

# **HEALTH AND FERTILITY CHALLENGES**

IN HIGH YIELDING DAIRY COWS DURING THE TRANSITION PERIOD

## **AND THE USE OF DIETARY FATTY ACIDS**

AS AN OPTIMIZATION STRATEGY



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AND THE USE OF DIETARY FATTY ACIDS  
AS AN OPTIMIZATION STRATEGY**

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Health and Fertility Challenges in High Yielding Dairy Cows during the Transition Period  
and the Use of Dietary Fatty Acids as an Optimization Strategy

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*“Misinformation is a weapon of mass destruction”*

M. Fraser



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# LIST OF ABBREVIATIONS

ABD	Abdominal
ALG	Marine Algae
BCS	Body Condition Score
BHBA	Beta Hydroxy Butyric Acid
CE	Cholesterol Esters
CLA	Conjugated Linoleic Acid
DHA	Docosa Hexaenoic Acid
DIM	Days In Milk
DM	Dry Matter
DMI	Dry Matter Intake
EBAL	Energy Balance
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FF	Follicular Fluid
FPCM	Fat Protein Corrected Milk
HDL	High Density Lipoprotein
LA	Linoleic Acid
LCFA	Long Chain Fatty Acids
LDL	Low Density Lipoprotein
LNA	Linolenic Acid
MCFA	Medium Chain Fatty Acids
MD	Metabolic Disease
MFD	Milk Fat Depression
MUFA	Mono Unsaturated Fatty Acids
NEBAL	Negative Energy Balance
NEFA	Non Esterified Fatty Acids
OFS	Omental Fat Score
PL	Phospholipids
PUFA	Poly Unsaturated Fatty Acids
SCFA	Short Chain Fatty Acids
SFA	Saturated Fatty Acids
SUBC	Subcutaneous
TAG	Triacylglycerols
UFA	Unsaturated Fatty Acids
VLDL	Very Low Density Lipoprotein





# GENERAL INTRODUCTION

Modified from:

Hostens, M., and G. Opsomer. 2012. Interaction of metabolic challenges and successful fertility in high yielding dairy cows. Proceedings of the 25th World Buiatrics Congress. Lisbon, Portugal: 118-121.



## THE DAIRY INDUSTRY TODAY

Milk provides an easily accessible matrix, rich in a large variety of essential nutrients such as minerals, vitamins and easy digestible proteins with balanced amino acid profiles. Therefore dairy products are important to support overall body function (Steijns, 2008). Dairy products provide key nutrients in the daily human diet (Vandevijvere et al., 2009) and their consumption is associated with overall diet quality (Steijns, 2008) and human health implications (Table 1; Bauman et al., 2006). Although, over the last 40 years, the nutritional image has also suffered from its content of saturated fatty acids (SFA) and *trans* fatty acids (TFA) which increases serum cholesterol, and hence is considered a risk factor for cardiovascular disease (CVD) in humans. A meta-analysis study from 2003 (Mensink et al., 2003) concluded that replacing SFA and TFA from industrial origin by *cis*-unsaturated fatty acids (FA) reduces the risk most effectively. Most meta-analysis conclude that public health implications of consuming *trans* fats from ruminant products are relatively limited, possibly due to lower levels of the intake (less than 0.5% of total energy intake), different biologic effects of ruminant and industrial TFA, or the presence of other factors in dairy products which may cover negative effects of small amounts of TFA (Mozaffarian et al., 2006; Bendsen et al., 2011). A recent meta-analysis of 17 prospective studies even found a decrease in CVD risk with increasing milk intake (Soedamah-Muthu et al., 2011). Nevertheless, additional studies are needed to motivate or eliminate the consumers' concerns on dairy products.

As the number of inhabitants on our planet is currently exponentially increasing, we will be challenged to provide 9 billion of people with a sufficient amount of food of a high and safe quality by mid-21<sup>st</sup> century (UNPD, 2010). Tight supplies and rising food prices currently push more than 1 billion people around the world into food insecurity (Godfray et al., 2010). The latter imposes the need to make safe, affordable and abundant food a global right (Simmons, 2012). But how should we best resolve the need for an increased food production, with the desire to minimize its environmental impact? Green et al. (2005) demonstrated that up till now, data still support land sparing through increased yield over wild-life friendly farming in regions with long histories of agriculture such as Europe.

**Table 1.** List of bioactive components in dairy products that have human health implications (adapted from Bauman et al., 2006).

<i>Milk protein components</i>	<i>Milk fat components</i>	<i>Other</i>
	<b>Cancer</b>	
Whey proteins Casein Lactoferrin $\alpha$ -Lactalbumin Peptides	Conjugated Linoleic Acid Vaccenic Acid Sphingolipids Butyric Acid 13-methyltetradecanoic acid Ether lipids	Calcium Lactose Vitamin A & D Oligosaccharides Nucleosides Probiotics
	<b>Cardiovascular Health</b>	
Whey proteins Casein	Conjugated Linoleic Acid Stearic Acid Omega-3 Fatty Acids	Calcium Vitamin D
	<b>Hypertension</b>	
Whey proteins		Calcium Potassium
	<b>Immune Response</b>	
Whey proteins Milk-fat globule membrane	Conjugated Linoleic Acid	Probiotics
	<b>Bone Health</b>	
Peptides	Conjugated Linoleic Acid	Calcium Phosphorus Vitamin K

