production but decreases glycogen synthesis (Wu et al., 2003; Yamauchi et al., 2003; Ceddia et al., 2005). Especially the globular form of adiponectin increases the glucose uptake by stimulating the glucose transporter protein 4 (GLUT 4) translocation in muscle cells (Ceddia et al., 2005). Lipolysis in adipocytes is inhibited by adiponectin (Qiao et al., 2011; Wedellová et al., 2011). In contrast to most proteins which are produced by AT, adiponectin is negatively correlated with obesity (Hu et al., 1996; Arita et al., 1999). Furthermore, adiponectin is decreased in patients with diseases associated with insulin resistance like cardiovascular disease or hypertension (Hotta et al., 2000; Ouchi et al., 2003; Trujillo and Scherer, 2005).

Bovine adiponectin was first characterized by Sato et al. (2001). The amino acid sequence of the bovine adiponectin has 92 % identity with murine adiponectin and 82 % identity with human adiponectin. The MW forms of the murine adiponectin are similar to the bovine adiponectin MW forms (Sato et al., 2001).

The mRNA abundance of AdiopR1 and AdipoR2 is decreased after parturition compared to late gestation in cattle (Lemor et al., 2009). The adiponectin concentration in serum decreases before parturition with a nadir around calving and an increase thereafter (Giesy et al., 2012; Mielenz et al., 2013; Singh et al., 2014a). The decrease of the adiponectin concentration is related to the already known status of insulin resistance around calving and was therefore expected (Giesy et al., 2012; Mielenz et al., 2013; Singh et al., 2014a). Hence, adiponectin might support the increased supply of glucose to the fetus in late gestation and to the mammary gland in early lactation, respectively.

In contrast to humans, the adiponectin concentrations and their metabolic effects are not well studied in neonate and young ruminants. In the human fetus, adiponectin is detectable from week 24 of gestation (Kajantie et al., 2004). At birth the concentration is higher in neonates than in older children and adults, but a decrease of adiponectin occurs within 2 years of age (Iñiguez et

al., 2004; Kamoda et al., 2004; Kotani et al., 2004). Adiponectin serum concentrations are positively correlated with birth weight (Kamoda et al., 2004; Kotani et al., 2004), whereas in adolescents a negative correlation was observed between adiponectin concentrations and body weight (Arita et al., 1999; Cnop et al., 2003). Concerning sex differences contradictory results were reported in neonates (Arita et al., 1999; Kamoda et al., 2004; Erhardt et al., 2014).

Adiponectin is found in human milk in a range of $4 - 30.4$ µg/L (Bronský et al., 2006; Martin et al., 2006). The concentration is influenced by maternal factors, like obesity, duration of lactation or ethnicity (Martin et al., 2006). Several studies have shown the positive effects of breast milk in contrast to milk replacer with less obesity and improved metabolic health (Gartner et al., 2005; Owen et al., 2005, 2006). Adipokines in milk, especially adiponectin might be one reason for the positive influence of breast milk (Bronský et al., 2006; Martin et al., 2006), since AdipoR1 was found in the small intestine of neonatal mice and humans (Zhou et al., 2005; Bronský et al., 2012). In neonatal pigs a positive influence of leptin (another adipokine) in colostrum and milk was observed on the development of the small intestine structure and function (Woliński et al., 2003) and therefore might influence gut development and nutritional programming (Bronský et al., 2012).

In cows' milk the adiponectin concentrations are far above the concentrations measured in human milk. Singh et al. (2014a) reported milk adiponectin concentrations of 600 ± 30 µg/L. However, like in humans, a decrease during lactation was observed. Adiponectin and the adiponectin receptors AdipoR1 and AdipoR2 were detected in the bovine mammary gland (Ohtani et al., 2011; Saremi et al., 2014; Lecchi et al., 2015), indicating a functional role for adiponectin in metabolism and immunity of the mammary gland.

1.4   Gluconeogenesis in dairy cows

An important fuel for the energy supply in mammals is glucose. Several cells and tissues depend on regular glucose supply. Therefore, a steady level of glucose is a central element of homeostasis. Glucose can be stored in cells as glycogen, but can also be produced by gluconeogenesis mainly in the liver. In dairy cows gluconeogenesis is an important mechanism to supply sufficient glucose for the organism, especially in times of high glucose demand (Aschenbach et al., 2010). In humans and non-ruminants the glucose supply is ensured due to the direct intestinal absorption. Dairy cows only absorb little amounts of glucose in the small intestine (Aschenbach et al., 2010). The required glucose level in circulation is ensured by hepatic and renal gluconeogenesis. In dairy cows the main substrate for gluconeogenesis is propionate followed by lactate, glycerol, and glucoplastic amino acids (Seal and Reynolds, 1993). There are two different transporter types to support the cellular uptake of glucose: the sodium-dependent glucose transporters (SGLT) which are mainly found in the small intestine and the kidneys, and the glucose transporter proteins (GLUT) (Zhao and Keating, 2007). There are several GLUT, of which not all require insulin (e.g. GLUT 1, which is mainly located on brain cells, erythrocytes, kidney cells, and mammary gland, but also ensures the basal glucose demand of all cells (Mueckler et al., 1985)). On adipose cells and muscle cells the GLUT 4 is the dominant one, a glucose transporter which requires insulin (Fukumoto et al., 1989). The GLUT 4 ensures a higher uptake of glucose into the adipocytes and muscle cells during times of high nutrient intake. In times of low glucose levels in the blood stream, and therefore low insulin levels, glucose uptake is not stimulated (Wilcox, 2005).

In dairy calves, gluconeogenesis increases with age and therefore with the increase of propionate due to the degradation of carbohydrates in the developing rumen (Leat, 1971).

1.5   Insulin and insulin resistance in calves and dairy cows

One of the main hormones regulating the cellular uptake of glucose is insulin. Insulin is secreted by the β-cells (or B cells) in the Langerhans' islets of the pancreas to maintain a constant level of glucose in the circulation. Besides, insulin stimulates glycogenesis, protein synthesis and lipogenesis and also exerts mitogenic and antiapoptotic effects (Fig. 5; Dimitriadis et al., 2011). The synthesis and secretion of this hormone can be stimulated by nutrients like glucose (Wilcox, 2005).

Insulin

Muscle:
- glucose uptake ↑
- glycolysis ↑
- glycogen synthesis ↑
- protein synthesis ↑

Liver:
- glycogenolysis ↓
- gluconeogenesis ↓

Adipose Tissue:
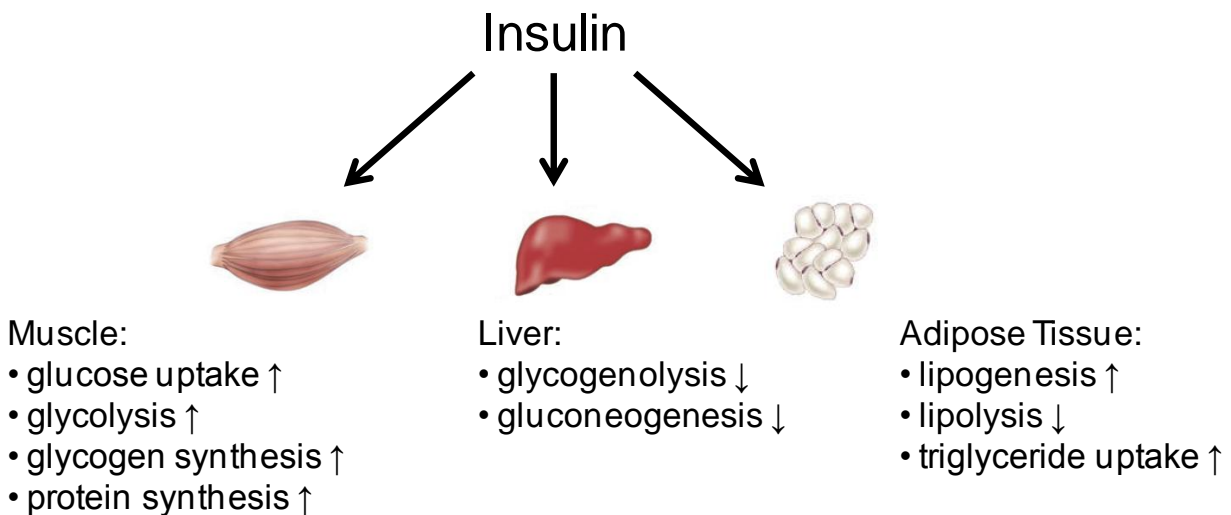- lipogenesis ↑
- lipolysis ↓
- triglyceride uptake ↑

Figure 5: Major effects of insulin on metabolism (modified from Dimitriadis et al., 2011).

Insulin binds to insulin receptors on the cell membrane and thereby stimulates the glucose transporters for the uptake of glucose into the cell. A reduced biological response (e.g. reduced uptake of glucose into the cells) to normal insulin concentrations is defined as insulin resistance (Kahn, 1978). Insulin resistance can be caused either by decreased insulin sensitivity or a decreased insulin response or both (Kahn, 1978). Insulin sensitive tissues need more insulin to achieve half of the maximal biological response (Fig. 6). In contrast reduced insulin responsiveness means that the biological response of the tissue is dampened even though the amount of insulin for reaching the half maximal response equals to the normal insulin response (Fig. 6) (Kahn, 1987; De Koster and Opsomer, 2013).
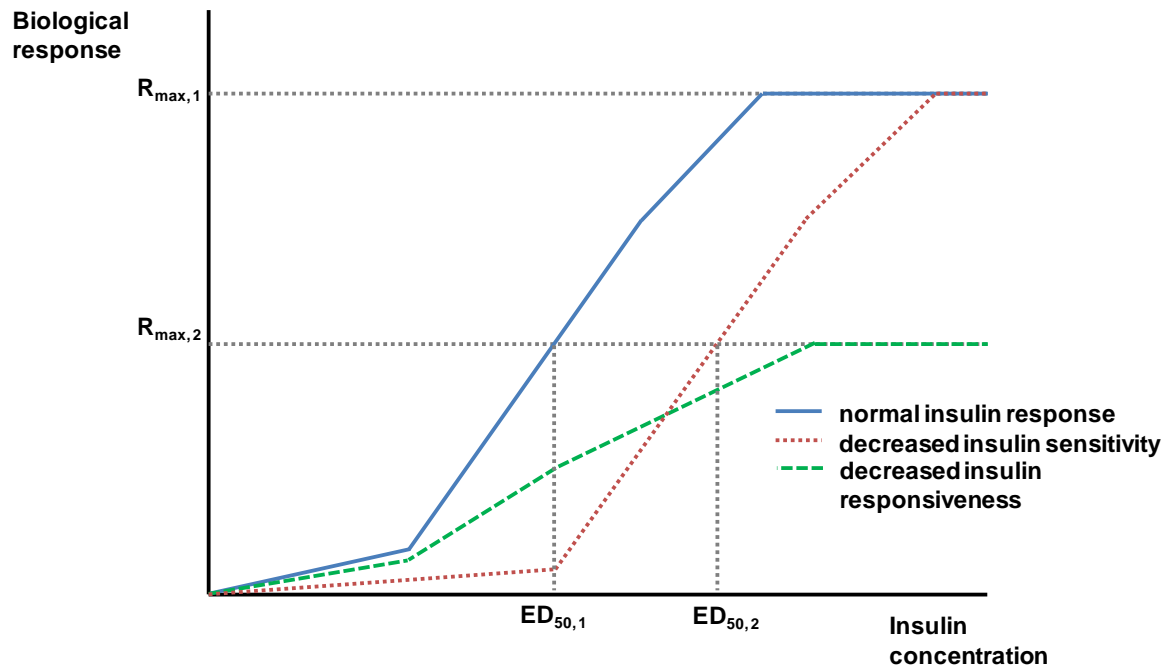
Figure 6: Differences between insulin sensitivity and insulin responsiveness. R_{max1} = maximal biological effect; R_{max2} = decreased biological effect; $ED_{50,1}$ = insulin concentration to elicit a half-maximal effect; $ED_{50,2}$ = increased insulin concentration to elicit half of the maximal effect (modified from Kahn, 1978 and De Koster and Opsomer, 2013).

In the newborn calf insulin secretory mechanisms are not fully developed (Grütter and Blum, 1991). However, the intake of colostrum by the calf leads to an increase of serum insulin concentrations (Malven et al., 1987). Furthermore, a positive correlation was observed with the increase of colostrum and insulin receptors in the intestinal mucosa (Hammon and Blum, 2002). At weaning the main source for the supply of energy changes from glucose to the short chain FA, in particular propionate.

The tissues of dairy cows seem to be more sensitive to insulin than tissues of non-ruminant animals (Brockman and Laarveld, 1986). The insulin sensitivity is different in different tissues, i.e. there are insulin-sensitive tissues, like the skeletal muscle and the AT and insulin in-sensitive tissues, like mammary gland, uterus, brain, and kidney (De Koster and Opsomer, 2013). During late gestation and early lactation, dairy cows experience an insulin resistant status to ensure a sufficient glucose supply to the fetus and the mammary gland. In late lactation the demand of the uterus for glucose increases to provide an adequate supply for the fetus. After parturition, when milk production increases rapidly, glucose is directed towards the mammary gland for lactose

production. During this time dairy cows develop insulin resistance and lipolysis increases. There are many methods to estimate the insulin resistance status. Beside the direct measurement of insulin sensitivity (Hyperinsulinemic Euglycemic Clamp (HEC) test), there are indirect methods, e.g. the intravenous glucose tolerance test (GTT) and the intravenous insulin tolerance test (ITT), to measure insulin sensitivity in an individual. The gold standard for measuring insulin resistance in humans and animals is the HEC test (Muniyappa et al., 2007; De Koster and Opsomer, 2013). However, the HEC test requires intensive animal experimentation and is labor and time-intensive, therefore several surrogate indices have been established to improve and simplify the estimation of the insulin sensitivity (Singh and Saxena, 2010). Those indices are based on a single blood sample and the concentrations of glucose, insulin, glycerol, non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB). The following table provides a list of some different indices suggested to assess insulin sensitivity (Tabl. 1).

Table 1: List of different indices to assess insulin sensitivity.

Designation	Abbreviation	Equation	Reference
Homeostasis model assessments	HOMO-IR	[glucose (mmol/L) x insulin (µU/mL)]/22.5	Matthews et al., 1985
Quantitative insulin sensitivity check index	QUICKI	1/[log glucose (mg/dL) + log insulin (µU/mL)]	Katz et al., 2000
Revised quantitative insulin sensitivity check index	RQUICKI	1/[log glucose (mg/dL) + log insulin (µU/mL)+ log NEFA (mmol/L)]	Perseghin et al., 2001
Revised quantitative insulin sensitivity check index including BHB	RQUICKI$_{BHB}$	1/[log glucose (mg/dL) + log insulin (µU/mL)+ log NEFA (mmol/L) + log BHB (mmol/L)]	Balogh et al., 2008
Quantitative insulin sensitivity check index including glycerol	QUICKI$_{Glycerol}$	1/[log glucose (mg/dL) + log insulin (µU/mL) + log glycerol (µmol/L)]	Rabasa-Lhoret et al., 2003

A low HOMO-IR indicates low insulin resistance whereas low values of QUICKI and RQUICKI indicate high insulin resistance, i.e. low insulin sensitivity.

Normally these parameters are tested in a fasting state in humans. In dairy cows this state is hard to achieve as the rumen is a long lasting nutrient reservoir. Additionally, the sensitivity of the tissues towards insulin changes in late gestation and during early lactation. Therefore these parameters should be used carefully (De Koster and Opsomer, 2013).

1.6 Metabolic Programming

Several studies have shown that the diet and the nutritional status of the fetus and the neonate in humans and animals can influence health and performance in later life (Lucas, 1991; Barker and Clark, 1997; Lucas, 1998; Ozanne, 2001; Gartner et al., 2005; Shamay et al., 2005; Guilloteau et al., 2009; Moallem et al., 2010; Kiezebrink et al., 2015). Permanent or long term changes on health and performance of individuals resulting from an early stimulus or insult at a critical or sensitive period were described by Lucas (1991) and defined as 'metabolic programming'. Especially hormones seem to play an important role by inducing long term effects (Lucas, 1991).

In contrast to humans, where the focus of metabolic programming is on long term health, in animals the focus is on later performance (Kaske et al., 2010). In dairy calves several studies have shown a positive effect of ad libitum (ad lib) feeding of whole milk or milk replacer on the later milk production in first lactation (Bar-Peled et al., 1997; Shamay et al., 2005; Zanton and Heinrichs, 2005; Raeth-Knight et al., 2009; Moallem et al., 2010; Davis Rincker et al., 2011; Soberon and van Amburgh, 2013; Kiezebrink et al., 2015). DeNise et al. (1989) have already demonstrated a positive relationship between the intake of immunoglobulins via maternal colostrum and the later milk production. In addition, they also observed a positive correlation between an increased culling rate because of low milk production and a lower intake of immunoglobulins with colostrum as calves.

In humans, several studies have shown the influence of early fetal or postnatal nutrition on the risk of developing diseases like insulin resistance, obesity or hypertension in later life (Martin et al., 2005; Owen et al., 2003, 2005, 2006, 2008; Martin-Gronert and Ozanne, 2012). All these diseases are primary symptoms of the metabolic syndrome (Symonds et al., 2009). The AT and

its adipokines might play a role in the development of later diseases after an early in utero or postnatal nutritional insult, as the number of adipocytes is determined in early life (Spalding et al., 2008; Mostyn and Symonds, 2009). Breast milk seems to have a positive influence on health in later life in humans (Martin et al., 2005; Owen et al., 2003, 2005, 2006, 2008). As to whether adipokines such as adiponectin, as bioactive components of colostrum and milk may have a role in protecting the organism against the metabolic diseases mentioned above is not clarified.

In rats, neonatal nutrition influences the development of the pancreatic islet cells (Aalinkeel et al., 2001; Srinivasan et al., 2003). After receiving a high carbohydrate formula in the preweaning period, rats developed hyperinsulinemia after weaning and an increase of β-cell proliferation was observed (Aarlinkeel et al., 2001; Srinivasan et al., 2003). The observation, that population growth of pancreatic islets occurs pre- and postnatally in rats, might indicate an influence of nutrition in the pancreatic cell development (Kaung, 1994).

In a study with male dairy calves, an increase of Langerhans islets was observed when calves were reared intensively as compared to the calves fed restrictively in the first three weeks of life (Prokop et al., 2015). This study might point out one reason for the observed increase in milk yield after intensive feeding (Moallem et al., 2010; Davis Rincker et al., 2011; Soberon et al., 2012). Taken together, health problems due to changes in the insulin action in later life might be triggered by nutritional stimuli in early pre- and postnatal life by affecting the development of the endocrine pancreas (Barella et al., 2014).

2   Objectives

Adiponectin is one of the most abundant adipokines and is positively associated with insulin sensitivity. However, in dairy calves the ontogeny of the circulating adiponectin concentrations was not characterized. Furthermore, the influence of different feeding regimen prior to weaning on the concentrations of adiponectin and its association with insulin sensitivity in dairy calves and further on their first lactation, were not studied before.

Therefore, the objectives of this thesis were:

1. To assess the changes of the circulating adiponectin concentrations in newborn dairy calves as affected by colostrum intake,

2. To characterize the effects of different feeding regimen in the first weeks of rearing on insulin sensitivity and the adiponectin serum concentrations (a) in dairy calves and (b) around first lactation, and,

3. To relate the findings to the metabolic profiles and performance data.

16

The rapid increase of circulating adiponectin in neonatal calves depends on colostrum intake

J. Kesser,* M. Hill,*† J. F. L. Heinz,* C. Koch,† J. Rehage,‡ J. Steinhoff-Wagner,*§ H. M. Hammon,§ B. Mielenz,*§ H. Sauerwein,* and H. Sadri*#[1]
*Institute of Animal Science, Physiology & Hygiene Unit, University of Bonn, 53115 Bonn, Germany
†Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, 67728 Muenchweiler a.d. Alsenz, Germany
‡University of Veterinary Medicine Foundation, 30173 Hannover, Germany
§Leibniz Institute for Farm Animal Biology, 18196 Dummerstorf, Germany
#Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, 5166616471 Tabriz, Iran

ABSTRACT

Adiponectin, an adipokine, regulates metabolism and insulin sensitivity. Considering that the transplacental transfer of maternal proteins of high molecular weight is hindered in ruminants, this study tested the hypothesis that the blood concentration of adiponectin in neonatal calves largely reflects their endogenous synthesis whereby the intake of colostrum might modify the circulating concentrations. We thus characterized the adiponectin concentrations in neonatal and young calves that were fed either colostrum or formula. Three trials were performed: in trial 1, 20 calves were all fed colostrum for 3 d, and then formula until weaning. Blood samples were collected on d 0 (before colostrum feeding), and on d 1, 3, 11, 22, 34, 43, 52, 70, 90, and 108 postnatum. In trial 2, 14 calves were studied for the first 4 d of life. They were fed colostrum (n = 7) or formula (n = 7), and blood samples were taken right after birth and before each morning feeding on d 2, 3, and 4. In trial 3, calves born preterm (n = 7) or at term received colostrum only at 24 h postnatum. Blood was sampled at birth, and before and 2 h after feeding. Additionally, allantoic fluid and blood from 4 Holstein cows undergoing cesarean section were sampled. Adiponectin was quantified by ELISA. In trial 1, the serum adiponectin concentrations recorded on d 3 were 4.7-fold higher than before colostrum intake. The distribution of the molecular weight forms of adiponectin differed before and after colostrum consumption. In trial 2, the colostrum group had consistently greater plasma adiponectin concentrations than the formula group after the first meal. In trial 3, the preterm calves tended to have lower concentrations of plasma adiponectin than the term calves

at birth and before and 2 h after feeding. Furthermore, the adiponectin concentrations were substantially lower in allantoic fluid than in the sera from neonatal calves and from cows at parturition. Our results show that calves are born with very low blood concentrations of adiponectin and placental transfer of adiponectin to the bovine fetus is unlikely. In conclusion, colostrum intake is essential for the postnatal increase of circulating adiponectin in newborn calves.
Key words: adiponectin, colostrum, milk-based formula, preterm-born, newborn calf

INTRODUCTION

Adiponectin is one of the most abundant adipocytokines in circulation and is well known for its insulin-sensitizing effects and its role in regulating lipid and glucose metabolism (Kadowaki et al., 2006). It is mainly expressed in adipose tissue, and the circulating concentrations are inversely associated with adiposity and inflammation (Cnop et al., 2003). Adiponectin in blood occurs as multimeric complexes of different molecular weights (**MW**): as low MW trimer, medium MW hexamer, and as a high MW complex (**HMW**; Waki et al., 2003).

In human fetal blood, adiponectin is detectable from wk 24 of gestation (Kajantie et al., 2004); the concentrations in newborns are higher than those in adults and are positively associated with birth weight (Kotani et al., 2004). In addition, the available body of evidence suggests that the concentration and the MW distribution of adiponectin differ in preterm and term infants (Siahanidou et al., 2007). The mRNA expression of adiponectin and its receptor in human and rat placental tissue has also been reported (Caminos et al., 2005). Humans have a hemochorial placenta type that allows for the transplacental transfer of maternal proteins of high MW. In ruminants with an epithelio-chorial placenta type, the transfer of such proteins is

hindered and therefore neonates depend on the transfer of HMW proteins as known from the acquisition of passive immunity through colostral immune globulins (Barrington and Parish, 2001).

Milk, in particular colostrum, contains a wide range of different biologically active compounds that influence both immediate and long-term metabolism and health of the offspring (Blum and Hammon, 2000). The presence of adiponectin has been documented for human milk (Martin et al., 2006; Bronskỳ et al., 2012). The concentrations of adiponectin in mature human milk (around 20 ng/mL; Bronskỳ et al., 2012) are far below the ones we recently reported for cow milk (610 ng/mL; Singh et al., 2014b), albeit the blood concentrations are comparable in the 1- to 2-digit µg/mL range in both species (Højlund et al., 2006; Singh et al., 2014a). In view of adiponectin's metabolic functions and of the expression of adiponectin receptor 1 in the small intestine of neonatal mice (Zhou et al., 2005), milk adiponectin may play an important role in infant development, both locally and systemically and may also exert a trajectory effect during later ages (Woo et al., 2012).

In consideration of the difference in placenta type and in milk concentrations between humans and ruminants, we hypothesized that in case of adiponectin, the blood concentration of adiponectin in neonatal ruminants will reflect their endogenous synthesis and might be influenced by intake of colostrum and milk. To test this hypothesis we used neonatal and young calves fed with either colostrum or formula and compared the time course of both the adiponectin plasma concentrations and the adiponectin MW distribution during the first days of life.

MATERIALS AND METHODS

Trial 1

Experimental Design, Animals, and Feeding. The experimental procedures performed in this study were in strict accordance with the German animal protection law and were approved by the relevant authority [Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (G 11–20–026.)]. Twenty German Holstein calves (10 male and 10 female) were weighed and transferred to individual hutches with straw bedding when born. After 8 d, the calves were kept in group pens equipped with an automatic feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany) until d 70. All calves received colostrum from their dam 2 times daily for 3 d, and then formula (Neumühle sauer, Trouw Nutrition Deutschland GmbH, Burgheim, Germany) from d 4 until weaning at 56 d with access

to a pelleted starter concentrate and ad libitum access to hay and fresh water. The formula was reconstituted (130 g/L of water) and restricted to 6 L per calf and day (780 g of powder per calf/d). The study covered the period from birth until d 110 after birth and was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany.

Sample Collection. Blood samples were taken from a jugular vein immediately after birth and before colostrum consumption (d 0), and on d 1, 3, 11, 22, 34, 43, 52, 70, 90, and 108 after birth. Blood samples were centrifuged within 1 h at room temperature at 3,000 × g for 20 min. The serum was obtained and frozen (−20°C) until analysis.

Trial 2

Experimental Design, Animals, and Feeding. The experimental procedures performed in this study were in accordance with animal care guidelines and were approved by the relevant authorities of the State Mecklenburg-Vorpommern, Germany (LALLF M-V/TSD/7221.3–1.1–014/07). The study was guided by the Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany, and calves were kept in single boxes at the Research Station of the University of Rostock. The calves in the present study were used in a feeding trial to investigate glucose metabolism as previously described in detail (Steinhoff-Wagner et al., 2011a). Briefly, 14 male German Holstein calves were separated from their dams at birth and were transferred to individual, straw-bedded boxes with free access to water. Calves were randomly assigned to 2 experimental groups, each consisting of 7 animals: (1) colostrum (**COL**), and (2) formula (**FOR**). Calves were bottle-fed either pooled colostrum obtained from d 1, 2, and 3 after parturition or milk-based formula (Bergophor Futtermittelfabrik GmbH, Kulmbach, Germany) with comparable nutrient composition as colostrum on the first 3 d of life. On d 4, calves received either colostrum of d 3 or formula of d 3 in groups COL and FOR, respectively. The daily amount of colostrum or formula fed was targeted to be 8% of BW on d 1 and 10% of BW on d 2 to 4. The calves in both groups were slaughtered on d 4 of life, 2 h after the last feeding.