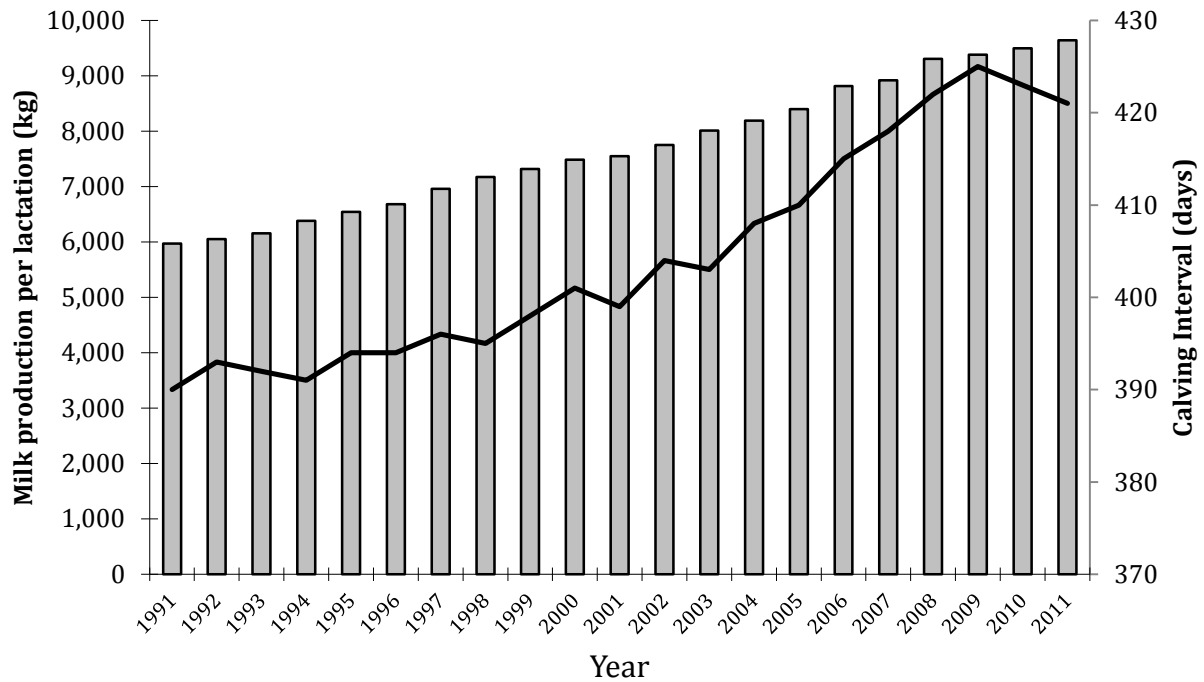
To keep up with the population growth, the dairy industry is facing a necessary increased production. As the world's resources are limited, the Food and Agriculture Organization of the United Nations estimates 70% of the additional food supply must be safeguarded by developing and applying efficiency-enhancing technologies (FAO, 2002; Capper et al., 2009). As an example, the dairy industry generally has adapted to the widespread use of genetic improvement and artificial insemination, thereby allowing producers to increase milk yield by 3,500 kg, milk fat by 130 kg, and milk protein by 100 kg per lactation in 20 years (Shook, 2006). Hence, the efficiency by which feed resources are converted, is largely influenced when expressed per unit of milk. Capper et al. (2009) estimated that production of the same milk quantity in 2007 as compared with 1944 required only 21% of the number of animals, whereas the use of feedstuffs, water and land were decreased to 23%, 35% and 10% respectively. Concurrently, the amount of waste products: manure and carbon footprint were decreased to 24% and 37% of the level valid for dairy industry at that time, respectively. Even though dairy cows are more efficient and have the ability to convert food unsuitable for human consumption (grass, forages and by-products) into high quality food (milk), their nutrient conversion remains low (20-30%) with 70-80% of nutrients being excreted. This results in Europe

in a dairy industry which contributes between 20-30% of methane emissions, 32% of nitrogen and 25% of phosphate excretions (Garnsworthy, 2011). Many strategies have been developed in order to mitigate the environmental impact of livestock production (Smith et al., 2008; Friel et al., 2009; Gill et al., 2010). More specifically in dairy, models have shown that management strategies which improve reproductive efficiency can be complementary to an increased production efficiency reducing methane and ammonia emissions up to 24% and 17%, respectively (Garnsworthy, 2004). This demonstrates that from an industry perspective, productive and reproductive efficiency need to align in order for dairy production to be sustainable in the near, mid- and long-term future.

## THE DAIRY COW: TRAPPED BETWEEN PRODUCTION AND REPRODUCTION

The aforementioned increased production in dairy cows has often been linked with a concomitant decrease in the cows' reproductive capacity and outcome (Royal et al., 2000; Butler, 2003). In these papers, graphs suggesting a causative relationship between production and reproduction have often been defended. At least for the Flemish part of Belgium, this kind of graphs do exist as well (Figure 1). In Flanders, milk production in dairy cattle has increased from 6,000 kg per lactation in 1991 to above 9,500 kg per lactation in 2011, while simultaneously the calving interval has increased by 31 days (CRV, 2011). As temporal associations do not imply causation and since many other aspects of dairy production (especially concerning management decisions) have changed during the same time, these figures and the concomitant conclusions warrant scrutiny (Leblanc, 2010a). Recent data suggest even that at least in the USA the downward trend in some of the reproductive measures has reversed and has begun to improve despite on-going increases in production per cow (Norman et al., 2009).