Sample Collection. Blood samples were taken from a jugular vein after birth, before first feeding of colostrum (d 1), from d 2 until d 4 before morning feeding and 2 h after feed intake on d 4. Tubes containing K_3EDTA (1.8 g/L blood) were placed on ice and centrifuged at 1,500 × g at 4°C for 20 min to harvest plasma. The plasma was stored at −20°C until analyzed.

Trial 3

Experimental Design, Animals, and Feeding. The animal ethical and study prerequisites were as described for trial 2. The calves were used in a study to investigate the maturation of endogenous glucose production in preterm and term calves. Details of this study were reported previously (Steinhoff-Wagner et al., 2011b). Briefly, 14 German Holstein calves, born preterm (**PT**; 6 male and 1 female) or at term (**T**; 7 male) were kept in individual boxes with straw bedding and free access to water. Calves in the T group were spontaneously born after normal gestation length. Preterm calves were delivered by caesarean section 9 d before the anticipated calving date. The calves in both groups were slaughtered 26 h after birth. They did not receive colostrum or milk during the first 24 h postnatum and were then fed with pooled colostrum from d 3 of lactation at 5% of BW, 2 h before slaughter.

Sample Collection. Blood samples were taken from a jugular vein immediately after birth, and before (24 h after birth) and 2 h after final feeding. Preparation of plasma was as described for trial 2.

Additional samples from 4 healthy German Holstein cows undergoing caesarean section at the Clinic for Cattle, University of Veterinary Medicine (Hannover, Germany) were obtained. Allantoic fluid (**AF**) was collected during surgery, and blood samples from a jugular vein of the cows immediately thereafter. Blood was collected into EDTA tubes (Sarstedt AG and Co., Nümbrecht, Germany), centrifuged (1,000 × g, 15 min, 4°C) and the plasma obtained was stored at −20°C until analysis.

Analysis of Adiponectin Concentrations and MW Distribution

Quantitative Assessment of Adiponectin by ELISA. Serum, plasma, colostrum, formula, and AF were assayed in duplicate for adiponectin using an in-house developed bovine-specific ELISA that is based on a polyclonal rabbit antiserum generated against adiponectin purified from bovine serum (Mielenz et al., 2013). The original protocol of this ELISA was slightly modified, i.e., (a) for assaying colostrum, the microtiter plates were coated with whey prepared from colostrum (final dilution 1:20,000) instead of serum, (b) the antiserum was affinity-purified before use in all assays to exclude potential IgG binding antibodies, (c) the working dilution of the antiserum was 0.1 µg/mL and incubation was 3 h at 20°C, and (d) the peroxidase-conjugated secondary antibody (A1949, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was used at a 1:20,000 dilution. Serum and plasma can be used in

the assay without difference. Assay accuracy was confirmed by linearity of diluted samples and parallelism of standard curve and dilution series. The measuring range of the assay was 0.07 to 1 ng/mL and the limit of detection was 0.03 ng/mL. The intra- and interassay coefficients of variation were 7 and 9%, respectively.

Western Blotting. To maintain the different multimer complexes, the samples were neither heat-denatured nor reduced before electrophoresis. The amount of adiponectin per lane that allows for optimal display of the different MW forms in each body fluid and in formula was initially assessed and the samples (serum, plasma, colostrum, and AF) subjected to electrophoresis and Western blotting were standardized for the same adiponectin concentrations based on prior ELISA results. The final amount of adiponectin loaded per lane to which all samples were standardized by diluting the samples with PBS was 0.5 ng. The diluted samples were mixed with sample buffer (final concentration: 0.064 M Tris HCl pH 6.8, 1% SDS, 0.01% bromophenol blue, 10% glycerol) and were centrifuged for 5 min at 10,000 × g and 4°C before loading on 8% SDS-PAGE gels. Proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Europe, Freiburg, Germany) using tank blotting with the Criterion Blotter System (Bio-Rad Laboratories, Munich, Germany). After blotting, the membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (**TBST**) and 1% Roti-Block (Carl Roth, Karlsruhe, Germany) for 60 min at room temperature. The membranes were exposed to the primary antibody (1 µg/mL, anti-bovine adiponectin polyclonal rabbit antiserum, same preparation as used in the ELISA) for 1 h at room temperature and washed 4 times with TBST. Likewise, the membranes were treated with the secondary antibody [i.e., a monoclonal anti-rabbit IgG (γ-chain specific) produced in mouse and conjugated with horseradish peroxidase (Sigma; A1949, 1:10,000)]. After washing, the immune complex was detected with an enhanced chemiluminescence detection system (GE Healthcare Europe GmbH, Amersham, UK) using the VersaDoc MP4000 imaging system with Image Lab software (Bio-Rad, Munich, Germany). The MW of the developed bands was assessed by comparison with a MW marker (Prestained Protein Marker, High Range, 12949, Cell Signaling Technology Inc., Danvers, MA).

Statistical Analyses

Data from trials 1, 2, and 3 were analyzed using the Mixed Model of SAS 9.2 (SAS Institute Inc., Cary, NC). In trial 1, the model included the effects of time, sex, and the interaction of sex and time as fixed effects and calf as random effect. The outcome of this preliminary
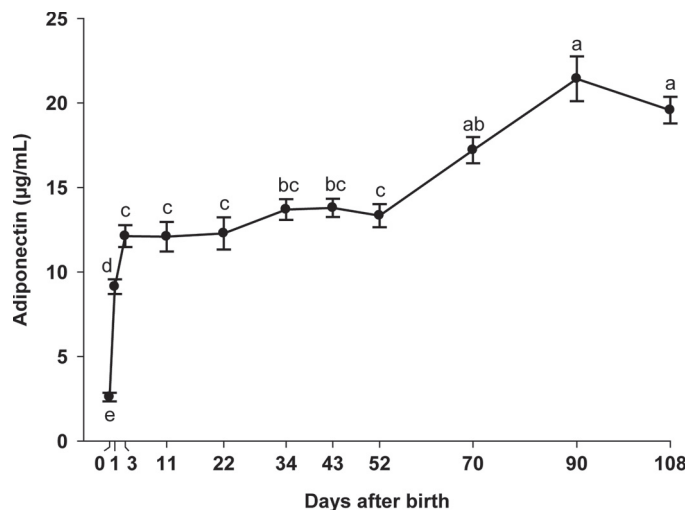
Figure 1. Concentrations of adiponectin in serum (means ± SEM, µg/mL) in Holstein calves (n = 20) from birth until 108 d of life. Different letters (a–e) indicate differences ($P < 0.05$) between the time points.

statistical evaluation did not show any significant effect of sex or an interaction of sex and time on the serum adiponectin concentrations. Therefore, sex and the interaction of sex and time were disregarded as effects in the model for the final statistical analysis. Pearson correlation coefficients were derived to identify potential correlations between serum adiponectin concentrations at birth and birth BW. In trial 2, the model included diet (COL or FOR), sampling time point, and diet by time interaction as fixed effects, and calf as random effect. In trial 3, the model included the fixed effects of group (PT, T), time, and group × time, and the random effect of calf. A Tukey-Kramer adjustment was used to account for multiple comparisons. Results are presented as means ± standard error of the mean. The threshold of significance was set at $P < 0.05$; trends were declared at $0.05 < P \leq 0.10$.

RESULTS

Adiponectin Concentrations in Serum and AF

The time course of the adiponectin serum concentrations in dairy calves during the first 108 d of life (trial 1) is shown in Figure 1. Serum adiponectin was changed during the course of the study ($P < 0.01$). Immediately after birth, and before colostrum intake, the concentrations of adiponectin were lowest as compared with all other sampling time points ($P < 0.05$). The adiponectin concentrations in serum increased 3.5- and 4.7-fold in the d 1 and 3 samples compared with d 0 (before colostrum intake). Until d 52, the concentrations remained

unchanged, but increased again thereafter until the end of the study on d 108 of life ($P < 0.05$). The serum adiponectin concentrations at birth and birth BW of calves (41.9 ± 0.82 kg) were not correlated.

In Figure 2 the plasma concentrations of adiponectin in COL and FOR calves (trial 2) from birth to d 4 of life are shown. In the FOR group, the plasma adiponectin concentrations slightly increased from birth until 72 h thereafter ($P < 0.05$). Before colostrum consumption (0 h), adiponectin concentrations were also very low in the COL group, comparable with the FOR group, but were substantially increased at 24 h after colostrum intake ($P < 0.05$). From that time onward, the calves in the COL group had consistently greater ($P < 0.05$) blood adiponectin concentrations than the FOR calves (Figure 2). The plasma concentrations of adiponectin before and 2 h after feeding on d 4 were not affected by time in both groups, but were also higher in COL calves than in FOR calves at both times ($P < 0.05$; data not shown).

The PT calves from trial 3 tended ($P = 0.10$) to have lower concentrations of adiponectin than the T calves at birth, and before and 2 h after feeding on d 2 of life (Figure 3). Plasma adiponectin concentrations did not change over time. Furthermore, no group by time interactions was observed for the plasma concentrations of adiponectin.
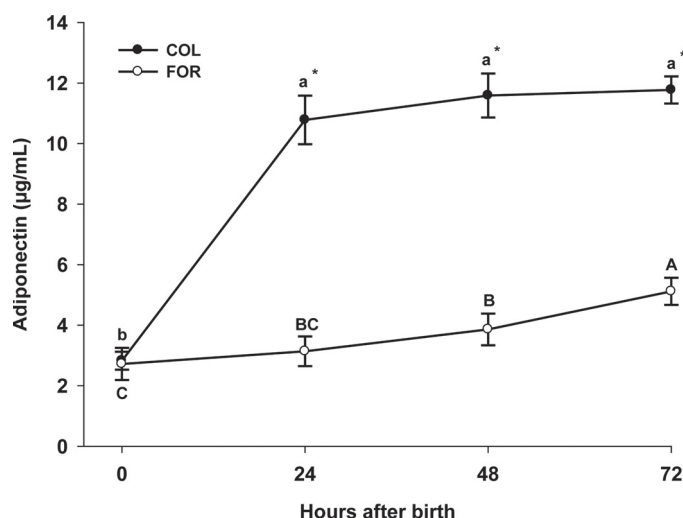
Figure 2. Concentrations of adiponectin in plasma (means ± SEM, µg/mL; n = 7 per group) in calves fed either colostrum (COL) or formula (FOR) for 4 d. Different lowercase letters indicate differences ($P < 0.05$) between the time points (a,b) in the COL calves. Different uppercase letters indicate differences ($P < 0.05$) between the time points (A–C) in the FOR calves. An asterisk (*) indicates a significant difference ($P < 0.05$) between COL- and FOR-fed calves at a given time point. Significant effects ($P < 0.001$) were time, diet, and diet × time effects.
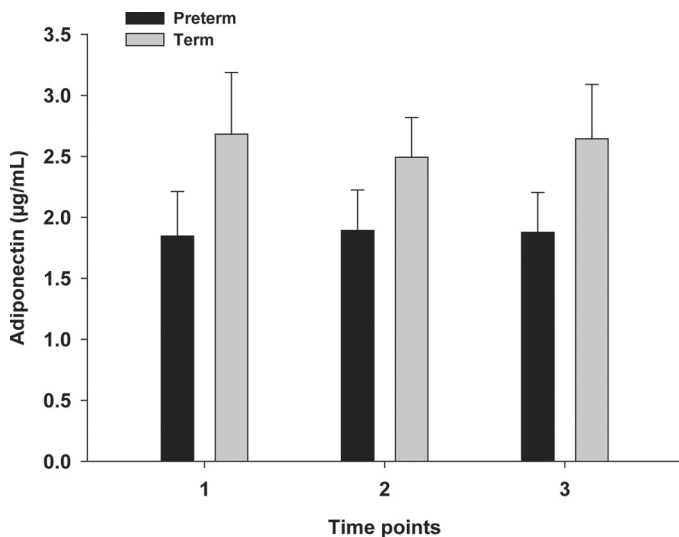
Figure 3. Concentrations of adiponectin in plasma (means ± SEM, μg/mL) immediately after birth (1) and before (2) and 2 h after feeding on d 2 of life (3) in preterm and term-born calves. Time effect, $P = 0.90$; group effect, $P = 0.10$; group × time effect, $P = 0.64$.

Table 1. Mean adiponectin concentrations in colostrum and formula

Item	Adiponectin (μg/mL)
Trial 1[1]	
Colostrum	
d 1	75.9 ± 4.19
d 3	3.32 ± 0.30
Formula	0.38
Trials 2 and 3	
Colostrum	
d 1	56.1
d 2	19.9
d 3	2.67
Formula	
d 1	0.38
d 2	0.36
d 3	0.27

[1]Colostrum data from trial 1 in which calves received colostrum from their own dams comprise 20 individual colostrums; all other adiponectin concentrations refer to pooled colostrum (trials 2 and 3) or pooled formula (trials 1 to 3) that was given to the calves.

The adiponectin concentrations recorded in AF collected during caesarean section were 2.60 ± 0.70 ng/mL and thus far below the ones observed in neonatal calves (2.55 ± 0.16 μg/mL; means from trials 1–3) and in cows at parturition (19.8 ± 0.70 μg/mL, trial 4).

Adiponectin Concentrations in Colostrum and Formula

The adiponectin concentrations in colostrum used in trials 1 and 2 were greater on d 1 than on d 2 and 3, respectively (Table 1). The adiponectin content in the formula was almost 200-fold lower than in d 1 colostrum in trial 1 and 165-fold lower in trial 2 (Table 1).

Adiponectin Multimeric Complexes in Blood, Milk, and AF

In trial 1, as displayed in Figure 4A, the adiponectin complexes differed in their distribution of HMW forms before and after colostrum consumption. Before colostrum intake, only faint bands were detected for the HMW isoforms of adiponectin in the serum, whereas after the intake of colostrum, the HMW complexes demonstrated a prominent band with a shift to the upper MW similar to the one in the corresponding colostrum samples. In trial 2, the same trend was observed in terms of the distribution of HMW adiponectin in the
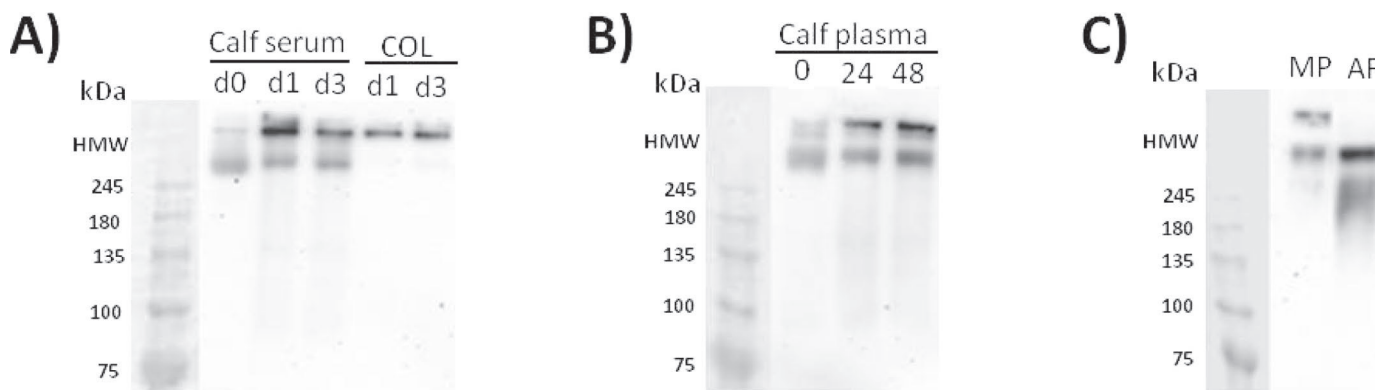
Figure 4. Representative Western blots of adiponectin multimeric complexes (A) in serum samples of calves from trial 1, before (d 0) and after receiving colostrum (d 1, 2, and 3) and corresponding colostrum (COL) samples (d 1 and 3); (B) in plasma samples of the calves from trial 2, before (0) and after receiving colostrum (24 and 48 h after birth); and (C) in maternal plasma (MP) and allantoic fluid (AF) from trial 4. HMW = high molecular weight form.

plasma before and after colostrum consumption (Figure 4B). The adiponectin HMW forms were also present in maternal plasma and in AF (Figure 4C).

DISCUSSION

The present characterization of the ontogeny of circulating adiponectin in neonatal and young calves documents that colostrum plays a critical role for the transfer of adiponectin from the dam to the neonate in cattle. Assuming that the low blood adiponectin concentrations in calves at birth represent the calf's endogenous secretion and originate from adipose tissue, low concentrations were to be expected in view of the low body fat content in neonatal calves. The blood adiponectin concentrations increased 3- to 4-fold after the first colostrum consumption. The changes observed for the MW distribution of circulating adiponectin in calves after intake of colostrum provided also qualitative support for colostrum being the major source of adiponectin in newborn calves. In umbilical cord blood from human neonates, the adiponectin serum concentrations range between 20 to 60 μg/mL (Sivan et al., 2003) and are thus greater than in the newborn calves before colostrum intake reported herein. The higher adiponectin serum concentrations in human neonates point to a placental transfer of maternal adiponectin to the fetus. However, the adiponectin serum concentrations in human neonates reportedly exceed the maternal ones (Kotani et al., 2004; Dawczynski et al., 2014), suggesting fetal origin of adiponectin (Lindsay et al., 2003; Sivan et al., 2003; Corbetta et al., 2005). In contrast, the adiponectin plasma concentrations in the calves at birth (trials 1–3) were substantially lower than those measured in the cows undergoing caesarean section or from other cows at parturition as reported earlier (Mielenz et al., 2013; Singh et al., 2014a,b). In addition, the very low concentrations of adiponectin in the AF suggest that the placental transfer of adiponectin in cattle is unlikely or negligible. Nevertheless, the concordance of the HMW adiponectin in AF and in blood from newborn calves before colostrum feeding supports that bovine fetuses do endogenously produce adiponectin, albeit at a low level. The higher endogenous production of adiponectin during fetal life in humans as compared with cattle might be related by species differences in body fat content because human infants have the highest body fat levels of any mammalian species (around 16%; Widdowson, 1950). Neonatal calves or lambs have body fat content of about 2% (Marple, 2003).

Human milk adiponectin concentrations have been reported to range from 4 to 88 ng/mL (Martin et al.,

2006). However, our present data about the adiponectin concentrations in bovine colostrum derived from the first 3 d of lactation (3 to 76 μg/mL) demonstrate substantially higher concentrations than reported for human milk in the first week postpartum (50 ng/mL; Ley et al., 2012). Therefore, unlike human neonates, calves receive a significant portion of their circulating adiponectin from their mother's colostrum.

Following the adiponectin serum concentrations beyond the time of colostrum feeding in the calves from trial 1, the values remained unchanged until d 52, but increased gradually thereafter to concentrations similar to the ones reported for lactating dairy cows (Mielenz et al., 2013) or breeding bulls of similar age (Heinz et al., 2015) until d 90 of life. In view of the biological half-life of adiponectin in circulation that is reportedly about 75 min in mice (Halberg et al., 2009), a decrease of the plasma adiponectin concentrations in the COL group from trial 2 would be expected after the initial rise with colostrum feeding. Surprisingly, the adiponectin concentrations in plasma remained fairly constant and were maintained when the intake of colostrum and thus adiponectin ceased due to gut closure around 24 h postnatum; also, 72 h after birth, no decline in adiponectin was observed. Adiponectin has structural homology with complement factor C1q (Okamoto et al., 2000) and C1q-adiponectin complexes were detected in human blood (Nakatsuji et al., 2013). The ELISA used herein to quantify adiponectin has negligible cross reactivity (<0.0001%) with the human C1q protein and other proteins such as albumin and collagen (Mielenz et al., 2013); therefore, we can exclude interference of C1q or C1q-adiponectin complexes in the assay. Moreover, the concentrations of C1q in bovine milk are very low (Rainard, 2003). The underlying mechanisms regulating plasma adiponectin concentrations in the neonatal calves are not yet known. It is likely that factors other than endogenous adiponectin secretion also affect the circulating concentrations of adiponectin in newborn calves. We thus speculate that the rate of turnover of colostral adiponectin might be slower as a compensatory effect to the lower rate of endogenous adiponectin production with regard to a very low percentage of body fat in neonatal calves (Marple, 2003). Nevertheless, in view of the consistently low concentrations in the FOR group compared with the COL group in trial 2, colostrum might indeed be indispensable for an early induction of adiponectin synthesis in neonatal calves. Colostrum intake might also have triggered the secretion of adiponectin from brown adipose tissue; the expression of adiponectin has already been reported in brown adipose tissue of humans and rodents (Viengchareun et al., 2002; Iacobellis et al., 2013). The serum adiponec-

tin concentrations in neonatal, prepubertal calves were not different between males and females; data on sex dependent variations of circulating adiponectin in human babies are contradictory (Ley et al., 2012). In our study, birth BW and serum adiponectin concentrations were not correlated. However, for humans, a positive correlation between adiponectin serum concentrations and birth BW was reported (Kotani et al., 2004).

Previous studies in human infants have shown that total adiponectin concentrations are significantly lower in preterm compared with full-term infants (Siahanidou et al., 2009) and this difference is probably due to decreased adiposity of preterm infants (Siahanidou et al., 2007). Adiponectin concentrations correlated positively with the degree of adiposity in neonates, whereas in adults inverse relationships are known (Kotani et al., 2004; Pardo et al., 2004; Tsai et al., 2004). In the current study, plasma adiponectin concentrations tended to be lower in PT calves compared with T calves. The calves in both groups received pooled colostrum from d 3 of lactation only 2 h before last blood sampling at 26 h of life. In both groups, no changes were observed in plasma concentrations of adiponectin 2 h after colostrum feeding compared with the values before feeding. Furthermore, the values measured were substantially lower than those observed in calves that received colostrum at birth (trials 1 and 2). Besides feeding colostrum with lower adiponectin concentrations, the timing of feeding (i.e., when gut closure occurred) is the most likely explanation for this difference. Taken together, adiponectin is unlikely to be transferred through the placental from the dam to the fetus in cattle. In contrast to human infants, fetal synthesis and secretion of adiponectin seem low in the bovine species. In confirmation of our working hypothesis, the blood concentrations of adiponectin in neonatal ruminants are indeed very low at birth but increase with intake of colostrum and milk. Besides increasing the circulating concentrations in the neonate, the high adiponectin concentrations in bovine colostrum and milk may also play a role for gut development because adiponectin receptors were already demonstrated both at the mRNA level and the protein level in intestinal tissue of fetal mice (Zhou et al., 2005) and humans (>9 mo of age; Bronský et al., 2012). Moreover, as adiponectin is a glycoprotein, it might also have a role for pathogen protection in the gut, acting as growth promoter for genera of beneficial microflora (Gopal and Gill, 2000) and preventing adverse effects of bacterial toxins because adiponectin was demonstrated to bind bacterial lipopolysaccharide (Peake et al., 2006).

In conclusion, calves are born with very low blood concentrations of adiponectin and colostrum intake is crucial to supply blood adiponectin and may also be indispensable to trigger the endogenous adiponectin secretion in newborn calves.

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4   Manuscript II (submitted)

Interpretive summary: Different feeding intensities during the first 1 four weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation: By Korst et al. The effects of restrictive (milk replacer, 6.78 kg/d) versus ad libitum feeding (milk replacer or whole milk) of dairy calves during the first four weeks of life on growth rate, feed intake and on performance in their first lactation were tested and the economic outcomes were estimated. Differences in growth rate were limited to the time of differential feeding; heifers fed ad libitum in early calfhood had numerically greater milk yields in first lactation than those fed restrictively. The greater feed costs in ad libitum fed calves were more than compensated by the increased returns from milk.

RUNNING HEAD: Intensified feeding and performance in calves

Different feeding intensities during the first four weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation.

M. Korst,*† C. Koch,†[1] J. Kesser,* U. Müller,* F.-J. Romberg,† J. Rehage,‡ K. Eder,# and H. Sauerwein*

*Institute of Animal Science, Physiology & Hygiene Unit, University of Bonn, Bonn, Germany

†Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany

‡University of Veterinary Medicine, Foundation, Hannover, Germany

#Institute of Animal Nutrition and Nutritional Physiology, Justus-Liebig University, Giessen, Germany

[1]Corresponding author: Christian Koch; E-Mail: c.koch@neumuehle.bv-pfalz.de

Abstract

The aim of this study was to test the effects of ad libitum feeding (ad lib) of whole milk (WM) or milk replacer (MR) versus restrictive feeding (res) of MR during the first 4 wk of life on growth performance and on milk yield in the first lactation. Fifty-seven German Holstein calves (29 females, 28 males) were studied from birth until d 110 of life (Trial 1). The 28 females from Trial 1 were further studied during their first lactation (Trial 2). In Trial 1, all calves were randomly assigned at birth to either group MR-res (6.78 kg MR/calf/d, n = 20), or group MR-ad lib (n = 17) or group WM-ad lib (n = 20). All calves received colostrum ad lib from their dam until day (d) 3 of age. From d 4 - 27 calves were fed according to their group regimen. From d 28 - 55 all calves received the MR-res feeding and were then gradually weaned until d 69. Body weight (until d 110), feed intake (amount (g), metabolizable energy (ME) and frequency of liquid feed intake until weaning) were recorded. The profitability of the different feeding regimen was estimated taking the income from milk yield (Trial 2) and the feed costs during rearing into consideration. Trial 1: Considering the total ME intakes, the calves from WM-ad lib and MR-ad lib had 2.02 and 1.65 fold greater intakes than the MR-res group during the first 4 wk of life. In this period, concentrate intake did not differ between groups, but tended to be greater in the WM-ad lib calves from d 28 - 69 of age as compared to the MR-ad lib animals. The MR-res calves visited the automatic feeders more often than the ad lib fed groups during the time of differential feeding, but 70% of the visits were in vein (< 10% in the ad lib fed calves). When all calves were subsequently fed at the MR-res level, the average portion of futile visits was 65% in all groups. Average daily weight 46 gain and body weight (BW) were greater in MR-ad lib and WM-ad lib calves than in MR-res animals in the first 4 wk of life but not from d 1 to 110. In Trial 2, age at first calving, dry matter intake and BW over the first 10 mo of lactation were not different between the groups. Milk composition was also not different. Milk yields (305 d) were numerically, but not statistically greater in the ad lib-fed groups (+ 765 kg for WM-ad lib versus MR-res, + 612 kg MR-ad lib versus MR-res) during the first lactation. Feeding WM-ad lib and MR-ad lib was 1.37 and 1.21 fold more costly than MR-res, respectively, but amounted to 18, 15, and 13 % of the total estimated feed costs until calving in WM-ad lib, MR-ad lib and MR-res, respectively. Our study confirms that ad lib feeding is an attractive measure for rearing dairy calves, both for animal welfare and - with the caveat of sample size in Trial 2 that lead to insufficient power - economic profit from milk. **Key words:** calf, nutrition, growth, milk yield

Introduction

Calves are born as pseudo-monogastrics without a functional rumen and nutrients are mainly provided in liquid form, i.e. as whole milk (WM) or milk replacer (MR) during the first wk of life to achieve high growth rates (Baldwin et al., 2004; Khan et al., 2011). Over the past decades feeding strategies for dairy calf were focused on early weaning to stimulate the intake of solid feed and thus the development of a fully functional forestomach system (Baldwin et al., 2004; Khan et al., 2011, 2012, 2016). Restrictive feeding before weaning is considered to drive the intake of concentrate and thus the production of volatile fatty acids (VFA), in particular butyrate which are the primary drivers of rumen epithelial and rumen papillae development (Quigley et al., 1991). However, restricting the amount of liquid feed results in lower growth rates, in abnormal behavior and in negative effects on rumen development (Khan et al., 2011, 2016).