The abovementioned fertility 'drop' has been shown to be associated with the negative energy balance (NEBAL) high yielding dairy cows encounter during the transition period (van Knegsel et al., 2005; Dobson et al., 2007; Grummer, 2007). This transition period being generally defined as the time span from 3 weeks before until 3 weeks after calving, has drawn a lot of attention of applied research during the last decades (Drackley, 1999; Leblanc, 2010b).



**Figure 1.** The average milk production per lactation (kg; grey bars) and calving interval (days; black line) in Flemish herds from 1991 to 2011 (CRV, 2011).

During the transition period, substrate availability for gluconeogenesis is limited due to both a decreased DMI and the ongoing adaptation of the rumen (Grummer, 1993; Grum et al., 1996; Goff and Horst, 1997). A simultaneous increasing demand for milk lactose synthesis causes the cow to be unable to meet her maintenance, production and reproduction requirements especially in terms of glucose. The latter profoundly stresses the cows' endocrine, metabolic and physiological status, and the cow has to pass a period commonly referred to as the NEBAL (Drackley et al., 2005).

The NEBAL pushes the cows' metabolism to switch to a depletion of body reserves, releasing large amounts of non-esterified FA (NEFA) mainly from the subcutaneously and abdominally stored fat depots (up to 3.2 kg/d; Drackley et al., 2001). This body fat mobilization results in a steep increase in blood NEFA concentrations around parturition. Elevated NEFA concentrations impair metabolic and immune cell function in humans (Mora and Pessin, 2002), and are associated with impaired metabolic health in transition cows (Herdt, 2000; Jorritsma et al., 2003; Bobe et al., 2004). Leroy et al. (2004) showed a significant correlation between the concentrations of biochemical indicators of NEBAL ( $\beta$ -hydroxy butyric acid (BHBA), glucose, NEFA) in the serum and follicular fluid (FF) of high yielding dairy cows early post partum. Afterwards, the same research group mimicked the follicular environment in an *in vitro* maturation model and observed a marked deleterious effect on granulosa

cells (Vanholder et al., 2005) and developmental capacity of bovine oocytes (Leroy et al., 2005; Leroy et al., 2006). Only recently it was substantiated that such suboptimal micro-environment during oocyte maturation also significantly alters the quality of the pre-implantation embryo (Van Hoeck et al., 2011). These findings further build on the suggestions made by others (O'Callaghan and Boland, 1999; Horan et al., 2005) that the decline in fertility is mainly caused by an inferior oocyte and embryo quality. Britt suggested already in 1991 a carry-over effect of metabolic conditions in times of energy deficit early post partum, on pre-ovulatory follicles 2 to 3 months later (Britt, 1991). Therefore, the effect of the FF composition, the environment in which the oocyte matures before ovulation, cannot be neglected when evaluating the effect of the NEBAL in high yielding dairy cows.

Due to the massive loss of glucose via the high milk production, insulin concentrations remain low which prevents an increased expression in liver growth hormone receptors and thus an adequate IGF-I secretion, causing the homeorhetic control of the somatotrophic axis to be uncoupled (Lucy, 2008). As ovaries have been shown to carry receptors of metabolic hormones such as insulin (Bossaert et al., 2010) and IGF-I (Schams et al., 2002), disruption of these signalling metabolites during NEBAL will compromise normal follicular growth and ovarian function (see reviews by Roche, 2006; Chagas et al., 2007). The latter results in delayed resumption of cyclicity (Gutierrez et al., 1999; Opsomer et al., 2000) and prevents ovulation of the dominant follicle in dairy cows (Beam and Butler, 1999). Increasing peripheral insulin levels by dietary manipulations is therefore a practical tool to stimulate ovarian function during the early postpartum period (Gong et al., 2002). This methodology has been shown to have negative effects on early embryonic survival in heifers (Adamiak et al., 2005) which has questioned its applicability in lactating dairy cows (Fouladi-Nashta et al., 2005). However, sequential feeding of a glucogenic diet early post partum to hasten the onset of cyclicity followed by a lipogenic diet during the breeding season to lower insulin seems to increase oocyte quality and embryo development. This has been suggested as a strategy to increase fertility in dairy cows (Garnsworthy et al., 2009).

The NEBAL has furthermore been associated with increased oxidative stress in dairy cows, which may compromise the immune and inflammatory response of the cow (Sordillo and Aitken, 2009). This immunocompromisation might be exacerbated by the decreased DMI and subsequent mismatch between macromineral requirement and



availability in the diet (causing hypocalcaemia, hypomagnesaemia) in early lactation (Mulligan and Doherty, 2008; Walsh et al., 2011). Immunocompromised cows in return are at a higher risk to develop periparturient diseases such as retained placenta (Kimura et al., 2002), metritis (Hammon et al., 2006) and mastitis (Chagunda et al., 2006; Moyes et al., 2009). The incidence of these periparturient diseases is inter-related and the occurrence of one can predispose cows to related disorders, such as ketosis, acidosis and abomasal displacement (see reviews by Ingvartsen, 2006; Mulligan and Doherty, 2008). Besides their known negative effect on reproductive efficiency in dairy cows (Walsh et al., 2011), periparturient diseases cause profound economic losses for the dairy industry and have a serious impact on animal welfare (Ouweltjes et al., 1996; Ahmadzadeh et al., 2009).