The effects of increasing nutrient supply with WM or MR on feed intake, growth rate and milk yield in the first lactation were investigated recently (Soberon et al., 2012; Eckert et al., 2015; Kiezebrink et al., 2015). Increasing WM or MR intake decreased concentrate intake (Khan et al., 2007a, b; Raeth-Knight et al., 2009), delayed rumen development, and decreased BW at weaning (Suarez-Mena et al., 2011). However, Robelin and Chilliard (1989) and Moallem et al. (2010) found that increased ADG during the first 2 months of life resulted greater BW at 24 mo of age. Greater growth rates in early life reportedly improve gastrointestinal development at weaning (Eckert et al., 2015), reduce age at first calving (Raeth-Night et al., 2009) and increase first lactation milk yield albeit not always significant (Magerison et al., 2013; Soberon and van Amburgh, 2013).

Brown et al. (2005) documented that increasing the intake of energy and protein from 2 to 14 wk of age affected the development of the mammary gland in heifer calves, i.e., total parenchymal mass and parenchymal DNA and RNA increased, and the histological development was stimulated. A recent report (Geiger et al., 2016) confirmed these results and documented that intensified feeding over 8 wk of life resulted in increased organ weights, e.g., liver, mammary gland, spleen.

The "lactocrine hypothesis" emanated from the notion that milk-born factors may affect the development of specific tissues or physiological functions and thus exert long term effects (Bartol et al., 2008, 2013). Such findings were first described in neonatal pigs (Donovan and Odle, 1994; Burrin et al., 1997) and subsequently also in calves (Blum and Hammon, 2000; Rauprich et al.,

2000a, b, Blattler et al., 2001). Indeed, the results of these studies showed that neonates may undergo a programming by early nutrition with sustained long term effects e. g. on the gastrointestinal tract, liver, and mammary gland. Naturally milk born factors are constituents of WM, occurring at particularly high concentrations in colostrum (Blum and Hammon, 2000), whereas MR hardly contains such bioactive substances. Potentially sustained effects of early intensive WM feeding might thus be due to these bioactive substances but also to the level of energy and protein intake.

We herein aimed to test the following hypotheses: (1) Feeding WM or MR ad libitum (ad lib) for the first 4 wk of life and continuing thereafter on a restrictive regimen with MR until weaning at 10 wk of life will result in improved performance until d 110 of life, and thereafter during the onset and course of the first lactation. (2) Calves fed ad lib with WM will perform better in later life than calves receiving ad lib MR, and (3) the monetary costs of the 4 wk ad lib feeding will be balanced by the returns achieved with the lactating animals. The effects of the different feeding strategies on the metabolic and endocrine status from birth over the first lactation are described in the companion paper by Kesser et al. (submitted to Journal of Dairy Science, currently revised, JDS-16-11595).

Material and Methods

The animal experiments were performed in strict accordance with the German Law for Animal Protection and were approved by the relevant authority (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (G 11-20-026)). Two trials, one with calves and one with heifers recruited from the initial calf trial, were conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany.

Trial 1

Animals, housing, feeding and sampling

German Holstein calves (29 females; 28 males) were studied from April 2012 to January 2013 during their first 110 d of life. All calves were born spontaneously at term and received 10 mL iron suspension (Sinta fer-o-bac, 115 mg Fe3+/mL and 108 mg dextran/mL, Sinta GmbH, Schwarzenborn, Germany) per os. Colostrum milked from respective dams was provided ad libitum within 2 h after birth in the calving pen next to their dam. The calves were randomly allocated directly after birth to 3 feeding groups, but differential feeding was not started until d 4 of life, i.e. after the colostrum phase. The groups were **MR-res** (milk replacer restrictively, n=20; each 10 males and 10 females, birth weight: 41.9 kg ±0.8), **MR-ad lib** (milk replacer ad libitum, n = 17, 8 males and 9 females, 41.8 kg ±1.4) or **WM-ad-lib** (whole milk ad libitum, n = 20; each 10 males and 10 females, 42.3 kg ±1.3). The first colostrum intake was not different between the groups (MR-res: 2334 g ±211; WM-ad lib: 2349 g ±237; MR-ad lib: 2245 g ±211). From the second feeding time until d 3 of age all calves received colostrum and transition milk, respectively, ad lib from their dam. From d 4 to d 27 of age calves were fed according to their group regimen, i.e. the calves of the MR-res group received MR (11.5 % solids; 42 °C mixing temperature and 39 °C drinking temperature) limited to 6.78 kg/d; the calves of the MR-ad lib and the WM-ad lib group had free access 24 h/d to MR (13.8 % solids) and WM, respectively. The acidified MR was provided from Trouw Nutrition Deutschland GmbH, Burgheim, Germany (Table 1). The WM was saleable bulk tank milk from Hofgut Neumuehle (in average 3.9 % fat and 3.3 % protein) and was acidified with 2 mL acidifier per L of WM (Schaumacid, H. W. Schaumann GmbH, Pinneberg, Germany) to pH 4.6. In addition, WM was supplemented with a mix of trace elements and vitamins (1 mL Milkivit Quick-Mix trace elements/L whole milk and 1 ml Milkivit Quick-Mix vitamins/L whole milk; Trouw Nutrition Deutschland GmbH). For the first 7 d of age all calves were kept in individual straw bedded hutches (FLIXBOX, Mayer Maschinenbaugesellschaft mbH, Tittmoning, Germany). During this time calves were fed twice

daily by teat buckets. The MR-res group received 3.4 kg MR each in the morning and in the evening. Both ad lib groups were offered 9 kg WM or MR each in the 142 morning and in the evening and the buckets were accessible all day to achieve free access. From d 8 to d 70 of age calves were housed in straw bedded group pens with an automatic feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany) and had free access to water, hay, and concentrate. The latter was also offered by an automatic feeding system (Vario Kombi, Förster-Technik GmbH). The calves of the different groups were mixed in the pens and differential feeding was achieved by transponder collars through which the calves had access to their group-specific diet. From d 25 to d 27 of age the calves of the MR-ad lib and the WM-ad lib group were gradually adapted to the MR-res feeding regimen (11.5 % solids, maximal daily allowance was 6.78 kg) on which they continued until d 55. All calves were then stepped down from 6.78 kg MR/d to 2 kg on d 69. From d 70 of age onwards MR supply was entirely stopped and all calves had free access to a total mixed ration (TMR, Table 1) for lactating dairy cows until the end of the trial at d 110 of age. The calves were housed in group pens irrespective of their rearing group. All calves were subjected to blood samplings from the jugular vein immediately after birth (d 0) until d 108 of age (total number of blood samples: 11). Liver biopsies were taken on d 19 and 100. In addition, glucose tolerance test (GTT) were performed in all calves on d 22, 52 and 108 and insulin tolerance tests (ITT) were performed on d 24, 54 and 110 in male calves only. Details of sampling, processing and storage of the samples obtained, the analyses done and the results therefrom are described in the companion paper by Kesser et al. (submitted to Journal of Dairy Science, JDS-16-11595 revised version submitted concomitantly with the present revision).

Feed intake

Daily MR and WM intake was documented individually from d 1 - 7 when all calves were kept in individually hutches. From d 8 – 69 of age MR, WM and concentrate intakes were recorded daily per individual via the automatic feeding system (Vario Kombi, Förster-Technik GmbH). The contents of ME in MR and WM were calculated according to Jentsch et al. (2000); the ME content of the concentrate was analyzed according to the methods of the Verband der Deutschen Landwirtschaftlichen Untersuchungs- und Forschungsanstalten (VDLUFA, 2007). The daily ME intakes via MR, WM and concentrate were calculated by multiplying the individual daily intake of MR, WM and concentrate by the mean ME content (MJ/kg DM) of MR (16.3), WM (19.3) and concentrates (11.6) for each calf, and are presented as average intake (± SEM) per group from d 2

- 27 and d 28 - 69. Beside the amounts consumed, the number of visits in the automatic feeders was recorded and subclassified in successful visits, i.e. when feed was dispensed, and attempts in vein, i.e. without receiving feed when the daily allowance had already been retrieved.

Body Weight

All calves were weighed after the first colostrum feeding and birth weight was determined by subtracting the amount of ingested colostrum. During Trial 1, BW was recorded weekly from birth until day 110 of age with a mobile scale (Tru- Test Ltd., Auckland, New Zealand).

Economic estimates

The total feed costs over the liquid feeding period were calculated with the average intake of MR, WM or concentrate consumption from d 4 until d 70 of age per group (MR-res, MR-ad lib, WM-ad lib) and the costs of the WM (= 0.456 Euro/kg, i.e. 0.43 Euro market price plus 0.028 Euro/kg for the vitamin and mineral supplement used for WM), MR (2.50 €/kg, respectively) and concentrate (0.38 €/kg) at that time.

Trial 2

Animals, housing and feeding

In 2014, the young heifers from Trial 1 (n = 28; MR-res: n = 10, MR-ad lib: n =9, WM-ad lib: n = 9) were allocated to a second trial. They were kept in straw bedded group pens and had ad lib access to a TMR for lactating dairy cows (Table 1) from d 70 until 7 mo of age. The heifers were then transferred to a loose-housing system with high boxes with rubber mattresses and were fed with TMR for heifers. The composition and nutrient contents of the diets are provided in Table 1. Heifers were artificially inseminated when having reached a minimum age of 15 mo and spontaneous estrus was detected using activity sensors (Rescounter, leg mounted, via Dairy Plan C 21, GEA Farm Technologies GmbH, Boenen Germany) and visual observation. Three weeks before the expected calving date, they were integrated into the herd of the lactating cows to get accustomed with the milking parlor (GEA Farm Technologies GmbH), and the weighing feed

troughs (Insentec B. V., Marknesse, Netherlands). During this time they had free access to a TMR for lactating dairy cows (Table 1). The heifers were transferred to individual straw bedded calving pens 5 - 7 d ante partum (a.p.). Immediately after calving colostrum was milked from the heifer and fed directly to the calf next to their dam in the calving pen. The heifers were kept in group housing with straw bedding and ad lib access to a TMR for the lactating cows (Table 1) for the first 5 d post partum (p.p.). Thereafter the heifers were transferred to group pens with straw bedded boxes with the lactating herd receiving the same TMR through the weighing troughs.

Table 1: Ingredients and nutrient composition of milk replacer (MR), concentrate, and TMR for heifers and lactating cows (Trial 1 and 2).

Item	MR*	Concentrate	TMR Heifer	TMR Lactating[a]	TMR Lactating[b]
Ingredient (% of DM)					
Grass silage	-	-	85.8	20.1	23.0
Corn silage	-	-	-	20.3	18.2
Pressed beet pulp silage	-	-	-	19.4	12.8
Wheat straw and hay	-	-	6.6	3.6	4.9
Barley	-	-	-	9.1	10.6
Grain maize	-	-	-	10.2	10.6
SES[1]	-	-	-	3.4	5.7
SER[2]	-	-	6.7	12.1	13.2
Vitamin and mineral mix	-	-	0.9	1.5	0.8
Urea	-	-	-	0.3	0.2
Dry matter (DM)	96.6	88.9	38.4	48.2	44.8
Crude protein (CP)	23.0	19.0	14.0	15.4	16.6
Crude fat (CL)	17.0	4.1	n.d.	n.d.	n.d.
Crude fiber (CF)	0.4	6.0	19.1	16.4	15.9
Crude ash (CA)	7.4	7.4	n.d.	n.d.	n.d.
ADF_{OM} (%)	n.d.	n.d.	31.8	31.5	19.7
$aNDF_{OM}$[3] (%)	n.d.	n.d	38.3	37.9	35.5
NE_L, MJ/kg DM[4]	n.d.	n.d.	5.8	6.8	7.0
Ca (%)	1.0	1.0	n.d.	n.d.	n.d.
P (%)	0.7	0.6	n.d.	n.d.	n.d.
Na (%)	0.4	0.3	n.d.	n.d.	n.d.
Lysine	1.8	n.d	n.d.	n.d.	n.d.
Methionine	0.5	n.d.	n.d.	n.d.	n.d.

[a]TMR which were fed from d 70 until the age of 7 mo; [b]TMR which received the heifers three wk before expected calving date and over the first lactation; n.d. not determined; [1]solvent extracted soybean meal; [2]solvent extracted rapeseed meal; [3]Neutral detergent fibre content, which was assayed with a heat stable amylase and acid detergent fibre content were expressed exclusive of residual ash; [4]calculated values from the analyses of all feedstuffs, *milk replacer.

Collection of samples and data

During lactation, daily individual feed intake was recorded. The TMR was provided every morning (0730 h). All cows had free access to drinking water. Samples of all feedstuffs were collected every second week and stored at -20 °C until analysis. Feed samples were analyzed for crude ash, crude protein (CP), crude fat and crude fiber, as well as aNDFOM and ADFOM (VDLUFA, 2007). The NE_L and CP contents of the diets were calculated according to the German Society of Nutrition Physiology (GfE, 2001). Cows were milked twice daily at 0500 and 1530 h. Daily milk yield was recorded electronically via the herd management system Dairy Plan C21 (GEA Farm Technologies GmbH) and milk samples were collected monthly over the first lactation (305 DIM) as combined aliquots from one evening and the next morning milking. Samples were treated with Bronopol (2-bromo-2-nitropropane-1,3-diol) and transported to the regional lab of the milk recording organization (Landeskontrollverband Rheinland-Pfalz-Saar e. V., Bad Kreuznach, Germany). Fat, protein, lactose and somatic cell count were analyzed via infrared analyzer (MilkoScan FT-6000, Foss Analytical A/S, Hillerod Denmark). Energy corrected milk (ECM) was calculated according to the equation provided by GfE (2001) which is adjusted to 4 % fat and 3.4 % protein. The energy balance (EB) was calculated individually from NE_L intake per d minus the energy requirement for maintenance ($BW^{0.75} * 0.293$) and minus the daily energy output via ECM (GfE, 2001). Blood samples were taken monthly (3, 2, and 1 month) a.p. and weekly (0 - 10 wk) p.p. from the jugular vein. Details and results from the blood analyses performed are reported in the companion paper by Kesser et al. (submitted to Journal of Dairy Science currently revised, JDS-16-11595).

Body weight, body condition and back fat thickness

BW was recorded every second mo from d 111 until calving with a mobile scale (Tru- Test Ltd.). After calving, BW was recorded twice daily after milking via an automatic scale (GEA Farm Technologies GmbH). Backfat thickness (BFT) was assessed by ultrasonography (Aloka SSD 500, 48 mm, UST 5820, 5 MHz, Aloka GmbH, Meerbusch, Germany) as described by Schröder and Staufenbiel (2006). Body condition was scored every second week (BCS, 5 point scale; Edmonson et al., 1989).

Economic estimates

The total feed costs from d 1 - 70 of age were calculated for all groups (MR-res, MR-as lib, WM-ad lib) as described for Trial 1. From d 70 until first calving, we used daily feed costs of 1.27 Euro. This number corresponds to the average from practical farms in Rhineland- Palatinate, Germany, at that time, as assessed by the extension services and authorized by the Ministry for Environment, Agriculture, Nutrition, Viticulture and Forestry in Rhineland- Palatinate (BZA Rind, 2013). Total feed costs from birth to first calving were calculated by summing up the respective costs from d 1 – 70 and the period thereafter until calving. To calculate the returns from milk, we used the average milk price in 2014 which was realized by Hofgut Neumuehle (0.43 Euro/kg milk) multiplied by the 305 d lactation milk yield per heifer. Milk returns over feed costs were accordingly calculated.

Statistical Analyses

Statistical analyses were done using SPSS (version 22.0 SPSS Inc. Chicago, IL). For the statistical evaluation, trial 1 was divided in 4 phases (P): P0 = d 0 - 1, P1 = d 2 - 27, P2 = d 28 - 69 and P3 = d 70 - 110 of age. At the end of each phase, differences in feeding and performance data between the feeding groups MR-res (n = 20), MR-ad lib (n = 17), and WM-ad lib (n = 20) were tested by ANOVA. The homogeneity of variance was checked by the Levene`s test (P > 0.10) and, in case of significance, the Kruskal-Wallis test for non parametric tests was used. The ADG and BW of the calves during the first weeks of life (Trial 1) were analyzed with linear mixed models: group, sex, time (week or phase) and the interaction group x time were included as fixed effects and calf as random effect. The performance data of the heifers (Trial 2) were also tested with linear mixed models: group, time (mo p.p.) and the interaction group x time were included as fixed effects and heifer as random effect. For multiple comparisons, the Bonferroni post hoc test was applied using the α-correction. Results are shown as means ± SEM. Significant differences were declared at *P < 0.05* and trends at *0.05 < P < 0.1*. It should be noted that the sample size in Trial 2, i.e. 9 – 10 animals per group, was actually too low to allow for sufficient power to correctly reject, or not, the null hypothesis.

Results

Trial 1: Growth performance and feed intake

The ADG and BW during the first 15 wk of life (Trial 1) are shown in Figure 1 and in Table 2, respectively. Birth weight was not different between the treatment groups. ADG was greatest in the ad lib fed calves ($P < 0.05$) in P1 and exceeded the gains of the MR-res group by a factor of 1.44 (WM-ad lib) and 1.58 (MR-ad lib), respectively. In P2, ADG tended to be greater ($P < 0.1$) in MR-res calves when compared with MR-ad lib, whereas ADG in MR-res versus WM-ad lib and MR-ad lib versus WM-ad lib were not different. Recorded BW was highest in group MR-ad lib ($P < 0.05$) at the end of P1 and was higher in group WM-ad lib than in group MR-res ($P < 0.05$). At the end of P2 and P3, BW was not different between the groups and also ADG during P3 was not different (Figure 1 and Table 2). Sex differences in BW and ADG were limited to P3 (Table 2 and Fig. 1 (footnote)) whereas feed intakes were not different between sexes. Feed intakes both as amounts and ME from the different phases of Trial 1 are presented in Table 3.
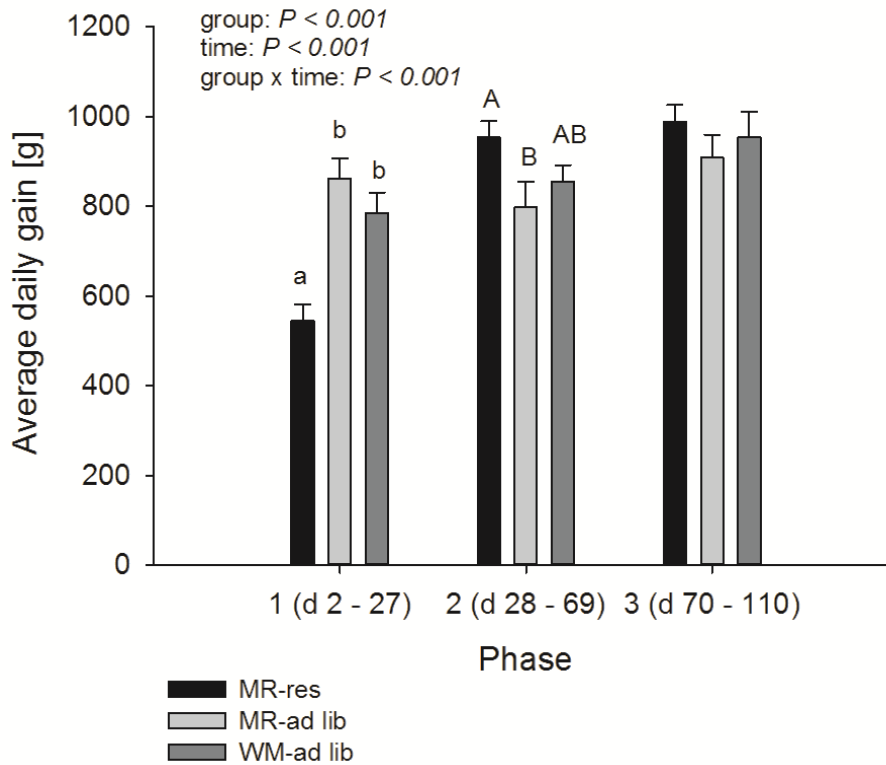
Figure 1: Development of ADG (means ± SEM) from birth until week 15 of life (Trial 1). Different letters indicate differences between groups within the respective phase (small letters: *P < 0.001*; capital letters: *P < 0.1*). Sex differences were limited to P3: *P < 0.05*; males: 1028 g ± 36; females: 876 g ± 44. Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum.

Table 2: Development of body weights (BW, kg ± SEM) during the first 110 d of life (Trial 1).

	Feeding		
BW	MR-res (n = 20)	MR-ad lib (n = 17)	WM-ad lib (n = 20)
Birth weight	41.9 (± 0.8)	41.8 (± 1.4)	42.3 (± 1.3)
BW at the end of phase 1 (d 27)	56.4[a] (± 1.0)	65.4[b] (± 1.9)	63.9[b] (± 1.3)
BW at the end of Phase 2 (d 70)	95.8 (± 1.9)	98.4 (± 2.9)	99.0 (± 2.2)
BW at the end of Phase 3 (d 110)*	131.0 (± 2.6)	131.0 (± 4.2)	133.0 (± 3.1)

Small letters indicate differences between groups (P < 0.05). Capital letters indicate trends (P < 0.10). Feeding groups (differential feeding was limited to d 4 – 27 of age): MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum

*Sex differences were limited to Phase 3: $P = 0.004$; males 137.6 kg ± 2.3; females: 127.0 kg ± 2.6

The intake of colostrum with the first meal (MR-res: 2334 g ± 211; WM-ad lib: 2349 g ± 237; MR-ad lib: 2245 g ± 211) and the amount of colostrum consumed per day from d 0 to 1 of age (P0) was not different between the groups (Table 3). In phase 1 (P1, d 2 − 27) MR and WM intake were approximately 1.4-fold greater (about 3 kg more) in both ad lib-fed groups than in the MR-res group (Table 3). During P1, the calves ate only very low and highly variable amounts of concentrate (0.07 - 0.25 kg/calf/d) that did not differ between the groups. In phase 2 (P2, d 28 - 69), i.e. after the differential feeding was ceased and all animals were fed according to the MR-res regimen, the intake of concentrate by the MR-ad lib calves tended to be less ($P < 0.1$) than in WM-ad lib animals; no differences in concentrate intake were detectable between MR-res and MR-ad lib and MR-res and WM-ad lib, respectively (Table 3). The daily energy intake (ME) via milk in P0 was the same in all groups. In contrast, the ME intake via milk in P1 was different ($P < 0.05$) between all groups with the greatest intakes in WM-ad lib exceeding those in MR-ad lib and MR-res by a factor of 1.8 and 2.1, respectively. In P2 the ME intake via milk was not different between the groups. The ME intake via concentrate in P1 was not different between the groups. In the subsequent phase of equal feeding (P2), the ME intake from concentrate was greatest in the WM-ad lib group without any difference when compared with group MR-res, but a

trend for greater intakes in MR-res than in MR-ad lib ($P < 0.10$). Total ME intake via milk and concentrate was greater ($P < 0.05$) in WM-ad lib than in MR-ad lib and MR-res in P1, but was not different between the groups in P2 (Table 3).
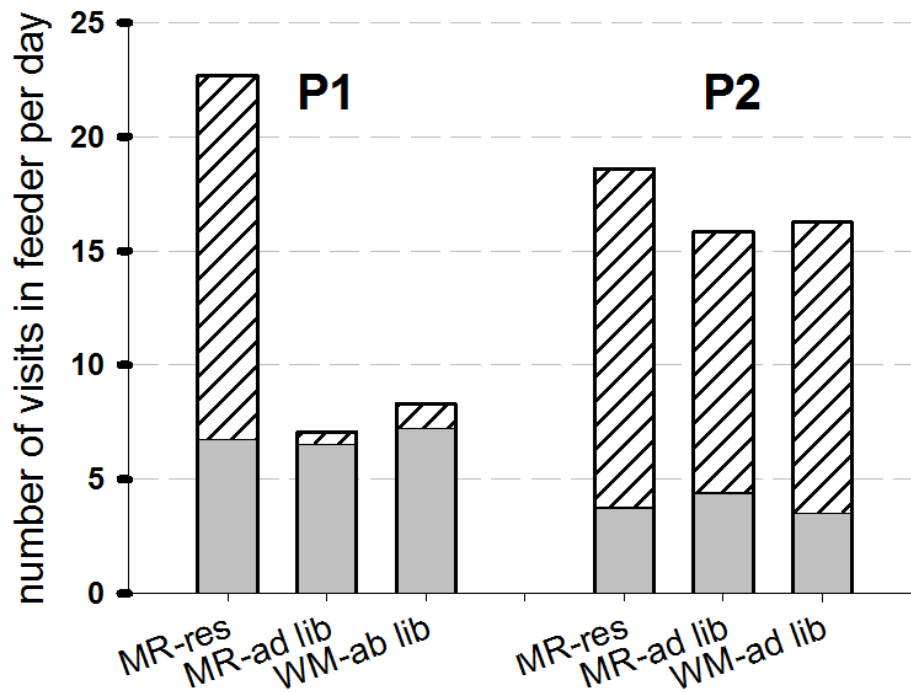

Figure 2: Daily visits at the automatic feeders by dairy calves feed at different intensities during the first four weeks of life (Trial 1). P1: phase of differential feeding from d 4 – d 27 of life; P2: phase when all calves were fed according to the restrictive regimen. Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum. Solid bars: mean number of visits when feed was obtained; hatched bars: mean number of visits during which no feed was obtained due to the restrictions set. The feeders were set to maximal allowances per visit of 5 and 2 L for the ad lib fed and the res fed animals; liquid feed was dispensed only if the calves had left at least 0.5 L (ad lib) or 1 L (res) of their daily allowance. Phase and the interaction group x phase were significant ($P < 0.001$).

The feeding frequency patterns, i.e. the daily number of visits in the automatic feeders, are shown in Figure 2. During P1, the number of total visits was greater in the MR-res fed group whereby the portion of futile visits, i.e., when the calves entered the feeder but feed was not dispensed since the daily allowance was already retrieved, amounted to 70% of total visits. The average number of visits with feed intake across all groups was 6.9/d during P1. Thereafter, when all groups were on the MR-res plane (P2), the total visits from calves of both ad lib groups were more than double as frequent than in P1, and reached 70 % futile visits, i.e. similar to the MR-res group during P1 and P2.

Table 3: Milk replacer (MR), whole milk (WM), concentrate and energy intake (ME) in the different phases of Trial 1 (means ± SEM).

	Feeding		
MR or WM (g[a])	MRr (n = 20)	MR-ad lib (n = 17)	WM-ad lib (n = 20)
Phase 0 (d 0 – 1)	4015 (± 243)	4827 (± 348)	4736 (± 410)
Phase 1 (d 2 – 27)	6385[b] (± 39)	9249[a] (± 150)	9470[a] (± 137)
Phase 2 (d 28 – 69)	5724 (± 50)	5833 (± 53)	5723 (± 50)
Concentrate (g)			
Phase 0	--	--	--
Phase 1 (d 2 – 27)	250 (± 20)	100 (± 10)	70 (± 10)
Phase 2 (d 28 – 69)	1230[AB] (± 20)	1050[B] (± 30)	1260[A] (± 20)
ME* intake (MJ/d) via milk			
Phase 0 (d 0 – 1)	12.5 (± 0.76)	15.0 (± 1.08)	14.4 (± 1.29)
Phase 1 (d 2 -27)	12.3[c] (± 0.09)	21.1[b] (± 0.34)	25.7[a] (± 0.37)
Phase 2 (d 28 – 69)	10.7 (± 0.09)	10.9 (± 0.10)	10.7 (± 0.09)
ME* intake (MJ/d) via concentrate			
Phase 0	--	--	--
Phase 1 (d 2 – 27)	1.04 (± 0.11)	0.32 (± 0.05)	0.23 (± 0.04)
Phase 2 (d 28 – 69)	12.6[A] (± 0.25)	10.8[Bb] (± 0.25)	12.8[a] (± 0.25)
Total ME* intake (MJ/d)			
Phase 0	--	--	--
Phase 1 (d 2 – 27)	13.0[c] (± 0.14)	21.5[b] (± 0.39)	26.2[a] (± 0.43)
Phase 2 (d 28 – 69)	23.3 (± 0.21)	21.7 (± 0.20)	23.5 (± 0.21)

[a]liquid intake; *ME: metabolizable energy; small letters indicate differences between groups ($P < 0.05$). Capital letters indicate trends ($P < 0.10$). Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum

Trial 2: Performance during the first lactation

Milk yield, DMI, EB and BFT recorded during 10 mo of the first lactation are depicted in Figure 3. In Figure 4, BW during lactation is shown. None of these variables was different between the animal groups originating from the different feeding regimens during calfhood. In addition, BCS was also not different between the groups (data not shown). When considering the ECM yields that are based on monthly milk recordings, the lactation curves of the heifers from the different groups were separating only during the last 2 mo; the P value for group was 0.168. When considering the 305 d milk yield (Table 4), the WM-ad lib heifers produced numerically more milk (+ 765 kg or + 9 %) than heifers reared on the MR-res feeding regimen. The milk yield in the first lactation of the MR-ad lib animals was 612 kg (+ 7 %) above the one of the MR-res heifers, but all comparisons were clearly below the level of significance (*P = 0.969*). Average milk fat %, protein %, fat and protein yield, and feed efficiency (kg ECM/kg DMI; Table 4) did also not differ between the treatment groups (Figure 2). Age at first calving was numerically lower in WM-ad lib reared heifers than in the other groups but the threshold of significance was also not reached (Table 4).

Trials 1 and 2: Economic considerations

 Economic estimates based on the feed costs for rearing until first calving and the performance in the first lactation are presented in Table 5. Means instead of individual intakes were considered since records of individual intakes were limited to the liquid feeding period; for the remaining time, we used an average value of rearing heifers in our region under comparable conditions. Variation of the recorded variables and statistical comparisons were already provided in the paragraphs above and we thus renounce on repeating these numbers after just being indued with a price factor. In average, the WM-ad lib regime was creating the greatest feed costs during the time of liquid feeding phase: 56 and 28 Euro more than for MR-res and MR-ad lib feeding, respectively. When relating the feed costs during liquid feeding to kg of BW gain during this time, the ranking was similar, i.e. the WM-ad lib feeding was 1.17 and 1.35 fold more costly than the MR-ad lib and the MR-res feeding, respectively. Considering the entire time of rearing until first calving, the portion of the costs for the liquid feeding phase in the total feed costs for rearing were 17.9, 15.1 and 12.7 % in WM-ad lib, MR-ad lib and MR-res, respectively. Taking the returns from milk sale into consideration that were (numerically) greater in group WM-ad lib

than in group MR-ad lib and MR-res, the WM-ad lib feeding yielded also 310 and 71 Euros more returns over rearing feed costs than the MR-res or the MR-ad lib regimens. When expressed per kg of milk, the difference would amount to 0.008 and 0.002 Euro, i.e. the returns over rearing feed costs in WM-adlib reared calves would be 2.5 and 0.6 % above the ones in heifers reared with MR-res or MR-ad lib.
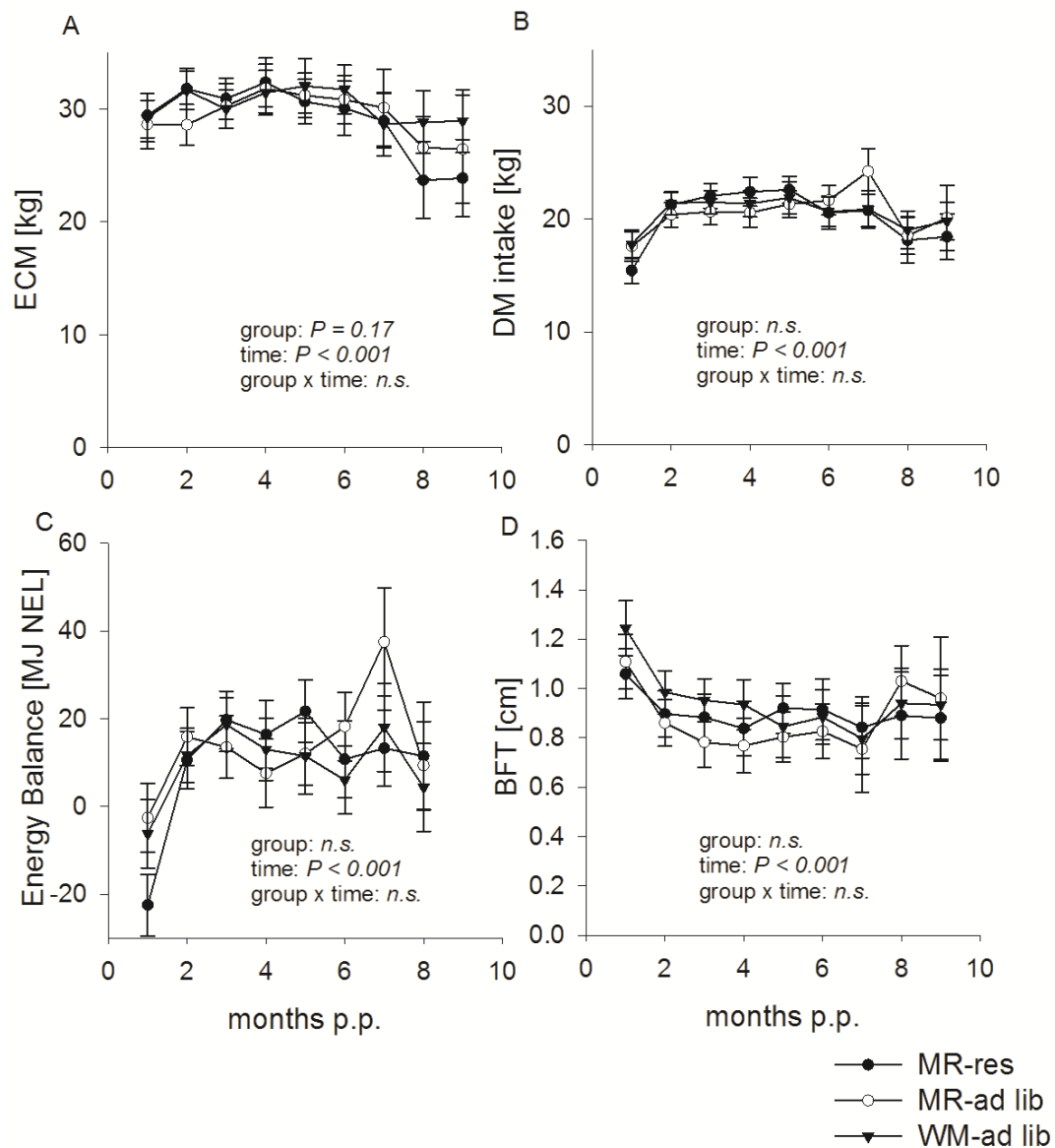

Figure 3: Development of ECM (energy corrected milk; A), DMI (dry matter intake; B), EB (Energy balance; C) and BFT (backfat thickness; C) in the first 10 months p.p. of the first lactation (mean ± SEM) (Trial 2). Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum.
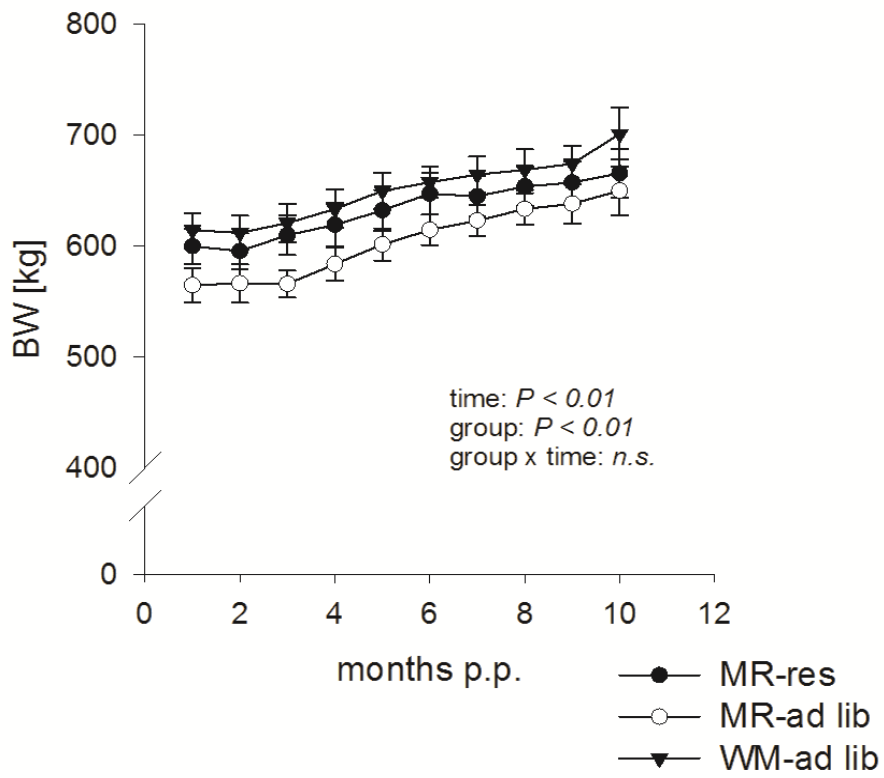
Figure 4: Development of body weight (means ± SEM) in heifers over the first 10 months of the first lactation (Trial 2). Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum; WM-ad lib = whole milk ad libitum.

Table 4**:** First lactation performance (means ± SEM) in heifers (Trial 2) reared at different feeding intensities over the first 4 weeks of life

First lactation performance	Feeding			p-value
	MR-res (n = 10)	MR-ad lib (n = 9)	WM-ad lib (n = 9)	group
Age at first calving (d)	775 (± 18.0)	773 (± 16.8)	745 (± 15.2)	0.969
305-d milk yield	8452 (± 402)	9064 (± 432)	9217 (± 475)	0.919
305-d Fat yield (kg)	329 (± 15.2)	358 (± 13.4)	347 (± 15.8)	0.925
305-d Protein yield (kg)	279 (± 14.4)	300 (± 12.8)	300 (± 17.5)	0.646
Fat (%)	3.85 (± 0.07)	3.77 (± 0.05)	3.83 (± 0.07)	0.171
Protein (%)	3.32 (± 0.04)	3.22 (± 0.04)	3.22 (± 0.05)	0.693
kg ECM[a]/kg DMI[b]	1.50 (± 0.05)	1.46 (± 0.04)	1.50 (± 0.03)	0.722

[a] ECM: energy corrected milk; [b]DMI: dry matter intake, Feeding groups: MR-res= milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum; gr: group

Table 5: Estimates of feed costs (Euro) for rearing heifers fed differently during the first 4 weeks of life in relation to the milk returns during their first lactation.

Item	Feeding		
	MR-res (n = 10)	MR-ad lib (n = 9)	WM-ad lib (n = 9)
Concentrate costs (d 4 – 70)	21.4	17.6	20.7
WM + MR costs	109.5	140.7	166.1
Total feed costs from d 1 – 70	130.9	158.3	186.8
Feed costs per kg BW gain from d 1 - 70	2.43	2.80	3.29
Total feed costs heifers*	1026	1051	1044
Milk returns from first lactation **	3634	3898	3963
Milk returns over rearing feed costs	2608	2847	2918
Milk returns over rearing feed costs per kg of milk	0.308	0.314	0.316

*Total feed costs from birth until date of first calving: total costs from d 1 – d 70 plus feed costs of 1.27 €/d from d 71 until first calving; **305-d milk yield multiplied with milk price (0.43 Euro/kg milk). Feeding groups: MR-res = milk replacer restrictive, MR-ad lib = milk replacer ad libitum, WM-ad lib = whole milk ad libitum

Discussion

Performance during calfhood

Several previous studies demonstrated that heifer calves fed WM or MR ad lib increased daily nutrient intake and had greater growth rates during the nursery period (Shamay et al., 2005; Moallem et al., 2010; Kiezebrink et al., 2015). In the current study ad lib feeding of WM or MR over the first 4 wk of life increased BW only during this period without any sustained difference at 15 wk of life. Similar results were observed by Morrisson et al. (2012) and Kiezebrink et al. (2015), where BW differences had disappeared by 84 to 126 wk of age and by 16 wk of age,

respectively. In our study, the calves were all fed MR restrictively after the first 4 weeks of life during which they were fed divergently. The increased growth rates from the previous ad lib MR or WM feeding could only be maintained if the energy intake via concentrate would have been accordingly increased and the development of the gastrointestinal tract would be adequate to digest the concentrate. It is well documented that starter intake and fermentation of starch in the rumen are responsible for rumen development (NRC, 2001) and ADG of calves between birth and 2 mo of age was positively related to starter intake (Heinrichs and Heinrichs, 2011; Bateman et al., 2012). Increased intakes of liquid feed reportedly decrease concentrate intake (Khan et al., 2007a; Khan et al., 2007b; Chapman et al., 2016). We were expecting the MR-res fed calves to have superior concentrate intakes since they were urged by the limited supply of MR to start earlier with concentrate intake, and to adapt their gastrointestinal function to solid feed earlier than the ad lib fed calves. However, we did not observe group differences in concentrate intakes during P1, probably because the amounts were low and highly variable. Concentrate intake starts only at about 14 d of age (Khan et al., 2011). During P2, the MR-res calves ate more concentrate but only when compared against the MR-ad lib group, but not to the WM-ad lib group that had similar concentrate intakes as the MR-res group during P2. However, taking the ME intakes from liquid and solid feed together at that time (P2), the groups were not different. There was a trend for greater gains in the MR-res group than in the MR-ad lib group in P2, indicating that MR-res feeding, but also the WM-ad lib feeding may have resulted in greater feed efficiencies. This trend for greater gains might have also resulted from the actual comparison with the previous MR-ad lib feeding group in which a dip in their growth curve might have been occurred whereas the MR-res were able to increase their growth rate. After weaning, when all calves had free access to a TMR for lactating cows (6.8 MJ NE_L/kg DM), maintaining increased growth rates via increasing feed intake might have been possible, but was not observed during the 39 d phase following weaning. Chapman et al. (2016) showed that the greater ADG and BW in calves fed at a high level before weaning, were not maintained but reduced thereafter. The latter aspect might be attributable to constraints of the digestive capacity for the diet fed after weaning (Chapman et al., 2016). Khan et al. (2016) concluded in a recently published review that the provision of high-starch and low-fiber starter feeds may negatively affect rumen development and that forage supplementation is beneficial for promoting gut development and rumination behavior in young calves. We could not quantify the intake of hay offered to the calves from d 4 of life and can thus not address this aspect. It is certainly important for maintaining high growth rates after weaning

that the diet fed thereafter would allow for this in terms of amount, nutrient concentration and also digestibility.

In view of the frequent feeder visits of group MR-res during P1, with the high portion of futile visits, it seems likely that this regimen left the animals unsatisfied and possibly frustrated by the futile visits which may in turn be stressful. This pattern was continued in P2, and occurred accordingly in the ad lib fed animals when these were transferred to the MR-res regimen. As to whether this level of suspected stress might have metabolic consequences above the mere nutrient intake remains unknown. However, albeit sustained effects seem improbable considering ADG and BW after the phases of liquid feeding, the restrictive feeding implies reduced welfare (Khan et al., 2016).

Our findings that calves had equal growth rates after weaning are not in support of a sustained programming of overall growth rate through feed intake or feed efficiency by feeding regimens during the first weeks of life. Nevertheless, there might have been alterations at the level of individual organs showing effects only in later life, or being too subtle to translate into growth performance. A greater feeding intensity (1.2 kg/d of MR) during the preweaning period has been demonstrated to improve nutrient intake, growth rates, and gastrointestinal development at weaning (Eckert et al., 2015). Geiger et al. (2016) showed in a recent report that higher growth rates over the first 8 wk of life increased organ weights per kg of BW (e. g. liver and mammary gland).

Performance during the first lactation

We were pursuing the performance of the heifer calves from Trial 1 until the end of their first lactation in Trial 2 albeit the sample size was basically too small to allow for sufficient power. In a meta-analysis of studies testing the effects of an enhanced supply of nutrients from WM or MR to dairy calves on milk yield in first lactation, Soberon 412 and Van Amburgh (2013) showed that the overall milk yield response was 435 ± 117 kg/lactation (*P < 0.001*). The individual studies analyzed had not equivocally reported significant effects and were mostly also underpowered. However, when using the equation elaborated from the meta-regression for the effect of ADG during preweaning on milk yield by Soberon and Van Amburgh (2013), on our data, the predicted increase in milk yield in our study amounts to a 330 kg for both ad lib fed groups in average. The actual (numerical) increase we had was in average of the two ad lib fed

groups 688 kg, which is more than double than the calculated value. However, regardless of the absolute numbers, our study is – albeit not significant - in line with the general notion and our starting hypothesis that early intensive feeding with increased growth rates has beneficial effects on milk yield in later life. Based on the lack of differences between all groups and the marginal numerical difference in 305 d milk yield between the MR-ad lib and the WM-ad lib group (+ 153 kg or 1.12 % more), the source of nutrients in early life seems rather not important for milk yield in later life. This is in line with earlier studies as summarized by Soberon and Van Ambergh (2013). Our hypothesis that WM-fed calves will perform better than MR-fed ones in later life was thus not substantiated. However, when considering the data on concentrate intake and thus ME intake after the differential feeding, WM-fed calves had some advantage over the MR-fed ones at least during calfhood

Economics

Total feed costs over the preweaning period were greater in both groups fed ad lib, similar to the results presented by Brown et al. (2005) and Raeth-Knight et al. (2009). Total costs per calf were higher in the group WM-ad lib, and also when related to a kg gain and compared against the MR fed groups. The reason for the somewhat lesser total feed costs for rearing until first calving in the WM-ad lib group than in the MR-res group is due to the numerically lower AFC in WM-ad lib calves. The returns from milk were greatest in the WM-ad lib group. When related to the total rearing feed costs, the returns over feed costs seem to be able to compensate the additional expenses during the preweaning phase. These results support the positive effect of the higher investment over the nursery period without any negative effect on economics in heifers over the first lactation.

CONCLUSION

Ad libitum intake of whole milk or milk replacer over the first 4 wk of life increased ADG and BW until d 28 but not thereafter. Restrictive feeding of MR stimulated concentrate intake to the same extent as did ad libitum feeding of whole milk. Accelerated BW gain over the first 28 d of life did not impair milk yield, milk ingredients, DMI, EB, BW, body condition or back fat thickness in the first lactation. The numerical but not statistically significant increase in milk yield is in line with previous findings and supports the notion that intensified feeding strategies may improve lactation performance. Further research is needed to identify an optimal transition from liquid to solid feed with focus on the development of a healthy rumen, gut, well adapted microbiome and all bodily functions allowing for a long productive life span.

ACKNOWLEDGEMENTS

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5   Manuscript III (submitted)

Interpretive Summary: Different feeding intensities during the first four weeks of rearing in dairy calves: Part 2: Effects on the metabolic and endocrine status during calfhood and around the first lactation. Kesser et al. The effects of three different feeding regimens in the first weeks of life of dairy calves on the metabolic and endocrine status until the first lactation were tested. Calves received either milk replacer restrictively or milk replacer or whole milk ad libitum. Differences in NEFA, glucose, insulin, adiponectin and a marker of insulin sensitivity were limited to the time of different feeding but were not sustained thereafter and when entering lactation.

RUNNING HEAD: Rearing effects on the endocrine status of heifers

Different feeding intensities during the first four weeks of rearing in dairy calves: Part 2:

Effects on the metabolic and endocrine status during calfhood and around the first

lactation.

J. Kesser,* M. Korst,*† C. Koch,† F.-J. Romberg,† J. Rehage,‡ U. Müller, * M. Schmicke,‡ K. Eder,# H. M. Hammon,§ H. Sadri,* and H. Sauerwein,*[1]

*Institute of Animal Science, Physiology & Hygiene Unit, University of Bonn, Bonn, Germany

†Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany

‡University of Veterinary Medicine, Foundation, Hannover, Germany

§Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany

#Institute of Animal Nutrition and Nutritional Physiology, Justus-Liebig University, Giessen, Germany

[1]Corresponding author: Helga Sauerwein; E-Mail: sauerwein@uni-bonn.de

Abstract

Feeding dairy calves at high intensity has been demonstrated to increase milk yield in later life. We aimed to investigate the effect of three different feeding regimens in the preweaning period on the metabolic and endocrine status during calfhood and in heifers at the onset of their first lactation. In Trial 1, 60 German Holstein calves were allocated to 3 different feeding groups: **MR-res** (milk replacer restricted to 6.78 kg/calf/d, 11.5 % solids, n = 20); **MR-ad lib** (MR 13.8 % solids, ad libitum (**ad lib**), n = 17) and **WM-ad lib** (whole milk ad lib, n = 20). All calves received ad lib colostrum for the first 3 d post natum (p.n.). From d 4 to 27 all calves were fed according to their respective feeding regimen resulting in average intakes of 6.38, 9.25 and 9.47 kg/d in MR-res, MR-ad lib and WM-ad lib, respectively. Thereafter all calves were fed according to the MR-res regimen until weaning at d 55 (gradually until d 69 p.n.). Blood samples were collected on d 0 before the first colostrum intake and on d 1, 3, 11, 22, 34, 43, 52, 70, 90 and 108 p.n. Liver biopsies were taken on d 19 and 100, and on d 22, 52 and 108 p.n. intravenous glucose tolerance tests (GTT) were performed. The male calves (n = 8 to 10 per group) underwent also an insulin tolerance test (ITT) on d 24, 54 and 110 p.n. The females (n = 28) from Trial 1 were further reared and bred as common practice, and were enrolled in Trial 2 when being in last trimester of pregnancy. Blood samples were collected monthly ante partum starting 91 d before calving and weekly (0 − 70 d) post partum. Trial 1 was subdivided into 4 phases (P): P0 (d 0 - 1 p.n.), P1 (d 2 - 27 p.n.), P2 (d 28 - 69 p.n.), and P3 (d 70 - 110 p.n.). In Trial 1, the leptin and adiponectin concentrations increased with colostrum intake. Differences in non-esterified fatty acids (**NEFA**), insulin, adiponectin, revised quantitative insulin sensitivity check index (**RQUICKI**) and variables from the GTT were largely limited to P1. The MR-res group had greater RQUICKI and NEFA values, and lower insulin and, as a trend, lower adiponectin concentrations than one or both ad lib groups. These differences were partly sustained in P2 (NEFA, adiponectin and RQUICKI), and in P3 (adiponectin). The hepatic mRNA abundance of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and pyruvatcarboxylase increased from d 19 to d 100. None of the blood variables were different between the groups when tested in pregnancy and lactation. Our results are not in support of a sustained deflection of metabolic regulation by rearing at different feeding intensities, nevertheless the differences

observed during rearing might influence nutrient utilization in later life or the cellular development of organs such as mammary gland and thereby affect milk yield. Further studies involving greater animal numbers and thus improved power will help to sort out the mechanisms of programming body function in later life via nutrition in early life.