Some researchers have documented that at least a part of the mobilization of body reserves in early lactation is genetically driven (Friggens et al., 2007). Therefore, body reserve mobilization is not expected to hamper fertility until it becomes predominantly environmentally driven which is defined as that which will occur in response to an environment that is constraining, limiting the energy intake of cows (Friggens et al., 2010). This might be one of the reasons for experiments indicating that high individual milk production can even be positively related to high fertility (Lopez-Gatius et al., 2006). Interestingly, besides the effects of parity, the final model of Lopez-Gatius et al. (2006) also accounted for the parameter whether the cow had suffered retained placenta or not. While many imply that high production may lead to poorer reproduction, these data support an alternative hypothesis that cows can combine high milk production with sound reproduction when combined with optimal management targeting minimal periparturient diseases (Leblanc, 2010a).

Taken together, the marked changes in the physiological status during early lactation and its effect on subsequent fertility all explain why many strategies have been developed to alleviate the extent of NEBAL of high yielding dairy cows. Fatty acids have played an essential role in both nutritional and management strategies (Friggens et al., 2004; Grummer, 2008). Of particular interest is their capability to interfere directly with the reproductive performance in dairy cows (Wathes et al., 2007). However, many aspects of mobilized and dietary FA on the metabolism of early lactating dairy cows remain unclear and form the main objective of this dissertation.

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# The Fabulous Destiny of Fatty Acids

## A Review



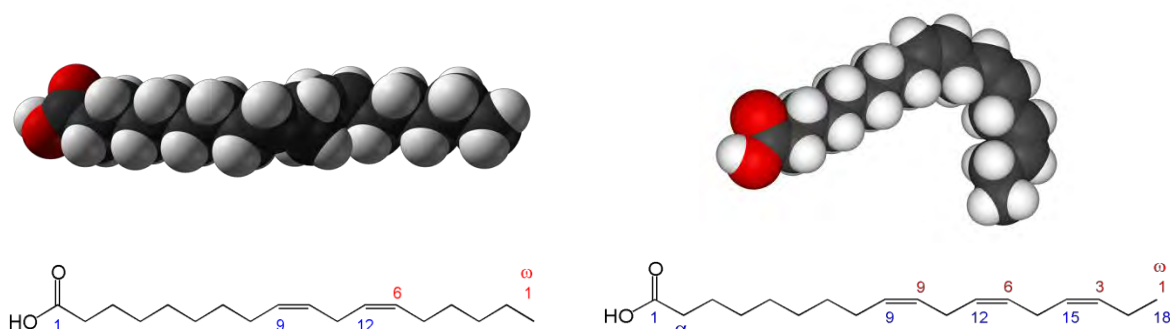
## NOMENCLATURE OF FATTY ACIDS

Lipids are a group of naturally occurring simple to complex molecules which are soluble in organic solvents. Within the dairy cow, especially cholesterol and lipids such as triacylglycerols (TAG) and phospholipids (PL) are of particular interest. Phospholipids are major components of cellular membranes, and are a source of fatty acids (FA) for the synthesis of a variety of effector molecules such as the eicosanoids, a group of compounds that includes prostaglandins, thromboxanes and leukotrienes (Wathes et al., 2007). Triacylglycerols serve as the most important energy storage in the animal whereas cholesterol is another component of the cellular membrane and is a main precursor for the synthesis of steroid hormones (Mattos et al., 2000).

The main compound of lipids are FA that mainly exhibit their function through the specific length of the hydrogenated acyl chain (2 up to 30), the number of double bonds in the chain, and the type of isomers formed by each double bond (Semma, 2002; Wathes et al., 2007). Fatty acids containing double bonds in the acyl chain are referred to as unsaturated fatty acids (UFA) in contrast with saturated FA (SFA). A FA containing one double bond is called a mono-unsaturated FA (MUFA) whereas a FA containing more than one double bond is called a poly-unsaturated FA (PUFA; Semma, 2002; Wathes et al., 2007). Often, FA are also classified according to their chain length into short-chain (<8 carbon atoms, SCFA), medium-chain (8 to 16 carbon atoms, MCFA) and long chain FA (>16 carbon atoms, LCFA; Chilliard et al., 2000).

According to their specific structure, FA have common and systemic names (Table 2; Calder and Yaqoob, 2009). As a shorthand nomenclature, the official International Union of Pure and Applied Chemistry and Molecular Biology (IUPAC-IUBMB) nomenclature for FA is commonly used where the carbon length of the FA chain is indicated by the number before the colon, and the number of the double bond is indicated by the number after the colon (Fahy et al., 2005). For example, all FA with 18 carbon atoms in the acyl chain and 2 or 3 double bonds are classified as 18:2 and 18:3, respectively. The position of the double bond relative to the methyl end is used to classify the FA into different omega families. Members of the omega-6 (e.g. 18:2n-6; linoleic acid; LA) and omega-3 (e.g. 18:3n-3; linolenic acid; LNA) family have the first

double bond at the sixth and third position respectively from the methyl end (Mattos et al., 2000; Calder and Yaqoob, 2009).



**Figure 2.** The spatial model (top) and chemical structure (bottom) of 18:2 *cis*-9, *cis*-12 or 18:2n-6 linoleic acid (left) and 18:3 *cis*-9, *cis*-12, *cis*-15 or 18:3n-3 linolenic acid (right).