Key words: dairy calves, metabolic programming, insulin sensitivity, adiponectin, RQUICKI

Introduction

Metabolic programming is defined as a permanent or long lasting change in the structure or function of an organism arising from a stimulus or insult which acts during a sensitive or critical period in early life (Lucas, 1991). In dairy cows, nutrition during fetal or neonatal life can influence health and performance in later life (Bach, 2012). Feeding increased amounts of whole milk or milk replacer in the first weeks of rearing was reported to lead to greater milk yields in the first lactation as compared to the common practice of feeding calves restrictively (Shamay et al., 2005; Moallem et al., 2010). In addition, increased growth rates due to increased intake of concentrate in the first months of life were positively correlated with later milk yields (Bach and Ahedo, 2008; Heinrichs and Heinrichs, 2011). A meta-analysis of 12 studies on the topic demonstrated that long-term productivity benefitted from increased nutrient intake from milk or milk replacer during the preweaning period; the authors also stated that many studies were underpowered to appropriately test such effects (Soberon and Van Amburgh, 2013).

The current concepts about the mechanisms underlying the increased milk yield in intensively reared dairy calves comprise mainly three different aspects: (a) Improved gastrointestinal function and liver metabolism resulting in greater feed digestibility and better nutrient utilization (Baldwin et al., 2004; Khan et al., 2011), (b) stimulated the development of the mammary parenchyma which may in turn give rise to a greater capacity for milk production (Brown et al., 2005; Geiger et al., 2016), and (c) tuning of the endocrine regulation of metabolism in favor of milk synthesis in later life. In the latter context, insulin and insulin sensitivity are of central importance: the reduced insulin sensitivity of peripheral tissues observed in late pregnancy and early lactation facilitates the partitioning of nutrients, in particular glucose, towards the mammary gland in which glucose uptake is largely independent of insulin (Bell and Baumann, 1997). There

is evidence from both animal models and epidemiological studies in humans that early nutrition may affect insulin action in later life (Martin-Gronert and Ozanne, 2012; Duque-Guimarães and Ozanne, 2013). Results from rat studies suggest that the early environment can also affect β-cell mass and function, and hence insulin secretion (Tarry-Adkins and Ozanne, 2011). Intensive feeding of male Holstein calves during the first 3 wk of life has been demonstrated to increase the numbers of islets of Langerhans and the circulating concentrations of insulin at 8 mo of age (Prokop et al., 2015). In this study, we focused on the endocrine and metabolic alterations potentially induced by the feeding regimen in early life. We hypothesized that intensive feeding during the first 4 weeks of life will elicit sustained changes of metabolic hormones that will continue until lactation and promote milk production. In addition, we hypothesized that ad libitum feeding of whole milk will be more effective to yield a metabolic profile in favor of milk production as compared to milk replacer. To test these hypotheses and to elucidate the mode of action of the beneficial effects reported for increased feeding of dairy calves during the first weeks of life on their later lactational performance, we aimed to (1) characterize their metabolic and endocrine status during differential feeding (d 4 - 27 of life), and (2) to evaluate whether potential differences might be sustained during the following 12 wk and also during their later pregnancy (last trimester) and the first 70 d of lactation.

Material and Methods

The animal experiments were performed in strict accordance with the German Law for the Protection of Animals and were approved by the relevant authority (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (G 11-20-026)). Two trials were conducted: Trial 1 was focused on the effects of different preweaning feeding regimen in calves; the female calves from this trial were studied as heifers in Trial 2 during late pregnancy and the first 70 d of lactation. Both trials were performed at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany. The experimental design and the gross outcomes in terms of performance are presented in the companion paper by Korst et al. (submitted to JDS, JDS-16-11594, currently revised). In brief, the experimental designs were as follows:

Trial 1

German Holstein calves (29 females and 28 males) were studied from April 2012 to January 2013 during their first 110 d of life. All calves were born spontaneously at term and received colostrum from their dam within 2 h after birth in the calving pen next to their dam. After the 3 d colostrum phase, the differential feeding was started on d 4 post natum (p.n.). The calves were randomly allocated to 3 different feeding groups balanced for sex and body weight (BW): group MR-res received milk replacer (MR, Neumühle sauer, Trouw Nutrition Deutschland GmbH, Burgheim, Germany) restricted to maximally 6.78 kg/d (11.5 % solids; n = 20, each 10 males and females), group MR-ad lib had ad libitum access to MR (13.8 % solids; n = 17, 8 males and 9 females), and group WM-ad lib had ad lib access to whole milk (acidified tank milk, 1 mL Schaumacid®/L, H. W. Schaumann GmbH, Pinneberg, Germany; supplemented with a mix of trace elements and vitamins (1 mL/L Milkivit Quick-Mix®/L, Trouw Nutrition Deutschland GmbH); n = 20, each 10 males and females). For the first 7 d p.n., all calves were kept in individual straw bedded hutches (FLIXBOX, Mayer Maschinenbaugesellschaft mbH, Tittmoning, Germany) and were fed twice daily by a teat bucket. From d 8 until d 69 p.n. calves were kept in straw bedded group pens with an automatic feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany). All groups had free access to hay and water from d 8 p.n. onwards, and concentrate was available for all calves from d 8 until d 69 p.n. by an automatic feeding system (Vario Kombi). Differential feeding was continued until d 27 p.n. Thereafter the calves of the MR-ad lib and the WM-ad lib group were gradually adapted (within 2 d) to the feeding regime of the MR-res group and all calves continued on this regimen until d 55 p.n. when gradual weaning was done until d 69. From d 70 until the end of the trial at d 110 p.n. calves were housed in group pens and had free access to a total mixed ration (TMR) for milking cows (Korst et al., submitted as companion paper).

Recordings and samplings. Birth weight was recorded and thereafter the calves were weighed weekly and also before a tolerance test (see below) was performed. Health status and eventual medical treatments were recorded regularly. As visualized in Figure 1, blood samples were taken immediately after birth (d 0) before colostrum consumption and on d 1, 3, 11, 22, 34, 43, 52, 70, 90 and 108 p.n. from the jugular vein and serum and plasma were prepared. All blood samples from ≥ d 1 p.n. were collected after calves were suspended from access to liquid and solid feed 2 h before sampling. Samples were stored at -20 °C until analyses.
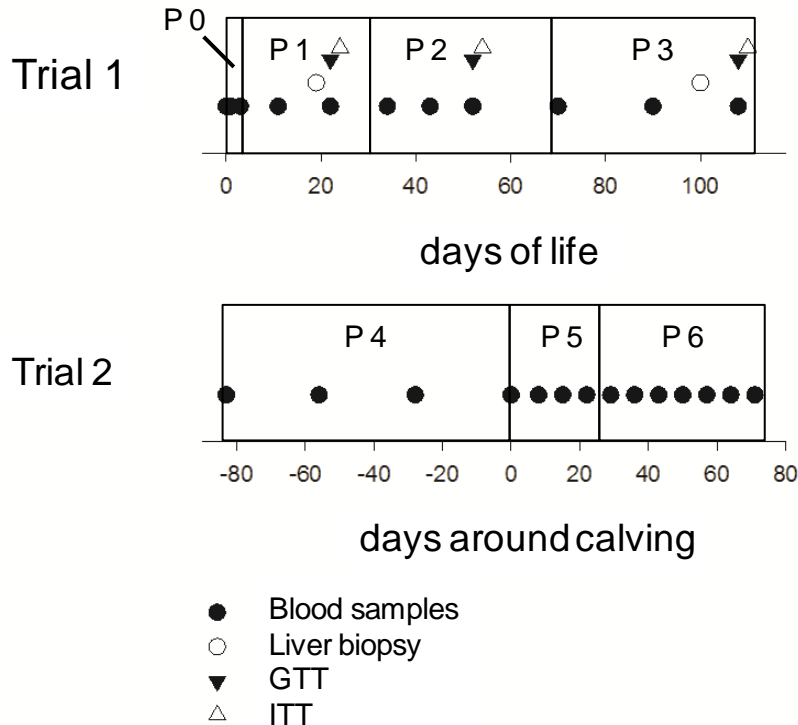
Figure 1: Sampling scheme in Trial 1 and 2. Phases (P): P0 = d 0 – 1; P1 = d 2 – 27; P2 = d 28 – 69; P3 = d 70 –110; P4 = d 91 – 30 ante partum; P5 = calving until d 21 post partum; P 6 = d 28 – 70 post partum. GTT: glucose tolerance test; ITT: insulin tolerance test

Liver biopsies were taken on d 19 and 100 p.n. After shaving, disinfection and local anesthesia (5 mL Isocaine 2 % (Selectavet Dr. Otto Fischer GmbH, Weyarn/Holzolling, Germany)), a small incision was made with a scalpel between the 11th and the12[th] rib on a line between the olecranon and the tuber coxae, and biopsies (~50 mg) were obtained with sterile 14G biopsy needles (Dispomed Witt oHG, Gelnhausen, Germany). The samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. After the biopsy, the puncture site was treated with antiseptic spray (Oxytetracycline spray blue, Bayer Health Care AG, Leverkusen, Germany).

In addition, intravenous (IV) glucose tolerance test (GTT) were performed in all calves on d 22, 52 and 108 and IV insulin tolerance tests (ITT) were performed on d 24, 54 and 110 in the male calves only. The protocols used were described earlier by Bossaert et al. (2009) and Oikawa and Oetzel (2006). At least 4 h before the tests, calves had no access to milk, hay and TMR. For the

GTT, the calves were IV infused with glucose (150 mg/kg BW, Glucose 40%, Selectavet Dr. Otto Fischer GmbH). Blood samples were collected from the jugular vein -10, -5, 4, 8, 12, 18, 25, 36, 45, 60, 90 and 120 min relative to the glucose infusion. For the ITT, the calves were IV infused with 0.05 IU/kg BW of human recombinant insulin (Actrapid® Penfill®, Novo Nordisk A/S, Bagsværd, Denmark) and blood was sampled at -15, -5, 15, 30 and 45 min relative to the infusion.

For testing the relationship between the concentration of adiponectin in colostrum and milk with the serum concentrations we collected blood samples from 22 additional German Holstein calves before their first colostrum intake on d 0 and thereafter on d 1, 2 h after the last colostrum feeding. In total, i.e. including the calves from Trial 1, we had serum samples from these 2 d available from 79 calves. Colostrum from the day of calving and milk samples (7th d in milk) from the respective dams (n = 79) were also collected and all samples stored at -20 °C until analysis.

Trial 2

After finishing Trial 1, the heifer calves (n = 28) were kept in straw bedded group pens and had ad lib access to a TMR for milking dairy cows and were then transferred to a loose-housing system with high boxes in the stable for the milking cows at 5 - 6 mo of age. When having reached 15 mo of age, estrus detection was started using activity sensors (foot rescounter via Dairy Plan C 21, GEA Farm Technologies GmbH, Boenen Germany) and visual observation. Pregnancies were confirmed by veterinary rectal palpation. In average, heifers were first inseminated at 18 ± 1 mo of age and received a TMR for dry cows until 21 d before expected calving when they were integrated into the herd with the lactating dairy cows. During this time they had free access to the TMR for high yielding dairy cows offered in weighing troughs (Insentec B. V., Marknesse, Netherlands). The heifers were transferred to individual calving pens 5 - 7 d ante partum (a.p.). After calving, the calves were separated; the heifers were milked twice daily (0500 a.m. and 0330 p.m.) and kept in a group pen with ad lib access to the TMR for the first 5 d post partum (p.p.). For the remaining lactation, the heifers were returned to the weighing troughs and received a TMR for high yielding cows.

Data recorded and samples collected. Milk was sampled monthly over the first lactation (305 DIM) as combined aliquots from the evening and the next morning milking. Samples were

stabilized with Bronopol (2-bromo-2-nitropropane-1,3-diol) and transported to the regional lab of the milk recording organization (Landeskontrollverband Rheinland-Pfalz-Saar e. V., Bad Kreuznach, Germany) where milk fat, protein, lactose and somatic cell counts were analyzed via infrared analyzer (MilkoScan FT-6000, Foss Analytical A/S, Hillerod, Denmark).

Body weight was recorded every second month starting 111 d p.n. After calving, BW was recorded twice daily after milking via an automatic scale (GEA Farm Technologies). Health status and eventual medical treatments were recorded regularly.

Blood samples were collected (*V. coccygealis*) monthly before the expected calving date, starting 91 d a.p., at calving and thereafter in weekly intervals until d 70.

Analyses

The concentrations of non-esterified fatty acids (NEFA), glucose and beta-hydroxybutyrate (BHB) were determined in the serum samples obtained in both trials by an automatic spectrophotometer (ABX Pentra 400, Horiba ABX, SAS, Montpellier, France). The following kits were used: Trial 1: glucose (#553-230, Idstein, Germany), NEFA (#434-91795, WAKO Chemicals GmbH Neuss, Germany), and BHB (# RB 1007, Crumlin, UK); Trial 2: glucose (#A11A01667, Horiba ABX); NEFA (#434-91795, WAKO Chemicals GmbH); urea (#LT-UR 0010, Labor + Technik, Berlin, Germany) and BHB (# RB-1008, Labor + Technik, Berlin, Germany). The concentration of total plasma protein (TPP) in the calves' samples was measured by a handheld refractometer (RF.5612, Euromex Microscopen B.V., Arnhem, NL).

Hormone analyses. For Trial 1, a RIA was used for determining the insulin concentrations (IM3210, Insulin IRMA KIT, Immunotech, Beckman Coulter, CA). The intra-assay coefficient of variation (CV) was 7.6 % and the inter-assay CV was 10.7 %. The limit of detection (LOD) was 3.95 µU/mL.

For Trial 2, insulin was measured via a RIA for porcine insulin (PI-12K, Linco Research, St. Charles, MO) that has been used with bovine serum previously (Bellmann et al., 2004). The intra-assay CV was 8.2 % and the inter-assay CV was 4.3 %. The LOD was 1.61 µU/mL and the specificity for bovine insulin was 90 %.

Leptin in serum, colostrum and milk was measured by ELISA (Sauerwein et al., 2004). The intra- and interassay CV were 3.6 and 7.8 %, respectively. The LOD was 0.3 ng/mL.

Adiponectin in serum, colostrum and milk was measured by a modified in-house developed ELISA specific for bovine adiponectin (Mielenz et al., 2013; Kesser et al., 2015). Assay accuracy was confirmed by linearity and parallelism of diluted samples. The LOD was 0.03 ng/mL. The intra- and interassay CVs were 7 and 9 %, respectively.

mRNA abundance of Pyruvate carboxylase (PC) and Phosphoenolpyruvate carboxykinase (PCK1) in liver samples. Quantitative PCR (qPCR) was carried out using a Mx3000P cycler (Stratagene, Agilent Technologies, CA) after total RNA extraction and cDNA synthesis as described earlier (Saremi et al., 2012). For qPCR an inter-run calibrator and a negative template control and for cDNA a negative template control and a no reverse transcriptase control were included in each run. For each PCR reaction, a cDNA standard curve with serial dilutions was used to calculate efficiency-corrected relative quantities of the targets. Data were normalized with the geometric mean of the reference genes selected by qBASE[plus] 2.0 (Biogazelle, Ghent, Belgium) as described earlier (Hosseini et al., 2010). The 3 reference genes identified as the most stable ones were eukaryotic translation initiation factor 3, subunit K (EIF3K), low-density lipoprotein receptor–related protein 10 (LRP10), and Hippocalcin-like (HPCAL1). The primer sequences and accession numbers of the target and the references genes are provided in Table 1.

Table 1: Characteristics of primers and real-time polymerase chain reaction conditions.

Gene[*]	Sequences (5'-3')	NCBI Accession No.	bp	Concentration (nM)	Annealing (s/°C)[4]
1 EIF3K					
Forward Reverse	CCAGGCCCACCAAGAAGAA TTATACCTTCCAGGAGGTCCATGT	NM_001034489	125	400	45/59
2 HPCAL					
Forward Reverse	CCATCGACTTCAGGGAGTTC CGTCGAGGTCATACATGCTG	NM001098964	99	400	30/60
3 LRP10					
Forward Reverse	CCAGAGGATGAGGACGATGT ATAGGGTTGCTGTCCCTGTG	BC149232	139	400	30/61
4 PC					
Forward Reverse	ATCTCCTACACGGGTGACGT TGTCGTGGGTGTGGATGTGCA	NM_177946	214	1000	30/60
5 PCK1					
Forward Reverse	AACTCACGGTTCTGCACTCCA GGTCGTGCATGATGACTTTGC	NM_174737	229	800	30/60

*EIF3K: Eukaryotic translation initiation factor 3; HPCAL: Hippocalcin-like 1; LRP10: Lipoprotein receptor-related protein 10; PC: Pyruvate carboxylase; PCK1: (cytosolic) Phosphoenolpyruvate carboxykinase

Calculations and Statistical Analyses

Glucose and insulin tolerance tests. For the GTT, the means of the concentrations from -10 and -5 min before the glucose infusion were considered as basal for glucose (G_B) and insulin (I_B), respectively. The difference between the basal and the peak concentrations was defined as $\Delta_{Peak-Basis}$. The area under the curve (AUC) was calculated with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) using the increase of the glucose and insulin concentrations above the basal values until 120 min after the infusion.

For the ITT, the insulin-stimulated blood glucose response (ISBGR, %) was calculated based on the equation of Oikawa and Oetzel (2006): ISBGR (%) = $[(G_B - G_{30}) / G_B]$ x 100, whereby G_B is the basal glucose concentration (calculated as the mean between the glucose concentration in the samples taken before the insulin infusion (-10 min and -5 min) and G_{30} is the glucose concentration at 30 min thereafter.

Insulin sensitivity was estimated by the revised insulin sensitivity check index (RQUICKI) (Perseghin et al., 2001; Holtenius and Holtenius, 2007) according to the following equation: RQUICKI = 1 / [log (Glucose, mg/dL) + log (Insulin, μU/mL) + log (NEFA, mmol/L)]. A low RQUICKI index indicates decreased insulin sensitivity.

For the statistical comparisons, data from Trial 1 and 2 were divided into phases (P): P0: d 0 - 1 p.n.; P1: d 2 - 27 p.n.; P2: d 28 - 69 p.n.; P3: d 70 - 110 p.n.; for Trial 1. For Trial 2 the phases were P4: d 91 - 30 a.p., P5: calving until d 21 p.p., P6: d 29 - 70 p.p. However, in case of Trial 2, the number of animals that could be considered (28 in total, i.e. 9 to 10 per group) was insufficient to allow for an adequate power and therefore results must be considered as preliminary. Data were analyzed using the linear mixed model from SPSS version 22.0 (SPSS Inc. Chicago, IL). Normal distribution was tested by the Kolmogorov-Smirnov test and the Levene's test was used to test the homogeneity of variances. The linear mixed model with Bonferroni Post Hoc tests was used for the metabolite and hormone concentrations as dependent variables to identify group, time and sex (only Trial 1) differences. Group, sex and time and the interaction between group and time were included as fixed effects and the animal as random effect. Differences between groups at each time point were tested with an ANOVA (normal distributed and homogeneity of variance) or Kruskal Wallis Test (not normal distributed and no homogeneity of variance). Student's t-test was used for the liver biopsies and the milk samples to identify differences between time points. Results are shown as means ± SEM. Correlations were calculated by Spearman analysis (= ρ). Significant differences were declared at $P < 0.05$ and trends at $P < 0.1$.

Results

Growth performance and milk yields. Detailed information about performance data is presented in the companion paper (Korst et al., submitted). In brief, differences in BW, feed and energy intake between the calves groups from Trial 1 were observed mainly during P1. During this time, the calves in the MR-res group were lighter when compared with the ad lib fed animals. Albeit their consumption of concentrate at that time was numerically but not statistically higher, the energy intake from both liquid and solid feed was only 60 and 80% of the ME intake in the MR-ad lib and WM-ad lib group, respectively. When all calves were fed according to the MR-res regimen for the remaining time of liquid feeding (P2), energy intakes with concentrate were greater in the WM-ad lib group than in the MR-ad lib group but were not different when compared with the MR-res group. At that time the ADG of the MR-res group tended to greater values than in the MR-ad lib group, but was not different from the WM-ad lib group. At the end of Trial 1 and in Trial 2 these differences had disappeared. In Trial 2, there were no differences in p.p. DMI, energy balance, BW, or milk composition. In addition, 305 d milk yield of heifers from the WM-ad lib group was numerically, but not statistically, greater than in the MR-res and the MR-ad lib group (+ 765 L and + 153 L, respectively).

Metabolites. The comparisons of the hormone and metabolite concentrations in the different phases of both trials are presented in Table 2. The time courses of the circulating concentrations of NEFA, glucose and BHB are shown for both the calves and the heifers in Figure 2. In Trial 1, the NEFA concentration was lower in the MR-ad lib than in the MR-res and WM-ad lib group ($P < 0.05$). From d 11 to 34 p.n. the glucose concentrations in the MR-res group were lower ($P < 0.05$) than in the MR-ad lib and the WM-ad lib group. The BHB concentration was greater in the MR-res than in the MR-ad lib group in Trial 1. There were no sex differences for NEFA, glucose and BHB. The concentrations of TPP increased about 1.2- fold after the first intake of colostrum ($P < 0.001$) until d 3 p.n. There were no group or sex differences for TPP throughout the entire trial. In Trial 2, the heifers originating from the different rearing protocols did not differ in NEFA or glucose. Only urea and BHB were different before parturition (P4). The WM-ad lib heifers had lower urea concentrations than the MR-res and MR-ad lib groups ($P = 0.1$ and $P < 0.05$, respectively) and tended to have lower BHB concentrations then the MR-ad lib group ($P = 0.1$). Urea decreased around calving ($P < 0.001$) and increased towards the end of the trial ($P < 0.001$).

Table 2: Comparison of the metabolic and endocrine blood variables (means ± SEM) during different phases (P) of life in calves and heifers.

	P1 (d 2 – 27 p.n.)			P2 (d 28 - 69 p.n.)			P3 (d 70 - 110 p.n.)			P4 (d 91 – 30 a.p.)			P6 (d 29 - 70 p.p.)		
	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib
NEFA [µmol/L]	332^b ± 30.4	204^a ± 16.5	299^b ± 18.9	337^{AB} ± 41.6	287^B ± 26.1	412^A ± 44.8	194 ± 25.9	177 ± 17.5	219 ± 27.7	190 ± 19.5	148 ± 16.1	174 ± 17.7	243 ± 17.7	259 ± 25.5	216 ± 20.3
Glucose [mmol/L]	6.1 ± 0.2	6.6 ± 0.2	6.5 ± 0.2	4.7 ± 0.2	4.9 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	4.9 ± 0.2	5.0 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.3 ± 0.1
BHB [mmol/L]	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.17^{ab} ± 0.01	0.14^b ± 0.01	0.19^a ± 0.01	0.29 ± 0.02	0.29 ± 0.02	0.29 ± 0.02	0.31^{AB} ± 0.02	0.33^B ± 0.02	0.27^A ± 0.02	0.53 ± 0.03	0.52 ± 0.02	0.49 ± 0.02
Urea [mmol/L]	-	-	-	-	-	-	-	-	-	4.1^B ± 0.2	4.3^{bB} ± 0.2	3.6^{aA} ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.1
TPP [g/dL]	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.8 ± 0.1	5.8 ± 0.1	5.9 ± 0.1	-	-	-	-	-	-
Insulin [µU/mL]	12.1^a ± 1.2	27.6^b ± 4.7	28.5^b ± 4.0	7.2 ± 0.1	7.1 ± 0.5	6.5 ± 0.7	9.8 ± 0.7	9.8 ± 0.6	10.8 ± 1.0	18.3 ± 2.1	19.8 ± 1.3	19.2 ± 2.1	9.6^a ± 0.5	12.6^b ± 1.0	10.5^{ab} ± 0.6
Adiponectin [µg/mL]	11.7^B ± 0.7	14.2^A ± 1.1	12.7^{AB} ± 0.5	14.1^a ± 0.5	16.2^b ± 0.4	17.4^b ± 0.7	19.3^a ± 0.8	23.5^b ± 1.1	22.8^B ± 1.3	23.0 ± 0.9	24.4 ± 0.8	24.8 ± 0.8	23.0^A ± 0.5	23.8^{AB} ± 0.6	24.9^B ± 0.6
Leptin [ng/mL]	2.7 ± 0.2	2.7 ± 0.2	2.8 ± 0.3	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	2.6 ± 0.2	2.5 ± 0.1	-	-	-	-	-	-
RQUICKI	0.41^a ± 0.01	0.39^{ab} ± 0.01	0.37^b ± 0.01	0.49^a ± 0.01	0.47^{ab} ± 0.01	0.46^b ± 0.01	0.47 ± 0.01	0.50 ± 0.01	0.47 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.27 ± 0.01	0.26 ±0.01	0.27 ± 0.01

No differences between groups in any variable were observed during P0 and P5, therefore results from these phases are not shown. Different small letters indicate differences between groups (a, b) ($P < 0.05$) within each time point, different capital letters letters indicate trends ($P < 0.1$). p.n. = post natum; a.p. = ante partum; p.p. = post partum; MR-res = milk replacer, restricted; MRad lib = milk replacer ad libitum; WMad lib = whole milk ad libitum; NEFA = non-esterified fatty acids; TPP = total plasma protein; RQUICKI = revised quantitative insulin sensitivity check index

Hormones: The concentrations of adiponectin and insulin during both trials are shown in Table 2 and Figure 3.
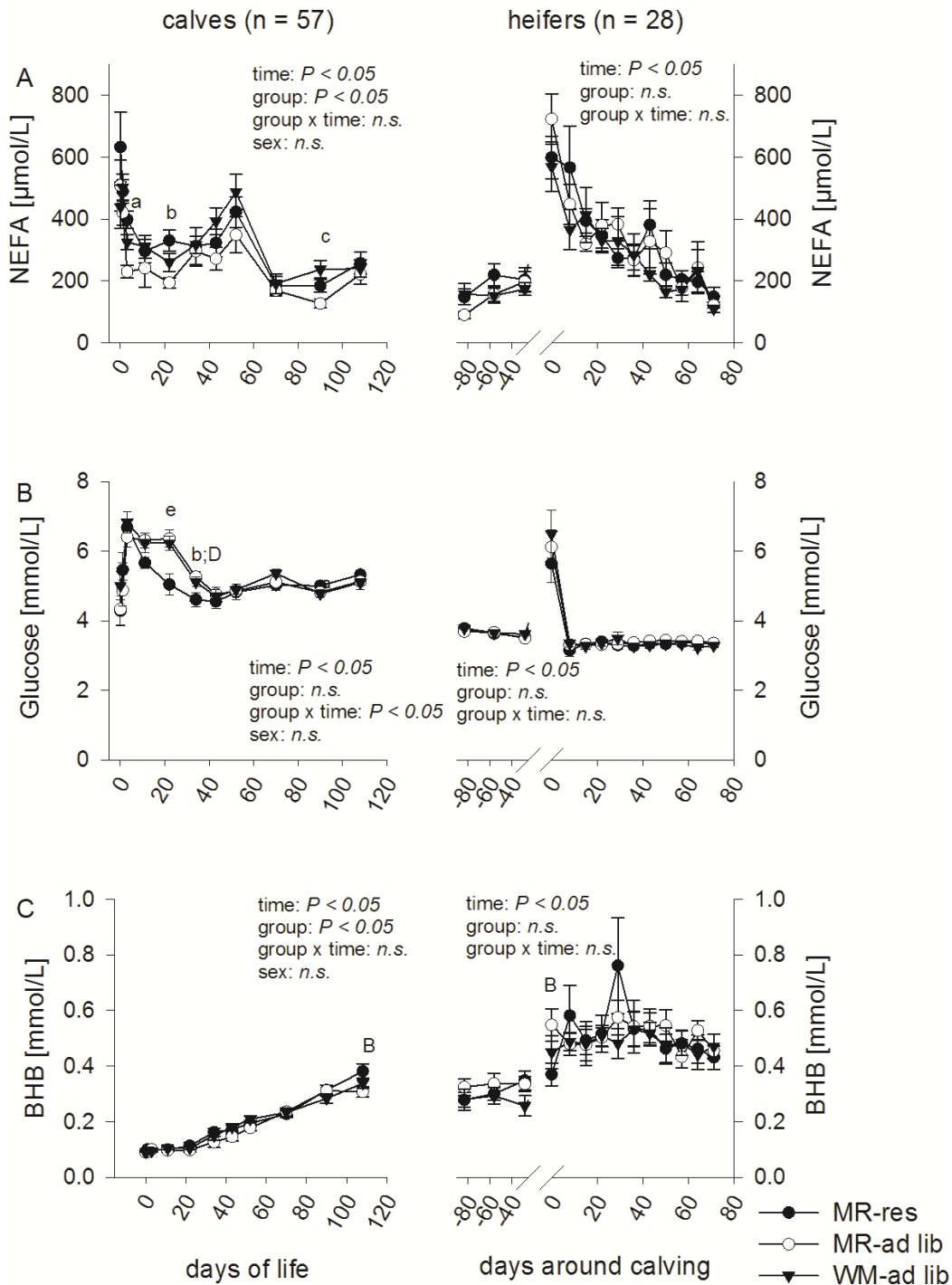

Figure 2: Time dependent changes of NEFA (A), glucose (B) and BHB (C) (means ± SEM) from Trial 1 (calves) and Trial 2 (heifers). Small letters indicate differences between groups ($P < 0.05$), capital letters indicate trends ($P < 0.1$). a/A = MR-ad lib vs. MR-res and WM-ad lib; b/B = MR-res vs. MR-ad lib; c/C = MR-ad lib vs. WM-ad lib, d/D = MR-res vs. WM-ad lib; e/E = MR-res vs. MR-ad lib and WM-ad lib.