Within the PUFA and uniquely found in the adipose tissues and milk of ruminant animals, conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of LA with two conjugated (one single bond in between) double bonds at various carbon positions in the FA chain (Nagpal et al., 2007; Crumb and Vatter, 2011). Each double bond can be *cis* or *trans*, but especially those with one *trans* double bond are bioactive (Jensen, 2002).

The presence of conjugated double bonds in FA was first demonstrated in food products derived from ruminants by Booth and Kon (1935), working with milk fat from cows turned out to spring pasture. Due to their wide range of physiologic effects in animal models, various CLA isomers ever since have gained a lot of interest (Pariza et al., 2000; Crumb and Vatter, 2011). More specifically for dairy products, a specific CLA isomer, i.e. *cis*-9, *trans*-11 has been proposed as a functional food (Bauman and Lock, 2010). In the dairy cow, this isomer is formed as an intermediate during ruminal biohydrogenation of 18:2n-6 to 18:0 by *Butyrivibrio fibrisolvens* (Kepler and Tove, 1967) and other rumen bacteria (Kritchevsky, 2000), or via endogenous conversion in the mammary gland by delta-9 desaturase enzymes from 18:1 *trans*-11 and other 18:1 intermediates of biohydrogenation of 18:2n-6 or 18:3n-3 (Griinari and Bauman, 1999; Corl et al., 2001). Recently however, the formation of CLA from biohydrogenation of 18:3n-3 has been reported as well (Lee and Jenkins, 2011).

## DIETARY FATTY ACIDS FOR DAIRY COWS

Usually fat compromises less than 5 % of the ruminant diet even though ruminants mainly depend on non-glucose metabolites for their energy provision (Palmquist and Jenkins, 1980). As reviewed by Palmquist and Jenkins (1980), fat has been supplemented to dairy cows as early as the first decade of the 20<sup>th</sup> century, but at that moment they were found not beneficial on milk and fat yield (Kellner, 1907). Between late 1920's and the mid 1940's, the first positive effects of fat supplementation on milk production were described when increasing the fat content of concentrates from 1-3% to 4-7% (Maynard et al., 1940; Loosli et al., 1944; Lucas and Loosli, 1944). Observations in 1960's showing a decreased fiber digestibility and a rather limited superiority of fat (mainly vegetable oils) in terms of energy contribution when compared to cereal grains, decreased the support for fat inclusion in dairy rations (Warner, 1960). As from the early 80's the steeply increased potential to reach great milk yields required the inclusion of higher amounts of digestible energy in the rations of lactating cows (Palmquist and Jenkins, 1980). Net Energy for Lactation (NE<sub>L</sub>) of rolled barley grain and tallow are estimated at 1.86 and 4.53 Mcal/kg DM, respectively (NRC, 2001). Increasing the starch intake by the cows is limited due to the negative effects of excessive grain on rumen health (Chamberlain and Wilkinson, 1996), which has led to a renewed interest in the use of fat to increase the energy intake by the cow. A wide variety of fats and oils have since then been used in dairy rations. Oils are not often used in their free form and most commonly fed as oilseeds (canola, cottonseed, soybeans, flaxseed; Drackley, 1999). Animal fats such as tallow and grease have been widely implemented but their use has been restricted in the European Union in order to stop the spread of bovine spongiform encephalopathy (BSE; Woodgate and Van Der Veen, 2004). Furthermore, a wide variety of processed dry "inert" or "bypass" fats are nowadays commercially available.

Fat as feed additive has historically gained interest beyond its energy content. As reported by Santos et al. (2008), Burr and Burr described a new disease in rats fed a fat free diet as early as 1929. They observed that growing rats ceased growing and experienced more health problems and an irregular ovulatory pattern, which was reversed by re-introducing fats in the diet. The FA fraction of the fats was one year later identified as that part of the fats effective in curing and preventing the disease (Burr and



Burr, 1930). Specific long chain fatty acids (LCFA), such as 18:2n-6 and 18:3n-3 and not fat *per se*, have ever since gained general recognition as being essential for reproduction in all mammalian species as well, being necessary precursors for metabolic regulation and cell membrane function (Staples and Thatcher, 2005).

**Table 2.** Common fatty acids (adapted from FAO, 2008)

Common Name	Systematic Name	Abbreviations
<b>Saturated Fatty Acids</b>		
Butyric Acid	Butanoic Acid	4:0
Caproic Acid	Hexanoic Acid	6:0
Caprylic Acid	Octanoic Acid	8:0
Capric Acid	Dodecanoic Acid	10:0
Lauric Acid	Dodecanoic Acid	12:0
Myristic Acid	Tetradecanoic Acid	14:0
Palmitic Acid	Hexadecanoic Acid	16:0
Stearic Acid (SA <sup>1</sup> )	Octadecanoic Acid	18:0
Arachidic Acid	Eicosanoic Acid	20:0
Behenic Acid	Docosanoic Acid	22:0
Lignoceric Acid	Tetracosanoic Acid	24:0
<b>Mono Unsaturated Fatty Acids</b>		
Palmitoleic Acid	<i>cis</i> -9-Hexadecenoic Acid	16:1 <i>cis</i> -9
Oleic Acid (OA)	<i>cis</i> -9-Octadecenoic Acid	18:1 <i>cis</i> -9
Vaccenic Acid	<i>trans</i> -11-Octadecenoic Acid	18:1 <i>trans</i> -11
<b>Poly Unsaturated n-6 Fatty Acids</b>		
Linoleic Acid (LA)	<i>cis</i> -9, <i>cis</i> -12-Octadecadienoic Acid	18:2n-6
$\gamma$ -Linolenic Acid	<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-Octadecatrienoic Acid	18:3n-6
Arachidonic Acid (AA)	<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-Eicosatetraenoic Acid	20:4n-6
<b>Poly Unsaturated n-3 Fatty Acids</b>		
$\alpha$ -Linolenic Acid (LNA)	<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatrienoic Acid	18:3n-3
Eicosapentaenoic Acid (EPA)	<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-Eicosapentaenoic Acid	20:5n-3
Docosapentaenoic Acid (DPA)	<i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19-Docosapentaenoic Acid	22:5n-3
Docosahexaenoic Acid (DHA)	<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19-Docosahexaenoic Acid	22:6n-3
<b>Conjugated Linoleic Acids</b>		
Rumenic Acid	<i>cis</i> -9, <i>trans</i> -11-Octadecadienoic Acid	18:2 <i>cis</i> -9, <i>trans</i> -11
<i>trans</i> -10, <i>cis</i> -12 CLA	<i>trans</i> -10, <i>cis</i> -12-Octadecadienoic Acid	18:2 <i>trans</i> -10, <i>cis</i> -12
<i>trans</i> -7, <i>cis</i> -9 CLA	<i>trans</i> -7, <i>cis</i> -9-Octadecadienoic Acid	18:2 <i>trans</i> -7, <i>cis</i> -9

<sup>1</sup> Abbreviation key used in this thesis

Typically, dairy cattle diets contain approximately 2% of LCFA, predominantly polyunsaturated (Staples et al., 1998). The main FA in most seed lipids is 18:2n-6, whereas 18:3n-3 predominates in most forage lipids (Palmquist and Jenkins, 1980). These FA cannot be synthesized by the mammalian cells as they lack desaturase enzymes to incorporate a double bond beyond the ninth carbon in the acyl chain (Wathes et al., 2007). Combined with the limited supply of UFA to the small intestine due to the extensive biohydrogenation in the rumen (Jenkins et al., 2008), UFA have been proposed by researchers to be included as nutraceuticals in the bovine diet, for

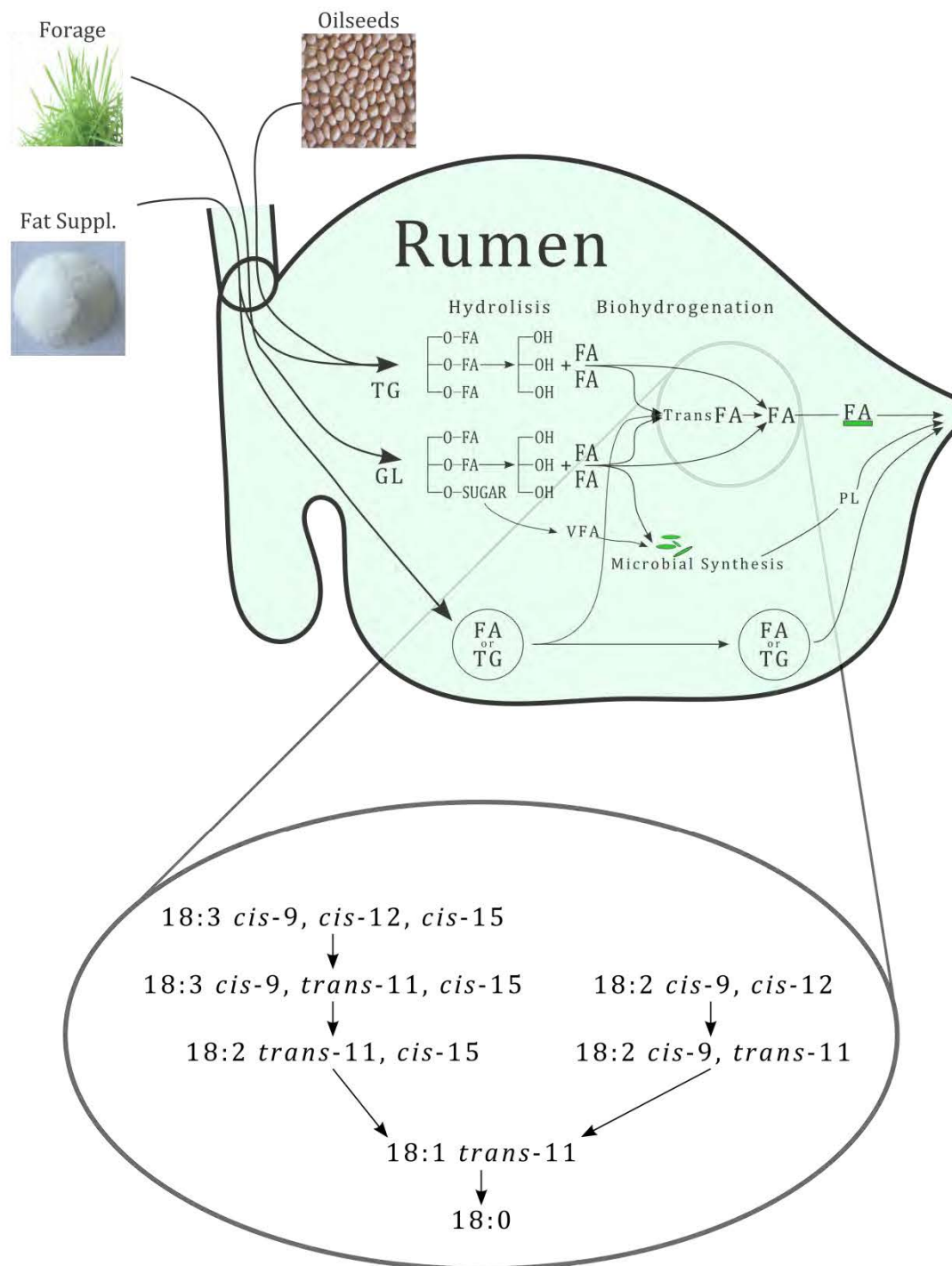
example to ameliorate dairy cow fertility (Santos et al., 2008; Silvestre et al., 2011; Thatcher et al., 2011).