Figure 3: Time dependent changes of adiponectin (A), insulin (B) and RQUICKI (C) (means ± SEM) from Trial 1 (calves) and Trial 2 (heifers). Small letters indicate differences between groups ($P < 0.05$), capital letters indicate trends ($P < 0.1$). a/A = MR-ad lib vs. MR-res and WM-ad lib; b/B = MR-res vs. MR-ad lib; c/C = MR-ad lib vs. WM-ad lib, d/D = MR-res vs. WM-ad lib; e/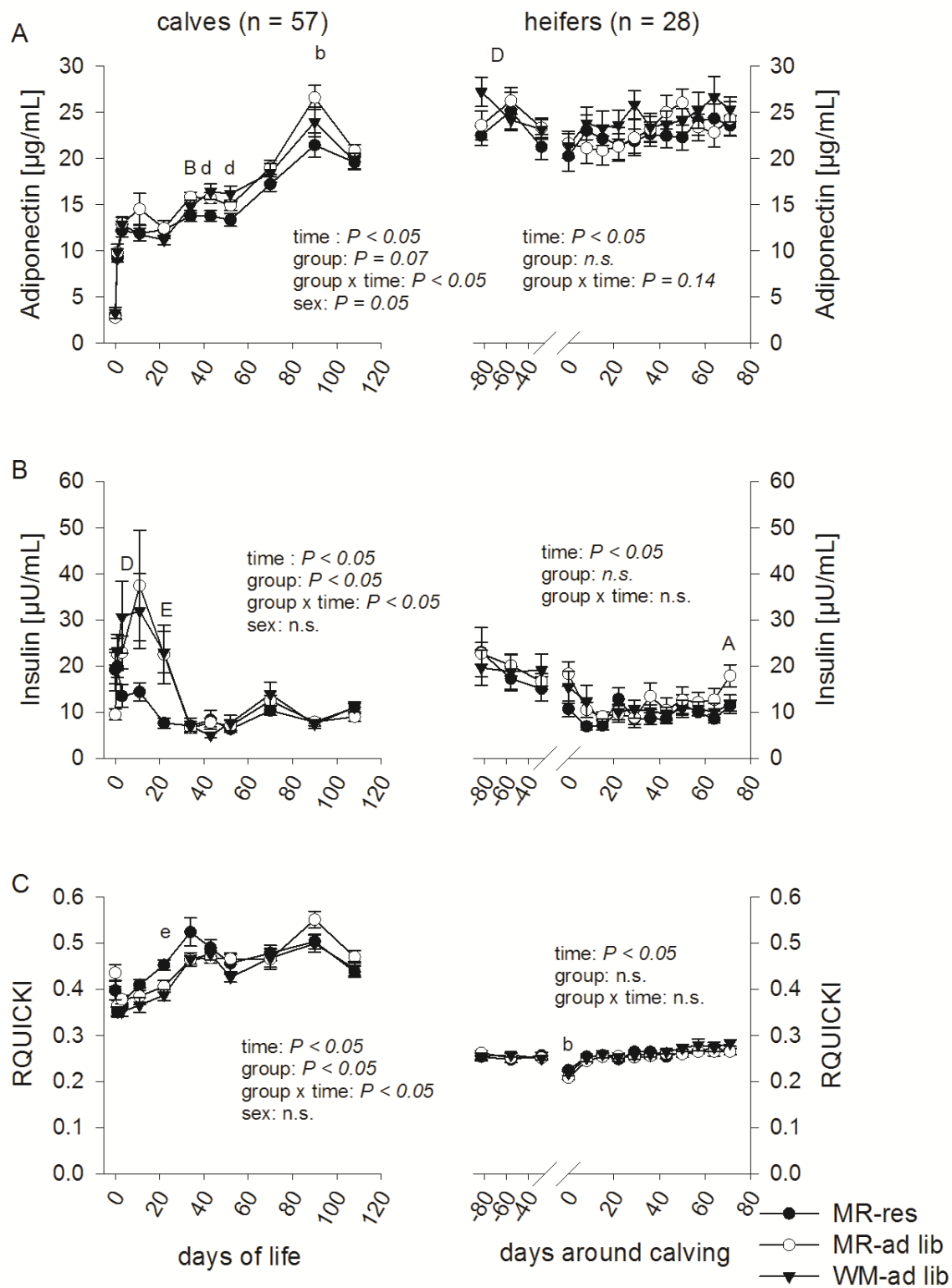E = MR-res vs. MR-ad lib and WM-ad lib. The adiponectin concentrations of the MR-res calves were reported previously (Kesser et al., 2015).

The MR-res group tended (*P = 0.07*) to have lower adiponectin concentrations than the MR-ad lib group during Trial 1. Female calves had greater adiponectin concentrations than male calves (*P = 0.05*).

The MR-res group had lower (*P < 0.05*) insulin concentrations in blood than the WM-ad lib group in Trial 1. On d 11 and 22 p.n. the MR-res group had lower (*P < 0.05*) insulin concentrations than the MR-ad lib and the WM ad lib group. In Trial 2, no group differences were observed for the insulin and adiponectin concentrations. The leptin concentrations, assessed only during Trial 1, were not different between the feeding groups and are shown in Figure 4 together with the mean leptin concentrations in colostrum and milk.
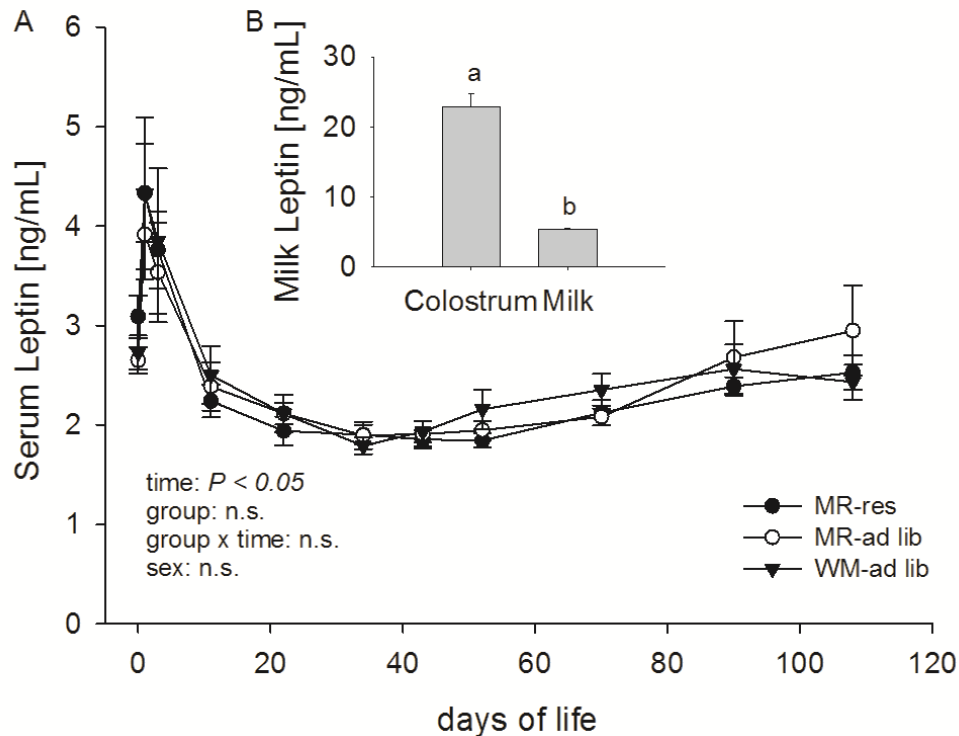

Figure 4: Time dependent changes of serum leptin (A; n = 57; Trial 1) and leptin concentration in colostrum and in milk from the corresponding dams (B) (means ± SEM). Small letters indicate differences between leptin concentration in colostrum and milk (*P < 0.01*).

The RQUICKI data are presented in Table 2 and Figure 3 C. The WM-ad lib group had lower RQUICKI values than the MR-res group ($P < 0.05$). No group differences were observed in Trial 2.

GTT and ITT. The basal concentration, the peak concentration, $\Delta_{Peak-Basis}$ and the AUC of insulin and glucose measured during the GTT are presented in Table 3.

Table 3: Variables (means ± SEM) from intravenous glucose tolerance tests (GTT) performed at different phases (P) of life from calves reared (d 4 – d 28 of life) at different feeding intensities.

	n = 51*	Basal concentration			Peak concentration			$\Delta_{Peak-Basis}$			Area under the curve (AUC)		
		MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib	MR -res	MR -ad lib	WM -ad lib
P1 (d 22)	Glucose [mmol/L]	4.9[bx] ± 0.23	6.3[ax] ± 0.20	6.2[ax] ± 0.23	9.3[xy] ± 0.69	9.4[xy] ± 0.24	10.3[xy] ± 0.74	4.5[Ax] ± 0.61	3.1[Bx] ± 0.11	4.0[ABx] ± 0.69	125 ± 11.1	112 ± 5.78	113 ± 8.73
	Insulin [µU/mL]	8.0[bx] ± 1.25	21.4[ax] ± 6.84	18.8[ax] ± 3.32	58.5[bx] ± 14.2	87.1[abx] ± 20.1	120[ax] ± 17.2	50.6[bx] ± 13.4	65.6[abx] ± 15.3	101[ax] ± 14.6	1,087[bx] ± 256	2,170[abx] ± 650	2,562[ax] ± 461
P2 (d 52)	Glucose [mmol/L]	4.9[y] ± 0.25	4.8[y] ± 0.15	4.9[y] ± 0.11	9.0[x] ± 0.27	9.1[x] ± 0.19	9.1[x] ± 0.22	4.1[xy] ± 0.22	4.2[xy] ± 0.12	4.2[xy] ± 0.24	122 ± 11.0	115 ± 7.49	112 ± 6.98
	Insulin [µU/mL]	7.09[y] ± 0.7	7.34[y] ± 1.1	6.23[y] ± 0.6	48.3[y] ± 7.3	68.1[y] ± 13.4	61.9[y] ± 7.4	41.2[x] ± 7.2	60.7[x] ± 12.8	55.7[x] ± 7.4	875[y] ± 122	1,161[y] ± 240	1,089[y] ± 119
P3 (d 108)	Glucose [mmol/L]	5.3[Y] ± 0.08	5.1[Y] ± 0.18	5.1[Y] ± 0.08	10.0[y] ± 0.11	9.7[y] ± 0.13	9.7[y] ± 0.08	4.6[y] ± 0.12	4.6[y] ± 0.18	4.6[y] ± 0.07	123 ± 5.20	139 ± 8.99	123 ± 4.95
	Insulin [µU/mL]	11.6[XY] ± 1.4	11.3[XY] ± 2.2	11.5[XY] ± 1.0	137[z] ± 14.7	108[z] ± 11.9	132[z] ± 13.5	125[y] ± 14.3	96.7[y] ± 12.5	121[y] ± 12.2	2,650[z] ± 274	2,465[z] ± 222	2,679[z] ± 256

P1: d 2 – 27 post natum (p.n.); P2: d 28 – 69 p.n.; P3: d 70 – 110 p.n.; *: at the time of the GTT planned, 6 calves were exempted from the tests due to minor health issues, data are thus limited to n = 51, not 57. a, b: different letters indicate differences between groups (a, b, c) or phases within each group (x, y, z) ($P < 0.05$); different small and capital letters indicate trends between groups (a, A, b, B) or phases within each group (x, X, y, Y, z, Z) ($P < 0.10$)

Differences between the groups were limited to P1. The basal concentrations of glucose and insulin were lower in the MR-res than in the MR-ad lib and WM-ad lib group ($P < 0.05$) and male calves tended to have greater basal insulin concentrations ($P < 0.1$). No differences were observed in the peak concentrations of glucose. However, the peak insulin concentration was lower in MR-res than in the WM-ad lib group ($P < 0.05$). $\Delta_{Peak-Basis}$ of glucose tended to be greater in the MRr than in the MRal group ($P < 0.1$) and the $\Delta_{Peak-Basis}$ of insulin was lower in the MR-res than WM-ad lib group ($P < 0.05$). The AUC of insulin was lower in the MR-res than in the WM-ad lib group ($P < 0.05$), however, no differences were observed in the AUC of glucose between groups and phases. In contrast, the AUC of glucose in male calves was greater than in female calves ($P < 0.05$). In table 4 the ISBGR from the ITT (done in males only) is shown. Only in the MRr group there was a decrease of the ISBGR from P1 to P3 ($P < 0.05$). No differences between the groups were observed and only a numerical decrease from P1 to P3 in the ad lib groups.

Table 4: The insulin-stimulated blood glucose response (ISBGR, means ± SEM) in response to an intravenous insulin tolerance test (ITT) performed at different phases (P) of life in male calves reared at different feeding intensities from d 4 to 27 of life.

	ISBGR [%]		
	MR-res (n = 9)	MR-ad lib (n = 7)	WM-ad lib (n = 7)
P1 (d 24)	$56.8^{x} \pm 4.8$	52.0 ± 3.2	52.8 ± 5.5
P2 (d 54)	$43.4^{y} \pm 3.3$	48.8 ± 3.3	45.0 ± 2.3
P3 (d 110)	$39.6^{y} \pm 4.1$	43.4 ± 3.6	43.2 ± 3.3

P1: d 2 – 27; P2: d 28 – 69; P3: d 70 – 110; MR-res = milk replacer restricted; MR-ad lib = milk replacer ad libitum; WM-ad lib = whole milk ad libitum. Different small letters indicate differences between phases ($P < 0.05$).

The mRNA abundance of PC and PCK1 is shown in Figure 5 A and B. Both mRNA abundance of PC and PCK1 increased from the first to the second biopsy ($P < 0.01$), but without any differences between feeding groups or sexes.
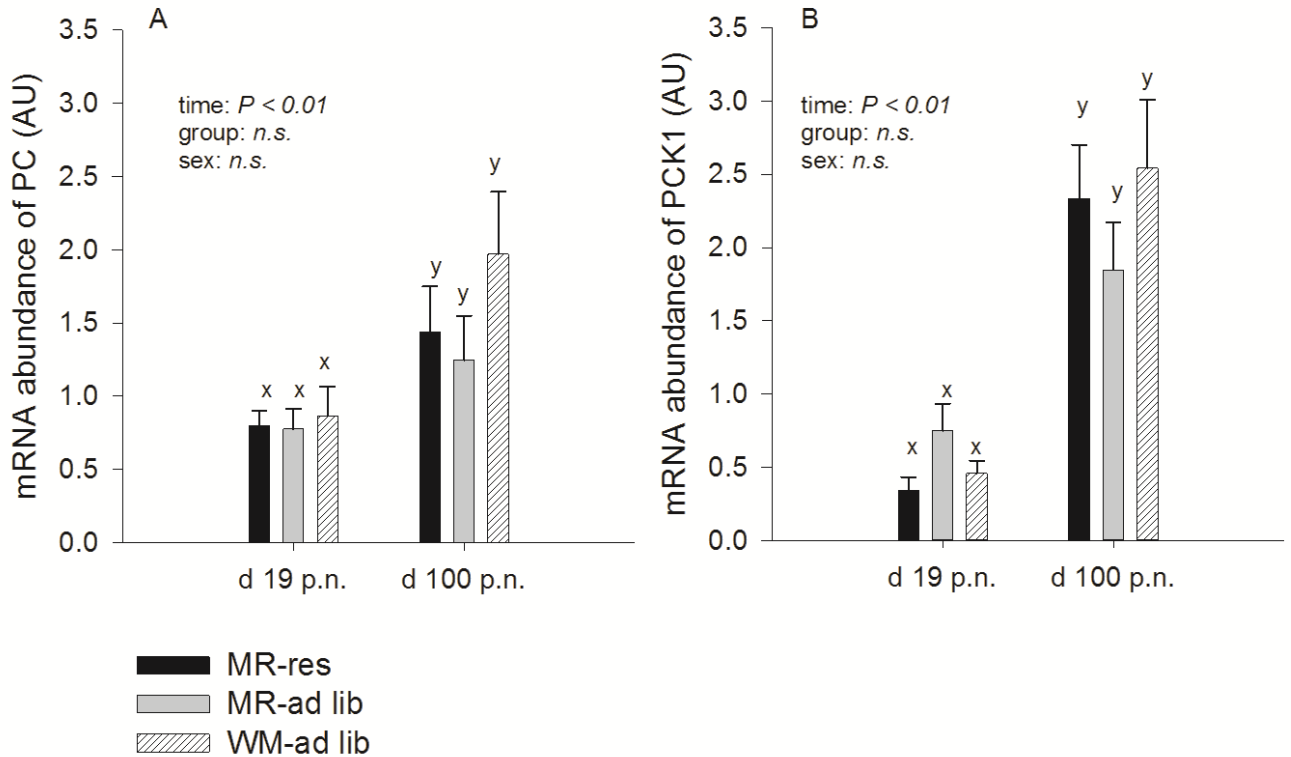
Figure 5: mRNA abundance of pyruvate carboxylase (PC; A) and phosphoenolpyruvate carboxykinase (PCK1; B) on d 19 and 100 post natum (p.n.). Different letters indicate differences between sampling time points ($P < 0.01$).

In Trial 1, there was a positive correlation between the RQUICKI values and the adiponectin concentrations across all samples ($\rho = 0.37$; $P < 0.01$). On d 0, before the first intake of colostrum, RQUICKI values and adiponectin were negatively correlated ($\rho = -0.32$; $P < 0.05$). This correlation changed to positive coefficients throughout the trial: on d 90 p.n. the correlation was $\rho = 0.42$ ($P < 0.01$). In Trial 2, RQUICKI and adiponectin were not correlated.

In colostrum, the adiponectin concentration were greater on d 1 than in milk from d 7 p.p. ($P < 0.001$; 76.7 ± 3.2 vs. 3.8 ± 0.3 µg/mL; n = 79; i.e. samples from Trial 1 and from 22 additional

animals). The correlation between the adiponectin concentration in colostrum and the serum adiponectin concentration of the calves after their first colostrum consumption was $\rho = 0.3$ ($P < 0.05$; n = 79; additional calves). The same correlation was seen with leptin in colostrum and in serum of calves after their first colostrum consumption ($\rho = 0.3$; $P < 0.05$, n = 57; Trial 1).

Discussion

The different feeding regimens tested in the present study affected the intake of both liquid and solid feeds as reported in the companion paper (Korst et al., submitted to JDS). In brief, the metabolizable energy (ME) from liquid feed during the differential feeding period (P1) was 1.9-fold higher in the ad lib fed groups than in the MR-res group. At the same time the intake of concentrate was low, thus confirming earlier findings that calves eat only little solid feed during the first wk of life (Khan et al., 2011). The intake of hay might have been different between the groups during calfhood and might thus have affected rumen development and performance as shown by Khan et al. (2012, 2016). Unfortunately we could not measure the intake of hay that was offered ad lib and thus we have to limit our discussion to the effects of concentrate intake. Albeit data about rumen and intestinal development could not be directly assessed, we have some indication from BHB concentrations in serum about rumen development as discussed below. In the lactating heifers, there were no differences in DMI, BW, body condition and energy balance, but the numerically greater milk yields in the ad lib groups may point to a more efficient nutrient utilization or stimulated development of the mammary gland in heifers that were reared ad lib, in particular those receiving WM during the first 4 wk of life. However, in view of the limited number of animals we could pursue until they were lactating, this consideration remains speculative.

Metabolic traits

The plasma concentrations of NEFA, glucose, BHB and TPP in the dairy calves are consistent with other studies (Hadorn et al., 1997; Hugi and Blum 1997; Hammon et al., 2002). Differences between groups were observed in P1 during the differential feeding. However, after all groups were adapted to the same feeding regimen, these differences disappeared in our study.

Similar adaptations were also reported in the literature (Hadorn et al., 1997; Rauprich et al., 2000; Prokop et al., 2015). The greater plasma glucose concentration occasionally observed in the ad lib fed dairy calves as observed in our study (Fig. 2) might be due to the higher intake of lactose, as observed in veal calves earlier (Hugi et al., 1997).

For BHB, differences between the feeding groups were limited to the time after differential feeding. When all calves were turned to the MR-res regimen (P2), the calves from the preceding WM-ad lib and MR-res feeding had greater BHB concentrations than the MR-ad lib group. Increasing BHB concentrations might indicate increased hepatic ketogenesis due to an increased supply or decreased oxidation of NEFA from mobilization of body fat. Pre-ruminant animals are basically capable of hepatic ketogensis when fasted, but the increase of BHB in the circulation is more likely related to the beginning ruminal production of ketones (Baldwin et al., 2004). The suitability of serum BHB as an indicator for grain intake and rumen development in calves was recently confirmed (Deelen et al., 2016). Indeed, the intakes of concentrate and the BHB serum concentrations when all calves were fed according to the MR-res protocol until weaning showed the same pattern: highest values in both variables were observed in the WM-ad lib and MR-res groups whereas the MR-ad lib group ate about 200 g/d less concentrate (Korst et al., companion paper), and had around 80% of the BHB concentrations. We had expected the MR-res group to have the fastest BHB increase and to differ from both ad lib groups since early restriction for liquid feed would stimulate the intake of concentrate. In contrast to this expectation, the BHB blood concentrations in calves from the WM-ad lib feeding group were not different from the MR-res group, but both groups had higher values than the MR-ad lib group. This finding might point to beneficial effects of WM feeding on rumen development by milk-borne stimuli. Rumen development could be directly or indirectly affected through promoting a rumen microbiome which in turn accelerates rumen development and might also concern the entire intestinal tract (Steele et al., 2016).

In the heifers, the metabolite concentrations in our study were typical for the transition period as observed earlier (Drackley, 2000; Wathes et al., 2007; Weber et al., 2013). There were no group differences in the plasma concentrations of NEFA or glucose. The BHB concentrations tended to be lower in the WM-ad lib group than in the MR-ad lib group in late pregnancy. In contrast to the findings during calfhood when BHB in blood likely reflects rumen development, BHB concentrations in blood of late pregnant and of lactating cows indicate mainly increased

ketogenesis. The observation of sporadically lower BHB concentrations in heifers reared on the WM-ad lib regimen might point to a greater capacity for the complete oxidation of NEFA in these animals, however, the values reached in all heifers were still in a normal range and the difference was transient only. Before calving, urea was lower in the WM-ad lib group than in the MR-res and MR-ad lib groups. It is unlikely that these differences resulted from feed composition and feed intake since all heifers received the same ration and the dry matter intake was not different (albeit intake could be recorded only post partum). The highest concentrations were observed in the MR-ad lib group and might point towards a less efficient ruminal microbiome. However, all values were well within the reference values suggested for late pregnant and Holstein cows (Brscic et al., 2015) and the difference between the groups disappeared after calving. Nevertheless the data may provide some support for a more efficient N utilization in WM-ad lib reared heifers. Taking the differences in blood urea a.p. together with the findings from BHB during calfhood, we speculate that WM feeding might thus be superior to both MR feeding regimens in terms of nutrient utilization. When taking the results from the metabolites together, they provide 418 some - albeit minor- support for our hypothesis that ab lib feeding of WM will be more beneficial than feeding MR ad lib. In general, the greater nutrient supply by ad lib feeding during the first weeks of life is now generally accepted as being beneficial for long term growth and productivity (Khan et al., 2011).