## CHALLENGES WHEN FEEDING FATTY ACIDS TO DAIRY CATTLE

### BIOHYDROGENATION, ABSORPTION AND DISTRIBUTION OF FATTY ACIDS

The dietary FA reflect the FA composition of pig and poultry products in a predictable way because they are absorbed unchanged before incorporation into the tissue lipids (Chesworth et al., 1998; Woods and Fearon, 2009). In contrast, increasing the amount of dietary UFA in the diet of ruminants is not linearly reflected in the body tissues as microorganisms in the rumen largely affect the composition of the FA (Doreau and Chilliard, 1997; Jenkins et al., 2008). The microorganisms are responsible for the isomerisation and hydrolysis of dietary lipids, and the conversion of UFA to various partially hydrogenated intermediates such as *cis*-9, *trans*-11 CLA or 18:1 *trans*-11, and other fully saturated derivatives such as 18:0 (Woods and Fearon, 2009). This process, also called ruminal biohydrogenation (Figure 3), causes the major FA exiting the rumen to be 18:0, although the main FA in most seed lipids and forages are 18:2n-6 and 18:3n-3 respectively (Palmquist and Jenkins, 1980). In general, rates of rumen biohydrogenation are faster with increasing unsaturation, showing hydrogenation rates of 18:2n-6 and 18:3n-3 up to 70-95% and 85-100% respectively (Lock et al., 2006). Biohydrogenation of 20:5n-3 and 22:6n-6 is still not well understood, but at present they are believed to be hydrogenated at the same extent as 18:2n-6 and 18:3n-3 (Shingfield et al., 2010). Due to the extensive biohydrogenation of FA, ruminants seem to have developed very efficient pathways for capturing and maintaining essential FA within the body as functional deficiencies do not occur (Drackley, 2005).

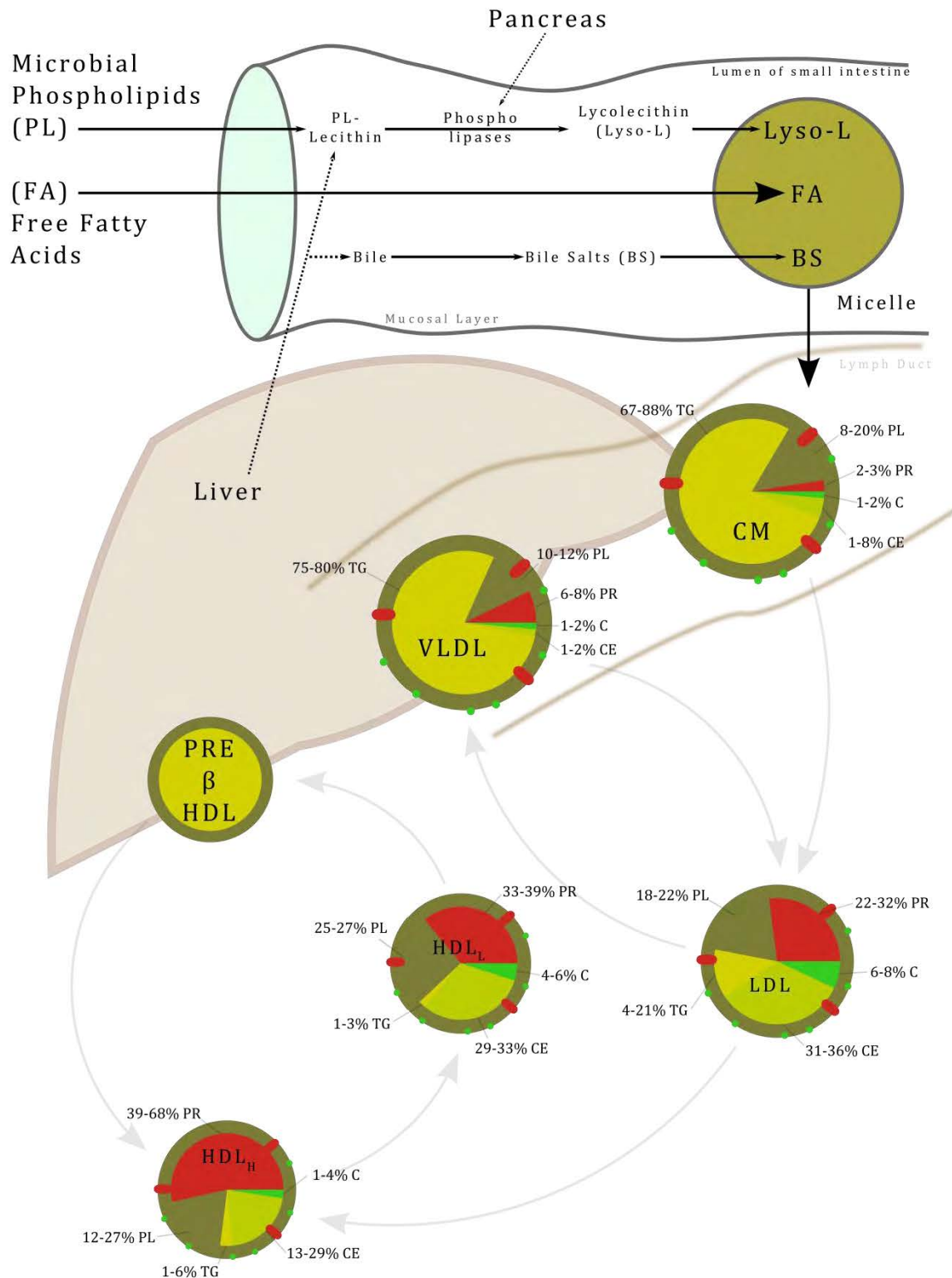
There is no significant absorption or hydrogenation of LCFA in the omasum or abomasums; the lipid material available for absorption in the small intestine is similar to that leaving the rumen (Moore and Christie, 1984). Approximately 80-90% of lipids are entering the small intestine as free FA attached to feed particles, bacteria and