Hormones

The serum leptin concentrations in neonatal dairy calves increased with the first intake of colostrum in our study. The colostral leptin concentrations decreased from d 1 to 3 by factor of 4. In neonatal piglets, Woliński et al. (2014) reported a 3-fold increase of the leptin concentrations in plasma after the first feeding of colostrum. In the corresponding samples of sows' colostrum and milk, the leptin concentrations increased from d 1 to 3 p.p. and then decreased to d 7 p.p. (Woliński et al., 2014). Casabiell et al. (1997) have shown that leptin is transferred from the maternal circulation into the milk and through the stomach of neonatal rats into the bloodstream without a loss of biological activity. Leptin from colostrum and milk was suggested to be important for the development of the small intestine, since the maturation of the small intestine was slowed down when only formula was fed (Woliński et al., 2003). However, the leptin concentrations in colostrum and in plasma in neonatal piglets (Woliński et al., 2014), whereas we observed a weak correlation ($\rho = 0.3$; *P < 0.05;* n = 57) in dairy calves. The role of colostral

leptin seems less clear since the increase of serum leptin observed in the present study upon colostrum intake was not observed in two other studies (Blum et al., 2005; Schäff et al., 2014), but this might have been due to the relatively small animal numbers in these studies which did not allow for picking up the small and transient increase we were able to show herein. When comparing calves receiving only MR with colostrum-fed ones, the leptin concentrations were lower in MR-fed calves (Schäff et al., 2014). However, it is improbable that individual adipokines such as leptin or adiponectin out of a plethora of other bioactive components contained in colostrum would alone mediate the beneficial (not immune globuline related) effects commonly associated with colostrum intake.

In contrast to our previous study involving only 10 males and 10 female calves (Kesser et al., 2015) in which no sex difference was established for adiponectin during the first 110 d of life, the female calves in the present study (n = 29) had greater adiponectin concentrations than the males (n = 28; $P = 0.05$). In human babies, data concerning sex differences were contradictory (Sivan et al., 2003; Kamoda et al., 2004; Erhardt et al., 2014).

The trend to lower adiponectin serum concentrations in the MR-res fed calves compared to the MR-ad lib calves indicated a reduced insulin sensitivity in the MR-res group, however, the results of the GTT (as discussed below) contradict this finding. The nadir of adiponectin around parturition in the young heifers is in line with other studies (Giesy et al., 2012; Mielenz et al., 2013; Singh et al., 2014) and may be interpreted as a support for the nutrient supply towards the mammary gland by decreasing the insulin sensitizing, gluconeogenesis and lipolysis inhibiting effects (Yamauchi et al., 2002; Kadowaki et al., 2006; Singh et al., 2014). In addition, the decreased adiponectin concentrations around calving might result from the increased secretion of blood adiponectin into colostrum (Singh et al., 2014). However, only a weak positive correlation ($\rho = 0.3$; $P < 0.05$; n = 79) was seen between the plasma adiponectin concentrations and the colostrum adiponectin concentrations.

In intensively fed calves the insulin concentration increases (Hadorn et al., 1997; Hammon and Blum, 1998; Kühne et al., 2000), but is commonly not sustained when the animals are moved to less intensive feeding regimen. The increased insulin concentration in the ad lib groups from the present study was probably due to the greater amounts of ingested energy compared to the MR-res group as reported earlier in veal calves (Hugi et al., 1997, Maccari et al.,

2014). Decreased insulin concentrations around parturition as observed in the heifers from our study and reported previously (Swali and Wathes, 2006; De Koster and Opsomer, 2013) point to reduced lipogenesis and protein synthesis and enhanced lipolysis and thus support the flux of glucose and amino acids to the mammary gland (De Koster and Opsomer, 2013). However, the insulin concentrations were not affected by the rearing conditions during the first weeks of life which is in contrast to studies in rats where intensive feeding in early life stimulated the development of the pancreatic cells and lead to higher insulin concentrations in later life (Srinivasan et al., 2003). In intensively fed male German Holstein calves an increase in the number of the islets of Langerhans was observed after 8 mo of life (Prokop et al., 2015). In early life the pancreatic cells as well as the adipose tissue (AT) continue to develop and to establish the total number of cells (Kaung, 1994; Spalding et al., 2008). Therefore, these tissues serve as potential targets of metabolic programming (Mostyn and Seymonds, 2009; Duque-Guimaraes and Ozanne, 2013; Barella et al., 2014).

However, greater milk yields after an intensive feeding regime in the preweaning period were reported in several studies (Bar-Peled et al., 1997; Moallem et al., 2010; Soberon et al., 2012). In our study, the milk yield of the WM-ad lib group was numerically higher as shown in our companion paper (Korst et al., submitted as companion paper). Another possibility for the increased milk yield after intensive feeding regimens in the preweaning period might be the increased mammary parenchymal mass, parenchymal DNA and RNA (Brown et al., 2005). A positive influence of intensive feeding regimen in the preweaning period on the mammary gland parenchyma was shown previously (Brown et al., 2005; Meyer et al., 2006; Geiger et al., 2016). Tucker (1981) stated that one of the primary determinants for milk production is the number of cells available for synthesis of milk. Before puberty, the mammary gland grows allometric (Esselburn et al., 2015) and therefore the mammary gland might be more sensitive to external stimuli compared to the remaining tissues. The influence of different feeding regimens before puberty might have long term programming effects on the development of the mammary gland and therefore on the life time milk yield production of dairy cows.

Variables describing insulin sensitivity

In the dairy calves from the present study, the GTT variables suggested a higher insulin sensitivity in the MR-res group compared to the ad lib groups in P1. The trend for higher RQUICKI values, as surrogate indicator for insulin sensitivity, in the MR-res group compared to the WM-ad lib group supported the latter result. The accordance between GTT variables and RQUICKI values in dairy calves was confirmed earlier (Bossaert et al., 2009).The ISBGR from the ITT did not differ between groups and in the MR-res group a significant decrease was only observed from P1 to P3 indicating decreased insulin responsiveness (Ohtsuka et al., 2001). In contrast, the trend for lower plasma adiponectin concentrations in calves of the MR-res group suggests lower insulin sensitivity in the MR-res group than in the ad lib groups. Some reports confirm that insulin sensitivity is decreased by more intensive feeding levels (Bach et al., 2013; Yunta et al., 201), whereas others could not confirm such effects (MacPherson et al., 2016). The divergent results from these studies including ours might be explained by differences in the age when the feeding regimens were started as well as the setting of the GTT performed i.e., the fasting time before starting the GTT and also the age when the GTT were performed. Additional factors likely influencing the response are the amount and composition of the MR used, and the feeding frequency (usually 2 times daily versus the free access in our study resulting in average meal sizes of about 1.3 kg).

In the heifers, no group differences in RQUICKI values were observed. However, a slight decrease around parturition might indicate a decreased insulin sensitivity and hence supports the previous findings (Holtenius and Holtenius, 2007; Singh et al., 2014). In dairy cows, Singh et al. (2014) demonstrated a positive correlation between plasma adiponectin concentrations and RQUICKI values. This was not confirmed in our study. However, in dairy calves, we found a negative correlation during P0, P1 and P2. In contrast to tissues of non-ruminant animals, the tissues of dairy cows seem to be less sensitive to insulin (Brockman and Laarveld, 1986). Adiponectin is known for its insulin sensitizing effects (Berg et al., 2002). However, the change from a negative correlation between adiponectin and RQUICKI values in calves to a positive correlation in dairy cows (Singh et al., 2014) probably occurs after weaning. The adiponectin system in pre-ruminant calves might thus be still developing and be not yet mature. Moreover, the adiponectin system might have different functions than increasing insulin sensitivity at that age. Therefore, measuring adiponectin in calves seems inappropriate to infer information about insulin sensitivity.

Taken together, even though high feeding planes were shown to alter insulin sensitivity and glucose metabolism in some studies including ours, if tested in later life, these effects seem transient only and there is no evidence for sustained effects. Our results about the various hormones are not supporting our initial hypotheses according to which the ad lib feeding, in particular of WM, will elicit sustained changes of metabolic hormones that will continue until lactation and promote milk production. Bearing in mind that the small sample size available for Trial 2 may have impeded the detection of sustained differences, transient nature of the hormonal and metabolic changes induced by the feeding during the first weeks of life, they might nevertheless have affected the development of target tissues, in particular the development of gastrointestinal tract and the mammary gland as discussed above.

mRNA abundance of PCK1 and PC

The increase of the mRNA abundance of PC and PC1K from the first to the second biopsy observed herein indicates an increase of gluconeogenesis that is probably related to the switch from the pre-ruminant to the ruminant stage. The main trigger of this switch is the change of substrates from lactose to short chain fatty acids, in particular propionate (Greenfield et al., 2000; Aschenbach et al., 2010; Steinhoff-Wagner et al., 2011). As observed in our study and in earlier ones (Scheuer et al., 2006; Steinhoff-Wagner et al., 2011), different feeding regimens in the preweaning period had no influence on the mRNA abundance of PC and PCK1, indicating that the endogenous glucose production was not affected.

CONCLUSION

In times of differential feeding (d 4 - 27 p.n.) differences in the circulating concentrations of some metabolites and hormones were observed between groups. However, in contrast to our working hypothesis, these differences were not sustained when all calves received the same feed later on. Moreover, when considering the animals as pregnant and then lactating heifers, their endocrine and metabolic patterns were not different. Therefore, no programming effects on metabolism and its endocrine regulators could be identified. However, in view of the numerically higher milk yield in heifers raised at ad lib-feeding during the first weeks of life, programming

effects on gastrointestinal function or the cellular development of the mammary gland may not be ruled out but should be addressed in further studies involving more animals.

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6   General discussion and conclusions

Circulating adiponectin in neonatal dairy calves

The adiponectin concentrations in dairy calves during their first three months of life were characterized in the present thesis. After birth, the adiponectin concentrations in our study were about 7-fold lower compared to dairy cows around parturition (3.0 µg/mL ± 0.3; Mielenz et al., 2013; Singh et al., 2014 a, b). With the intake of colostrum, the adiponectin concentrations increased rapidly. In contrast, in dairy calves receiving formula instead of colostrum, only a slow increase of the adiponectin concentration was observed. The high adiponectin concentrations in the first colostrum in comparison to formula underline the importance of colostrum for the transfer of adiponectin from the dam to the offspring, albeit the function remains unexplained. After colostrum consumption, the adiponectin concentration stayed at about the same level until d 52 p.n., thereafter the calves were weaned and an increase was observed. The increase in adiponectin might be because of the increase in insulin sensitivity due to the change from pseudo-monogastric to ruminants (Brockman and Laarveld, 1986), as adiponectin is positively associated with insulin sensitivity in humans and rodent models (Berg et al., 2002).

The half life of adiponectin in mice is between 4.5 h (hexamers) and 9 h (HMW) (Pajvani et al., 2003). However, the time between blood samples in Trial 2 of Manuscript I is about 24 h, hence a decrease of the adiponectin concentrations between the sequential blood samples was expected in consideration of colostral adiponectin being the dominant source for adiponectin in the circulation. Potential reasons for the sustained elevation of serum adiponectin might be that the half life of adiponectin taken up from colostrum is extended or the colostral adiponectin might trigger the endogenous production of adiponectin in AT whereby not only WAT but also BAT may be involved. The BAT plays the major role in neonates and is relevant for heat production. Like WAT, BAT is known for its endocrine function, e.g. the expression of adiponectin (Iacobellis et al., 2013; Villarroya et al., 2013). Whether the adiponectin secretion of BAT and/or WAT is triggered by hormones reaching the circulation from colostrum and milk intake is not clarified.

In a study by Kotani et al. (2004), adiponectin was positively correlated with BW in human neonates. The authors concluded that in contrast to adults, adiponectin increases with fetal fat mass and might depend on the adipocyte size and body fat distribution. In our study there was no correlation between the BW of dairy calves and the serum adiponectin concentration. However, in comparison to humans, who have the highest body fat content as neonates in mammals (around 16 %; Widdowson, 1950), dairy calves only have a low content (2 %; Marple, 2003).

Concerning the influence of sex on the adiponectin concentration we found contradictory results. As reported in Manuscript III, female calves had greater adiponectin concentrations than male calves, whereas in Manuscript I, in which only the control group fed according to the MRr from Manuscript III was integrated, no sex differences were observed. Contradictory observations concerning the differences between sexes on the adiponectin concentration were also made in humans (Erhardt et al., 2014; Lausten-Thomsen et al., 2015). However, the different results from Manuscript I and II are probably based on the larger animal numbers included in Manuscript III.

Estimation of the portions of adiponectin and leptin contained in colostrum reaching the circulation of newborn dairy calves

For getting an impression about the portion of adiponectin and of leptin that was ingested with colostrum and milk and actually reached the circulation as evident from the increase of the plasma concentrations observed in the dairy calves studied herein, the following estimates were done, using the assumptions explained below for adiponectin (results are shown as means ± SEM):

1. *Total amount of adiponectin in the plasma in the circulation* = plasma volume of the calf (9 % of BW (mL), Quigley, 2002) x plasma adiponectin (µg/mL)

2. *Total amount of adiponectin (µg) in the first ingested colostrum* = Total amount of ingested colostrum (mL) x adiponectin (µg/mL) in colostrum (in consideration of gut closure, only the first meal was considered as contributing to adiponectin in the circulation)

3. *Percentage of the increase of plasma adiponectin concentration (µg/mL) due to the first colostrum from the ingested adiponectin (µg/mL) concentration in the first colostrum (transfer rate)* = increase of plasma adiponectin (µg/mL) concentration due to the first colostrum / total amount of adiponectin (µg) in the first ingested colostrum x 100

The estimation was analogously done for leptin (ng).

The total intake of colostrum containing ∼76 µg/mL (± 4.2) adiponectin was in average around 2.3 L (with the first meal) and thus the uptake of adiponectin with colostrum amounted to 175 mg. The total adiponectin content in the circulation was about 26 mg, i.e. around 15 % of the ingested adiponectin.

For leptin, colostrum contained ∼23 ng/mL (± 1.9) in average and thus, the uptake of leptin with colostrum (∼2.3 L with the first meal) amounted to 0.05 mg. The total leptin content in the circulation was about 0.006 mg. This corresponded to 12 % of the ingested leptin.

The large variation from these calculations reflects the intra-individual variation. The weak correlations between the adipokine concentration in the first colostrum and the plasma adipokine concentration in the calves' circulation after the first colostrum intake (Manuscript III) are in support of a relationship between intake and the circulating concentration achieved, albeit with considerable variability.

Formula contained 0.38 µg/mL in average. In dairy calves receiving formula as a first meal instead of colostrum (Manuscript I, Trial 2), the uptake of adiponectin with formula (∼3.6 L with the first meal) amounted to 3.6 mg. The total adiponectin concentration in the circulation was about 12.6 mg. This means that the increase of plasma adiponectin was higher than the amount of ingested adiponectin in formula and may thus indicate that this slight increase of adiponectin in plasma might be due to endogenous production of adiponectin.

In calves born at term and preterm and receiving their first colostrum only after 24 h of life (Manuscript I, Trial 3), the adiponectin concentration is colostrum contained 56.1 µg/mL. The uptake of first colostrum (∼2.3 L and ∼2.1 L for at term and preterm born calves, respectively) amounted to 129.7 mg and 115.0 mg, respectively. In the blood circulation the amount of adiponectin was about 10.5 mg for calves born at term and 6.2 mg for calves born preterm. This indicates that the intestinal barrier was closed after 24 h and none of the adiponectin from colostrum could be found in the circulation of these calves.

However, it still needs to be elucidated to what extent adipokines, like adiponectin and leptin, in the milk have an influence on the development of metabolism of dairy calves, and how the endogenous production is triggered.

The influence of different feeding regimens on the circulating adiponectin concentrations in dairy calves

The influence of three different feeding regimens on the adiponectin serum concentrations of dairy calves was tested. The restrictive feeding of milk replacer (MR 130 g/L, 6 L per day) is the common practice in many countries. The other two groups were to compare two different intensive feeding forms. The MR-ad lib group received milk replacer at a higher concentration (160 g/L) ad lib and the WM-ad lib group received whole milk ad lib. Even if the ingredients were quite similar, there are a many components in the whole milk which have not yet been investigated, let alone the influence of the components on the further development of the offspring.

Dairy calves receiving MR in restricted amounts tended to have lower adiponectin concentrations compared to calves receiving milk replacer ad lib. During the time of differential feeding (P1), the adiponectin concentration was even significantly lower in the MR-res group than in the MR-ad lib and WM-ad lib group. In P1 the energy intake via milk in the WM-ad lib group was 1.8-fold and 2.1-fold greater than in the MR-ad lib group and in the MR-res group, respectively. In contrast, the energy intake via concentrates was greater in the MR-res group (3.4-fold and 5.3-fold greater than in MR-ad lib and WM-ad lib, respectively). In colostrum, much higher adiponectin concentrations than in milk (23-fold lower adiponectin concentrations) or milk replacer (190-fold lower) were observed whereby the concentrations in milk decreased with time as observed in human milk (Ley et al., 2012). As alike observed for leptin (Woliński et al., 2003; Bronský et al., 2012), the greater adiponectin concentrations in colostrum in milk might influence gut development, especially the number of adipoR1 in the small intestine (Zhou et al., 2005; Bronský et al., 2012). The lower milk intake in P1 and therefore lower adiponectin and milk energy intake might have resulted in lower adiponectin serum concentrations in the restrictively fed group. The greater milk and energy intake likely increased the higher average daily gain in the ad lib groups in P1 and might thus be another reason for the higher adiponectin concentration in these groups.

Relationship between adiponectin and insulin sensitivity in dairy calves and the influence of different feeding regimen

For identifying the influence of the different feeding regimen on the insulin sensitivity of the dairy calves, GTTs were performed during the trial. Except for P1, no differences were observed. However, in P1 the greater basal and peak concentrations in the ad lib-groups indicated a higher insulin secretion in both groups and assumingly lower insulin sensitivity than in the restricted fed group. The MR-res group had also the highest RQUICKI values thus supporting the results from the GTT.

The lower adiponectin concentrations of the MR-res group indicate opposite conclusions compared to the results of the GTT and the RQUICKI. Adiponectin is known for its insulin sensitizing functions (Berg et al., 2002). In dairy cows a positive correlation between adiponectin and RQUICKI was observed (Singh et al., 2014a, b). However, in the preweaning period of the calves, the adiponectin concentration was negatively correlated with RQUICKI values and by the end of the trial, when calves were fully weaned, the correlation of adiponectin and RQUICKI values turned to positive. These results lead to the assumption that adiponectin cannot be used as an indicator for insulin sensitivity in growing dairy calves.

During the process of weaning in dairy cows the main supply of glucose changes from the direct intestinal absorption to hepatic and renal gluconeogenesis. In contrast to tissues of non-ruminant animals, the tissues of dairy cows seem to be more sensitive to insulin (Brockman and Laarveld, 1986). However, the change from a negative correlation between adiponectin and RQUICKI values in calves to a positive correlation in dairy cows (Singh et al., 2014a) occurs during weaning and lead to the assumption that with the change to gluconeogenesis as the main supply of glucose the insulin sensitivity in tissues of dairy cows increases. Adiponectin in pre-ruminant calves might have different functions than increasing insulin sensitivity. Adiponectin might support the differentiation of adipocytes in the growing calves as observed in growing mouse 3T3-L1 fibroblasts cells (Fu et al., 2005). Another possible role for adiponectin might be an anti-inflammatory effect on the development of the digestive system from pre-ruminants to ruminants. Before weaning the main feed is milk and therefore lactose is converted to glucose. After weaning, the intake of concentrates and thus of starch increases and in consequence, the production of volatile fatty acids (i.e. acetate, propionate, and butyrate) in the rumen. The lower

adiponectin concentrations in the MR-res group might be because of the greater intake of concentrates and less intake of forage. In contrast to forage, concentrates with high starch content have negative effects on the rumen development (Williams et al., 1987). This might lead to an increase of pro-inflammatory cytokines which suppress the synthesis of adiponectin (Maeda et al., 2002; Fasshauer et al., 2003).

Influence of different feeding regimen on adiponectin and on variables indicative for insulin sensitivity around the first lactation

Most changes of the adiponectin concentrations in dairy cows occur during the transition period (Singh et al., 2014a). Until calving, the adiponectin concentration decreased, thereafter an increase was observed. The RQUICKI had similar characteristics around calving (Holtenius and Holtenius, 2007; Singh et al. 2014a). In contrast to Singh et al. (2014a) we observed no correlation between adiponectin and RQUICKI, although the RQUICKI was decreased around calving, too. Even though the RQUICKI and adiponectin have the same characteristics around calving, a correlation does not mean that the parameters are linked and can be replaced by each other. The RQUICKI is just a calculated ratio and several studies doubted its use as an indicator for insulin sensitivity in dairy cows with metabolic disorders (Kerestes et al., 2009; Schulz et al., 2014).

However, the decrease of adiponectin around parturition might support the glucose flux towards the uterus and the mammary gland by decreasing the insulin sensitivity in other peripheral tissues and thus the uptake of glucose, and by stimulating hepatic gluconeogenesis (Yamauchi et al., 2001, 2002; Singh et al., 2014a). The greater adiponectin concentrations in colostrum and milk might be due to an increased transfer of blood adiponectin to colostrum and milk (Singh et al., 2014a). Based on the very low abundance of adiponectin mRNA detectable in the mammary gland it is unlikely that the mammary gland itself contribute a significant portion of the concentrations in milk (Sauerwein and Häußler, 2016).

The feeding regimen in the first weeks of life had no sustained influence on the concentrations of adiponectin and of insulin and on the RQUICKI values around calving.

Nevertheless, greater insulin concentrations were observed in rats after an intensive feeding in early life (Srinivasan et al., 2003). The authors suggested that a stimulation of the pancreatic cells triggers the elevated insulin secretion (Srinivasan et al., 2003). Increased numbers of Langerhans

islets after an intensive feeding was reported recently in 8 months old male Holstein dairy calves (6 - 9 L milk/per day for the first 3 weeks p.n. compared to the control group: 4 L milk/per day for the first weeks p.n. and 6 L MR/per day for second and third week p.n.; Prokop et al., 2015). Adipose tissue as well as pancreatic cells are most relevant target tissues when considering metabolic programming, since the development of cells and their number is determined in early life in both tissues (Kaung, 1994; Spalding et al., 2008).

Based on the lack of differences in insulin and the other metabolic and endocrine variables after differential feeding in the preweaning period in our study, we suggest that no programming effects on these parameters were set in our study. However, the numerically higher milk yield in the WM-ad lib calves together with the increase of milk yield after an intensive feeding during early calfhood reported in literature (Bar-Peled et al., 1997; Moallem et al., 2010; Soberon et al., 2012) point towards mechanisms other than metabolic programming underlying the effects on increased milk yield in the dairy cow.

One of the primary determinants for milk production is the number of cells available for milk synthesis (Tucker, 1981). Several studies observed an increase in mammary gland parenchyma after dairy calves were fed intensively before puberty (Brown et al., 2005; Meyer et al., 2006; Geiger et al., 2016). Until puberty the mammary gland grows allometrical, thereafter an isometric growth is observed (Cowie, 1949). Esselburn et al. (2015) observed during the first 2 months of life a 6-fold increase of the mammary gland, whereas BW did not even double during this time, i.e. the mammary gland growing obviously allometrical. This intensive growing phase of the mammary gland parenchyma prior to puberty might be another starting point for programming effects on the later life's performance in dairy cows because the allometric growth of the mammary gland might be sensitive for external stimuli.

Male German Holstein calves are a kind of 'by-product' of the milk industry and therefore are reared as veal calves although their breed is bred for milk production. A liquid diet with high fat content ensures a faster growing until the required weight for slaughter is reached (Doppenberg and Palmquist, 1991). In veal calves, insulin resistance is an often observed status, due to the high intake of milk or milk replacer and therefore lactose (Hostettler Allen et al., 1994; Hugi and Blum, 1997). In a recently published study, feeding male Holstein calves with WM ad lib lead to an increase in pancreatic Langerhans islets after 8 months of life (Prokop et al., 2015). Higher

insulin concentrations and insulin resistance during calfhood may have an impact on health in adult animals. The higher concentration of insulin due to a higher number of Langerhans islets may also lead to an increasingly insulin resistant status in adult animals and therefore, comparable to humans, metabolic problems may occur. However, considering the productive life span of male cattle, this might be an issue only in breeding bulls.

Bossaert et al. (2009) suggested that there are breed specific differences in insulin sensitivity, since beef calves have a higher insulin sensitivity compared to German Holstein calves which are bred for milk production. They assumed that the intensive selection for either growth rate or milk yield in beef and dairy cows, respectively, was accompanied by a selection for a greater insulin sensitivity when aiming for tissue accretion (beef cattle); on the other hand a selection for higher milk yield (dairy cattle) implied a lower insulin sensitivity of the peripheral tissues (Bossaert et al., 2009). However, from the scientific point of view it would be interesting to investigate intensive feeding, e.g. in veal calves and therefore triggering an insulin resistant status in early life, and the influence on health in later life.

Influence of different feeding regimen on performance and metabolic and endocrine variables in dairy calves and later around their first lactation

Ad libitum feeding of WM or MR increased the average daily weight gain and the energy intake during times of different feeding regimen and the BW until 13 weeks p.n. Starter intake was not influenced by the higher intake of WM or MR. Although there was no difference in the feed intake during the first lactation, a numerically increase of the milk yield was observed after whole milk was fed ad lib during the first weeks of life. The observed greater milk yield of the WM-ad lib group could not be explained by the measured blood metabolites in our study. However, an increase of milk yield after in intensive feeding during in the preweaning period was also reported in literature (Bar-Peled et al., 1997; Moallem et al., 2010; Soberon et al., 2012). Parallel to the increase of milk yield in the last decades (Oltenacu and Broom, 2010), an increase in health problems, especially reduced fertility, lameness and mastitis, but also metabolic disorders were observed (Ingvartsen, 2006). Whether there is a positive correlation between increased milk yield due to intensive feeding and health problems is not yet known.

The intensified feeding strategies may have economic benefits improving the income from milk and thus the profit from the first lactation. However, milk price versus feed costs will certainly affect the final extent of the benefit, if any.

Only in times of differential feeding (d 1 - 27 p.n.) differences in the metabolic variables (NEFA, glucose, BHB and TPP) were observed. After the feeding regimens of all groups were adjusted to the MR-res protocol, the differences were leveled off. Leptin increased significantly after colostrum intake, indicating a colostrum-depending leptin supply as observed earlier for the adiponectin concentrations (Kesser et al., 2015). An increase of leptin due to the first intake of colostrum was also observed in piglets (Woliński et al., 2014). In heifers no group differences in NEFA and glucose were observed. Differences were seen before parturition (P4) in the urea and BHB concentrations. WM-ad lib heifers had lower urea concentrations than the MR-res and MR-ad lib groups ($P = 0.1$ and $P < 0.05$, respectively) and tended to have lower BHB concentrations then the MR-ad lib group ($P = 0.1$). The feed intake prior to calving was not measured. The lower urea concentrations might indicate an increased metabolisation of protein in the WM-ad lib group. The tendency of higher, but not clinical relevant BHB concentrations in the MR-ad lib group before calving, might indicate a greater ketogenesis due to a higher physiological energy demand before calving compared to the WM-ad lib group, perhaps because of a lower gluconeogenesis.

Future perspectives

The potential influence of preventing the uptake of adiponectin with colostrum in formula-fed calves on the development of the circulating adiponectin concentrations could not be investigated in the present work. The calves fed exclusively with formula were maximally 4 days old when euthanized for tissue sampling and thus no data from formula-fed calves at older ages were available. Further studies are needed to identify the development of adiponectin without the maternal supply of colostrum or milk for a longer duration of life to better understand the regulation of the endogenous production of adiponectin and the role of adiponectin in calves. However, conflicts may occur because colostrum-free rearing is certainly not animal friendly as the colostrum provides next to the passive immunity a wide range of important nutrients and non-nutritive bioactives for the offspring (Blum, 2006).

As we found contradictory results concerning the influence of sex on the adiponectin concentration, a focus on this aspect is recommend in further studies. However, the greater number of animals in Manuscript III as compared to Manuscript I might account for this result. The lack of significant differences in metabolic and endocrine variables as well as in the performance data, like milk yield may be due to the low number of animals in each group (min: n = 9; max: n = 10). Soberon and van Amburgh (2012) conducted a meta-analysis and evaluated the effects of preweaning nutrition on milk yield in the first lactation. They assumed that the missing significance in several studies for a positive influence of intensive feeding on milk yield was due to a low number of animals per group and therefore lack of power. Finally by taking all data together, they found a stimulating influence of milk intake in the preweaning period on milk production during lactation.

For identifying the effect of different feeding regimen on metabolism and the related endocrine systems as well as on performance, more data need to be generated in neonatal dairy calves and later in their life when lactating and thus further studies are recommended with a greater number of participants. Next to regular blood samples, biopsies of the mammary gland should be sampled for identifying possible feeding influences. For measuring or estimating the insulin sensitivity, direct measurements (e.g. HEC-Test) or indirect measurements (eg. GTT or ITT) of insulin sensitivity should be conducted. In addition, for getting a better insight into the metabolic programming of different feeding regimen in the preweaning period on the later milk production, dairy cows should be observed also for the following lactations.

7   Summary

In the transition from pregnancy to lactation, dairy cows exhibit a period of decreasing insulin sensitivity in the peripheral tissues to support the partitioning of glucose towards the uterus and the mammary gland. During this time the susceptibility for metabolic disease increases as the main priority is the support of the offspring. Improvements in nutrition, management and genetic merit achieved in the past, have led to an increase of milk production but the incidence of diseases increased simultaneously. An intensive feeding of dairy calves in the preweaning period showed positive effects on the later milk production. The influence of nutritional stimuli during critical periods in early life and its long lasting effects on later health and performance is called metabolic programming. Furthermore studies have shown that an intensive rearing program increases the number of Langerhans islets in rats and dairy calves but also the parenchymal mass of the mammary gland.

Adiponectin is one of the most abundant adipokines in the circulation and is known for its insulin sensitizing effects. In dairy calves, the circulating adiponectin concentrations were not investigated until now, whereas it was known that in dairy cows the adiponectin concentrations decrease around parturition, probably to support the glucose flux to the mammary gland. As to whether different feeding methods in the preweaning period may influence the circulating adiponectin concentrations in dairy calves and later around the first parturition was to be tested in this thesis.

Insulin supports the cellular glucose uptake. During the transition period, the glucose demand for the offspring and later on for milk production increases. For supporting the flux of glucose to the uterus and mammary gland, the insulin sensitivity of the muscle cells and adipocytes decreases, and higher insulin concentrations are commonly observed in the circulation.

In this thesis we investigated 1) the adiponectin concentration in dairy calves and the influence of colostrum, 2) the influence of different feeding regimen in the rearing period on performance data, and on metabolic and endocrine parameters, and 3) the relation between performance and the metabolic and endocrine profiles.

In the Manuscript I we aimed to characterize the adiponectin concentration in dairy calves and the influence of colostrum. For this purpose, samples of three trials were used. In the first trial

(Trial I-1), 20 German Holstein calves (10 males and 10 females) were fed right after birth with colostrum from their own dam for 3 d. On d 1 the calves had ad libitum (ad lib) access and on d 2 and 3 the amount was restricted to 6 L. Thereafter milk replacer (MR) was fed in a restricted form (130 g MR /L and 6 L per calf per day) until weaning on d 56 post natum (p.n.). On d 70 p.n. all calves were fully weaned and had ad lib access to a total mixed ration (TMR) until the end of the trial (d 110 p.n.). From d 4 p.n. until the end of the trial, all calves received concentrates and had ad lib access to hay and water. Blood samples were taken on d 0 before first colostrum consumption and on d 1, 3, 11, 22, 34, 43, 52, 70, 90, 108 p.n. Colostrum samples were taken from the dam on d 1 and 3 post partum (p.p.). In the second trial (Trial I-2) 14 German Holstein Calves were divided into two groups right after birth. The first group received colostrum and the second group a milk based formula on d 1 p.n. (8 % of body weight) and on d 2, 3 and 4 p.n. (10 % of body weight). The calves in both groups were slaughtered on d 4 p.n., two h after the last food intake. Blood samples were taken before the first feeding, from d 2 to 4 p.n. before morning feeding and additionally before slaughtering on d 4 p.n. On each day samples of the pooled colostrum or formula were taken. In the third trial (Trial I-3) 14 German Holstein calves were born preterm (9 days before anticipated calving date by cesarean section) or after normal gestation length at term. Both groups received pooled colostrum (5 % of body weight) 24 h p.n. After 26 h p.n. all calves were slaughtered. Blood samples were taken immediately after birth, before feeding and before slaughtering. Additionally allantoic fluid and blood samples were taken of 4 German Holstein cows undergoing caesarian section.

In all samples adiponectin was measured with in-house developed ELISA specific for bovine adiponectin and via Western blot the molecular weight forms (MW) of adiponectin were determined.

In the Manuscripts II and III we investigated the influence of different feeding regimen in the preweaning period on performance (Manuscript I) and on metabolic and endocrine variables (Manuscript III) in dairy calves and around the first lactation. In Trial II-1, 57 German Holstein calves (28 males and 29 females) were fed right after birth with colostrum from their own dam for 3 d p.n. Thereafter calves were allocated to three feeding groups: MR-res (Milk replacer restricted 130 g MR /L and 6 L per calf per day, n = 20, i.e. the same animals as studied in Manuscript I, Trial 1-I), MR-ad lib (milk replacer ad lib, n = 17) and WM-ad lib (whole milk ad lib). All calves had ad lib access on colostrum on d 1 and on d 2 and 3 the amount was restricted

to 6 L for the MR-res whereas MR-ad lib and WM-ad lib had ad lib access. Thereafter groups were fed according to their respective feeding regimen. From d 25 p.n. the ad lib groups were gradually adapted to the feeding regime of the MR-res group and continued on MR-res feeding until weaning started on d 56 p.n. all calves received the same feeding regime. On d 70 p.n. all calves were fully weaned and had ad lib access to a TMR until the end of the trial (d 110 p.n.). From d 4 p.n. until the end of the trial calves received concentrates and had al access to hay and water. Blood samples were taken on d 0 before first colostrum consumption and on d 1, 3, 11, 22, 34, 43, 52, 70, 90, 108 p.n. An GTT was done on d 22, 52 and 108 p.n. and an ITT (only male calves) on d 24, 53 and 110 p.n. A liver biopsy was taken on d 19 and 100 p.n. Colostrum samples were taken from the dam on d 1 and 3 post partum (p.p.). Additionally, regular data concerning daily weight gain, body weight, food intake, and economics were recorded.

In Trial II-2, 28 heifers from the 29 that were participating in Trial I-1, were further investigated in terms of performance, including ecomomic outcomes and in terms of various metabolic and endocrine variables. Blood samples were collected 3, 2, and 1 month ante partum (a.p.), at calving and from wk 1-10 p.p.

Both trials were divided in phases (P): P0: d 0 – 1 p.n.; P1: d 2 - 27 p.n.; P2: d 28 - 69 p.n.; P3: d 70 - 110 p.n.; P4: 3, 2 and 1 month a.p., P5: calving until 3 wk p.p., P6: 4 - 10 wk p.p.

In the blood samples of the calves, non-esterified fatty acids (NEFA), glucose, beta-hydroxybutyrat (BHB), total protein content (TTP), adiponectin, leptin and insulin were determined. In the heifers, NEFA, glucose, BHB, urea, adiponectin and insulin were measured.

In Manuscript I we characterized the adiponectin concentration in dairy calves. A low adiponectin concentration (3 µg/mL ± 0.3) was observed after birth. With the intake of colostrum the adiponectin concentration increased 3.5-fold and 4.7-fold from d 0 to 1 and 3 p.n. and remained constant until d 52 p.n. when a second increase was observed. In colostrum the adiponectin concentration was higher than in milk (23-fold difference) or even milk replacer (190-fold difference). Calves receiving formula instead of colostrum had only a slow increase of adiponectin. Preterm calves tended to have lower adiponectin concentrations than term calves. In the allantoic fluid the adiponectin concentrations were far below the plasma concentrations of neonatal calves and dairy cows at parturition. The high MW form (HMW) of adiponectin was

detectable in the circulation of dairy calves after the intake of colostrum, in which mainly the HMW form was observed, but also in maternal plasma and allantoic fluid.

In Manuscript II the influence of different feeding regimen on performance data (average daily weight gain, body weight, food intake, energy intake, milk yield and economic outcomes) was studied in dairy calves and later one around the first lactation. In P1 ad lib calves had a higher milk intake, energy intake and gaily weight gain compared to the MR-res group, however, differences were leveled off in P2 and later around the first lactation. Albeit the ad lib groups had the highest feeding costs, the (numerically) greater milk yield in their first lactation overcompensated these costs, in particular for the WM-ad lib group, and lead to a higher profit compared to the MR-ad lib and MR-res groups.

In Manuscript III the influence of different feeding regimen in the preweaning period on the metabolic and endocrine profiles of dairy calves and, later on, around their first lactation was investigated. In dairy calves, differences between groups in the metabolic and endocrine variables were mainly limited to P1. Calves in the ad lib groups appeared as less insulin sensitive. No group differences were seen in the glyconeogenetic enzymes. After P1, differences were leveled off and except for BHB and urea in P4, no group related differences were detectable around the first lactation.

Besides the characterization of the circulating concentrations of adiponectin in dairy calves, this thesis provides information about the influence of different feeding regimen in the preweaning periods on performance and metabolic and endocrine profiles during later life including the first lactation.

8 Zusammenfassung

Im geburtsnahen Zeitraum bei Milchkühen verringert sich die Insulinsensitivität im peripheren Gewebe, um dem gesteigerten Glukosebedarf der Placenta vor der Abkalbung und des Euters nach der Abkalbung gerecht zu werden. Die in den letzten Jahren zu beobachtende Steigerung der Milchleistung aufgrund verbesserter Fütterung, Haltung und Genetik der Milchkühe führte auch zu einer erhöhten Inzidenz metabolischer Erkrankungen. Intensive Fütterungsmethoden von Milchkälbern zeigten einen positiven Einfluss auf die spätere Milchleistung. Der Begriff metabolische Programmierung bezeichnet den Einfluss verschiedener Reize in der frühen Entwicklung und deren langfristigen Auswirkungen zum Beispiel auf die Gesundheit und Leistung eines Individuums. Eine intensive Kälberaufzucht hatte nicht nur positive Effekte auf die spätere Milchleistung, auch konnten Effekte auf die Anzahl der Langerhan'schen Inseln bei männlichen Milchkälbern sowie im Eutergewebe von weiblichen Tieren festgestellt werden.

Adiponektin, ein Adipokin mit sehr hohen Blutkonzentrationen, ist für seine insulinsensitivierenden Eigenschaften bekannt. Bislang war die Konzentration von Adiponektin im Blut bei Kälbern unbekannt. Bei Milchkühen hingegen konnte eine sinkende Adiponektinkonzentration um die Geburt herum beobachtet werden, vermutlich um den gesteigerten Glukosebedarf des Euters zu unterstützen. Ob die Adiponektinkonzentration in Kälbern und bei Färsen durch verschiedene Fütterungsmethoden in den ersten Lebenswochen beeinflusst wird, ist noch nicht bekannt.

Insulin unterstützt die Glukoseaufnahmen in die Zellen. Zum Ende der Trächtigkeit hin, steigt der Glukosebedarf für den Fötus an. Mit Einsetzen der Milchproduktion steigt der Glukosebedarf des Euters. Um die Zufuhr von Glucose zum Uterus und dem Euter zu unterstützen, sinkt die Insulinsensitvität der Muskel- und Fettzellen im peripheren Gewebe. Die Insulinkonzentration in der Blutzirkulation steigt an.

Ziel dieser Arbeit war es 1) die Adiponektinkonzentration in Kälbern und den Einfluss von Kolostrum zu messen, 2) den Einfluss verschiedener Fütterungsmethoden in der frühen Aufzuchtsperiode auf die Leistungsdaten, die metabolischen und die endokrinen Daten zu untersuchen und 3) die metabolischen Ergebnisse mit den Leistungsdaten in Zusammenhang zu stellen.

In Manuskript I wurden die Adiponektinkonzentration von Kälbern und der Einfluss von Kolostrum untersucht. Dafür standen uns Daten aus 3 verschiedenen Versuchen zur Verfügung. Im 1. Versuch (Versuch I-1)wurden 20 Deutsch Holstein Kühe (10 weibliche und 10 männliche) für die ersten 3 Lebenstage mit Kolostrum gefüttert. Am 1. Tag bekamen alle Tiere ad libitum (ad lib) Kolostrum, am 2. und 3. Lebenstag wurde die Menge auf 6 L pro Tag beschränkt. Vom 4. Lebenstag bis zum Absetzen am 56. Lebenstag bekamen die Tiere 6 L Milchaustauscher (MR) pro Tag (130 g MR/ L). Ab dem 70. Lebenstag waren alles Tiere vollständig abgesetzt und hatten bis zum Ende des Versuches am 110. Lebenstag ad lib Zugang zu einer Totalmischration. Ab dem 4. Lebenstag bekamen die Kälber Kraftfutter und hatten ad lib Zugang zu Heu und Wasser. Blutproben wurden vor der ersten Kolostrumaufnahme an Tag 0 sowie an den Lebenstagen 1, 3, 11, 22, 34, 43, 52, 70, 90, 108 gezogen. Kolostrumproben wurden von den Muttertieren am 1. und 3. Tag nach der Kalbung genommen.

Im 2. Versuch (Versuch I-2) wurden 14 Deutsch Holstein Kühe in 2 Gruppen unterteilt. Über einen Zeitraum von 4 Lebenstagen bekam die ersten Gruppe Kolostrum und die zweite Gruppe einen MR (Tag 1: 8 % des Körpergewichtes; Tag 2-4: 10% des Körpergewichtes). Am 4. Lebenstag, 2 h nach der Fütterung wurden alle Tiere geschlachtet. Blutproben wurden vor der ersten Fütterung sowie an den darauffolgenden Tagen vor der morgendlichen Fütterung und vor der Schlachtung genommen. Zusätzlich wurde jeden Tag eine Probe vom gepoolten Kolostrum und des MR gezogen.

Im 3. Versuch (Versuch I-3) wurden 7 Tiere nach normaler Trächtigkeitsdauer geboren und 7 Tiere kamen 9 Tage vor dem errechneten Kalbetermin per Kaiserschnitt zur Welt. Beide Gruppen bekamen einmalig nach 24 Lebensstunden Kolostrum (5 % des Körpergewichtes). Nach 26 Lebensstunden wurden alle Tiere geschlachtet. Blutproben wurden direkt nach der Geburt sowie vor der Fütterung und vor der Schlachtung genommen. Zusätzlich zu den erwähnten Versuchen wurden Fruchtwasserproben und Blutproben von 4 Deutsch Holstein Kühen während eines Kaiserschnitts entnommen.

In allen Blutproben wurde Adiponektin mittels eines im Institut entwickelten ELISAs, der spezifisch für bovines Adiponektin ist, gemessen und das Molekulargewicht von Adiponektin mittels Western Blot bestimmt.

In den Manuskripten II und III haben wir den Einfluss verschiedener Fütterungsmethoden in der Zeit vor dem Absetzen zunächst auf die Leistungsdaten (Manuskript II) sowie metabolischen und endokrinen Parametern (Manuskript III) bei Kälbern und in der Zeit um die erste Abkalbung dieser Tiere untersucht. Im 1. Versuch (Versuch II-1) wurden 57 Holstein Kälber (29 weibliche und 28 männliche Tiere) nach der Geburt in 3 Gruppen unterteilt: die 1. Gruppe bekam MR restriktiv (MR-res, 130 g / L; 6 L pro Tag, n = 20 (die selber Tiere, die auch in Manuskript I im 1. Versuch teilgenommen haben)), die 2. Gruppe bekam MR ad lib (MR-ad lib, n = 17) und die 3. Gruppe bekam Vollmilch ad lib (WM-ad lib, n = 20) In den ersten 3 Lebenstagen wurden alle Tiere mit Kolostrum gefüttert, wobei ab dem 2. Lebenstag die Menge in der Gruppe MR-res auf 6 L beschränkt wurde. Ab dem 4. Lebenstag wurden die Tiere mit der der Gruppe zugehörigen Fütterungsmethode gefüttert. Ab dem 25. Lebenstag wurde die Tiere der Gruppen MR-ad lib und WM-ad lib langsam auf das Fütterungsregime der Gruppe MR-res umgestellt. Bis zum Absetzen am 56. Lebenstag wurden alle Tiere gleich gefüttert. Am 70. Lebenstag waren alle Tiere vollständig abgesetzt und hatten ad lib Zugang zu einer Totalmischration bis zum Ende des Versuches am 110. Lebenstag. Alle Tiere bekamen ab dem 4. Lebenstag Kraftfutter und hatten ad lib Zugang zu Heu und Wasser. Blutproben wurden vor der ersten Kolostrumaufnahme genommen sowie an den Lebenstagen 1, 3, 11, 22, 34, 43, 52, 70, 90, 108. An den Lebenstagen 22, 52 und 108 wurde ein Glucose Toleranz Test durchgeführt, sowie an den Tagen 24, 54 und 110 nur bei den männlichen Tieren ein Insulin Toleranz Test. Eine Leberbiopsie wurde am 19. Lebenstag und am 100. Lebenstag gezogen. Kolostrumproben wurden von den Muttertieren am 1. und 3. Tag nach der Abkalbung genommen. Zusätzlich wurden regelmäßige Aufzeichnungen zum Gewicht, Gewichtszunahme, Futteraufnahme, sowie ökonomische Aspekte (Kosten, Gewinn) gemacht.

Im 2. Versuch (Versuch II-2), von den 29 zuvor schon als Kälber im ersten Versuch (Versuch II-1) teilgenommenen Färsen wurden 28 Färsen hinsichtlich ihrer Leistungsdaten sowie metabolischen und endokrinen Daten um den Zeitraum ihrer ersten Abkalbung untersucht. Blutproben wurden vor der Abkalbung monatlich gezogen, beginnend 3 Monate vor dem errechneten Abkalbetermin, und wöchentlich bis zur 10. Woche nach der Abkalbung.

Beide Versuche wurden in Phasen aufgeteilt: P0: Lebenstage 0 - 1.; P1: Lebenstage 2 - 27; P2: Lebenstage 28 - 69; P3: Lebenstage 70 - 110.; P4: 3, 2 und 1 Monat vor Abkalbung, P5: Kalbung bis zur 3 Woche nach Abkalbung, P6: 4 - 10 Wochen nach Abkalbung.

In den Blutproben der Kälber wurden nicht veresterte Fettsäuren (NEFA), Glukose, Beta-Hydroxybutyrat (BHB), Gesamtprotein (TPP), Adiponektin, Leptin und Insulin gemessen. Bei den Färsen wurde NEFA, Glukose, BHB, Harnstoff, Adiponektin und Insulin gemessen.

In Manuskript I haben wir die Adiponektinkonzentration in Kälbern bestimmt. Direkt nach der Geburt und noch vor der ersten Kolostrumaufnahme konnten wir eine sehr geringe Adiponektinkontzentration (3 µg/mL ± 0.3) beobachten. Nach der Kolostrumaufnahme stieg die Adiponektinkonzentration um das 3.5-fache bis zum 1. Lebenstag und das 4.7-fache bis zum 3. Lebenstag an und blieb dann bis zu einem zweiten Anstieg ab dem 52. Lebenstag konstant. im Kolostrum war die Adiponektinkonzentration 23 mal so hoch wie in der Milch am 3. Tag nach der Kalbung und 190 mal so hoch wie im Milchaustauscher. Bei Kälbern, die nur Milchaustauscher bekommen haben, stieg die Adiponektinkonzentration nur langsam an. Kälber, die vor dem errechneten Kalbetermin per Kaiserschnitt zu Welt kamen, tendierten zu einer geringeren Adiponektinkonzentration als Kälber, die nach einer normalen Trächtigkeitsdauer zur Welt kamen. Die Konzentration von Adiponektin im Fruchtwasser war viel geringer als die in den Kälbern und in den Muttertieren um die Kalbung. Nach dem die Kälber Kolostrum aufgenommen hatten, erschien die hochmolekulare Gewichtsform von Adiponektin im Blut, die hauptsächlich im Kolostrum vorzufinden ist sowie im maternalen Plasma und im Fruchtwasser.

In Manuskript II wurde der Einfluss verschiedener Fütterungsmethoden bei Kälbern auf die Leistungsdaten wie tägliches Gewicht, Gewichtszunahme, Futteraufnahme, Energieaufnahme, Milchleistung und Kosten und Gewinn bei den Kälbern und später in der ersten Laktation untersucht. Im Vergleich zu den restriktiv gefütterten Kälbern war die Milchaufnahme, die Energieaufnahme und die tägliche Gewichtszunahme bei den ad lib Kälbern in P1 höher. Diese Unterschiede haben sich jedoch in P2 und später in der ersten Laktation ausgeglichen. Die höheren Kosten für die Fütterung der ad lib Tiere wurde durch die numerisch höhere Milchleistung wieder ausgeglichen und führte sogar zu höheren Einnahmen der WM-ad lib Gruppe im Vergleich zu den MR Gruppen.

In Manuskript III wurde der Einfluss verschiedener Fütterungen in den ersten Lebenswochen auf die metabolischen und endokrinen Parameter bei Kälbern und um deren erste Abkalbung herum untersucht. Bei den Kälbern konnten nur in der Phase unterschiedlicher Fütterungen (P1) Unterschiede in den metabolischen und endokrinen Parametern festgestellt werden. Mit

Ausnahme von BHB und Harnstoff zeigten sich weder Unterschiede in den letzten Phasen des 1. Versuches noch um die erste Abkalbung herum im Bezug auf die metabolischen und endokrinen Parameter. Die Kälber in den ad lib Gruppen waren weniger insulinsensitiv. Keine Gruppenunterschiede konnten bei den glukoneogenetischen Enzymen festgellt werden.

Neben der Messung von der Adiponektinkonzentration bei Kälbern wird in dieser Arbeit auf die Unterschiede verschiedener Kälberfütterungen und deren Einfluss auf die Leistungsdaten sowie die metabolischen und endokrinen Daten bei Kälbern und später in der ersten Laktation eingegangen.

112

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120

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126

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11   Publications and proceedings derived from this doctorate thesis

1. **J. Kesser,** M. Hill, J.F.L. Heinz, C. Koch, J. Rehage, J. Steinhoff-Wagner, H. Hammon, B. Mielenz, H. Sauerwein & H. Sadri. 2015. *The rapid increase of circulating adiponectin in neonatal calves depends on colostrum intake.* J. Dairy Sci., 98, 7044-7051

2. M. Korst, C. Koch, **J. Kesser,** F.-J. Romberg, J. Rehage, M. Schmicke, K. Eder, and H. Sauerwein. *Different feeding intensities during the first four weeks of rearing in dairy calves and its effect on first lactation: Part 1: Effects on performance and production from birth over the first lactation. to be submitted.*

3. **J. Kesser,** M. Korst, C. Koch, F.-J. Romberg, J. Rehage, M. Schmicke, K. Eder, H. M. Hammon, H. Sadri, U. Müller, and H. Sauerwein. *Different feeding intensities during the first four weeks of rearing in dairy calves: Part 2: Effects on the metabolic and endocrine status around the first lactation.* to be *submitted.*

4. **J. Kesser,** M. Hill, C. Koch. H. Hammon, H. Sauerwein, H. Sadri**.** 2015. *Ontogeny of the circulating adiponectin concentrations in neonatal calves: the importance of colostrum intake.* Proceedings of the Society of Nutrition Physiology. Band 24.

5. **J. Kesser,** M. Hill, C. Koch, M. Piechotta, J. Rehage, K. Eder, H. Sadri, U. Müller, and H. Sauerwein (2015): *Effects of different feeding intensities during the first weeks of rearing on the metabolic status and on the circulating concentrations of adiponectin in dairy calves until 110 days of age.* Journal of Animal Science, 93/ J. Dairy Sci., 98, Page 706*.*

6. M. Korst, C. Koch, **J. Kesser**, F.-J. Romberg, J. Rehage, M. Schmicke, K. Eder and H. Sauerwein. 2016. *Different feeding intensities during the first four weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation.* Proceedings of the Society of Nutrition Physiology. Band 25.

7. **J. Kesser,** M. Korst, C. Koch, F.-J. Romberg, J. Rehage, M. Schmicke, K. Eder, H. M. Hammon, U. Müller and H. Sauerwein**.** 2016. *Different feeding intensities during the first four weeks of rearing in dairy calves: Part 2: Effects on the metabolic and endocrine status around the first lactation.* Proceedings of the Society of Nutrition Physiology. Band 25.