**Figure 3.** Hydrolysis and biohydrogenation of triglycerides (TG), glycolipids (GL) and fatty acids (FA) to FA bound on feed particles (FA) and phospholipids (PL) from microbial synthesis which become available for intestinal digestion (Adapted from Davis, 1990 and Shingfield et al., 2010).

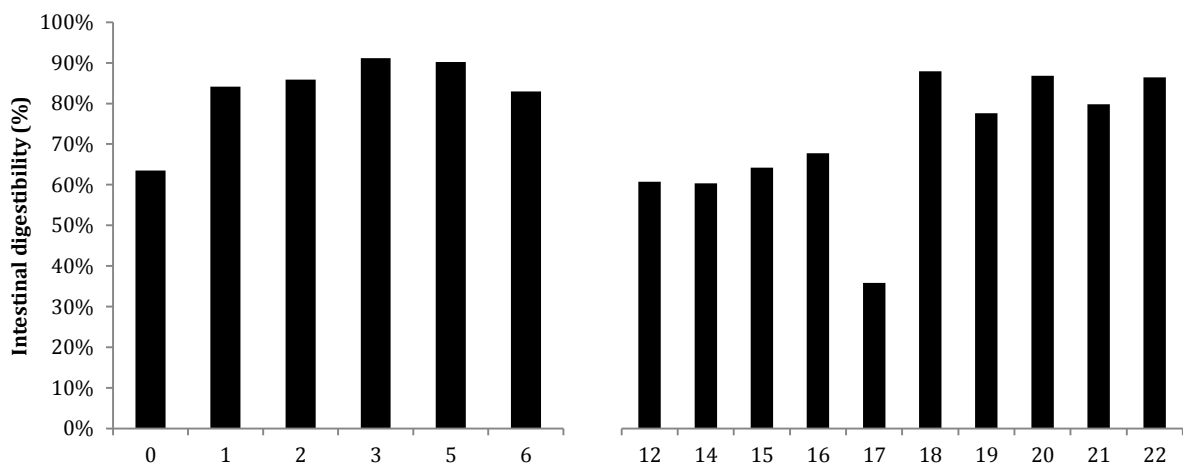
desquamated cells (Davis, 1990; Doreau and Chilliard, 1997). A smaller proportion are microbial PL and small amounts of TAG and glycolipids from residual feed stuffs which are subsequently hydrolysed by pancreatic lipases (Doreau and Ferlay, 1994). Bile salts and lysolecithins desorb FA from feed particles and bacteria which promotes micell formation which function to move across the water layer of the small intestinal epithelium. Inside the epithelial cells of the jejunum, FA are re-esterified into TAG and PL via the phosphatidate pathway to allow packaging into chylomicrons (Figure 4; Bauchart, 1993; Demeyer and Doreau, 1999). This packaging allows the resynthesized lipids to be exported to the lymph and plasma, and redistribute the FA to the different body tissues. In the bovine, chylomicrons and very low density lipoproteins (VLDL) mainly contain TAG, whereas the major components of high density lipoproteins (HDL) are PL and cholesterolesters (CE; Bauchart, 1993). As stipulated by Drackley (2005), the LCFA uptake and distribution is not believed to be hugely regulated at the assimilation site. It is in the animals' sake to take up all LCFA from the diet at any time, as food restriction might occur at some point. Therefore, the regulation mainly occurs at the site of deposition and storage and the capacity of the dairy cow to absorb and redistribute LCFA can exceed 1 kg per day (Doreau and Chilliard, 1997).

Absorption and apparent digestibility of FA differing in chain length and number of double bonds has been controversial as literature results have been biased because of ruminal FA loss, differences in esterification, biohydrogenation in the large intestine, and oxidation of UFA before analysis (Harvatine and Allen, 2006). Certainly, the absorption occurs with a higher efficiency compared to non-ruminants, and digestibility increases moderately with chain length and number of double bonds ranging from 80% for SFA to 92% for UFA in low fat diets (Figure 5; Woods and Fearon, 2009). A meta-analysis estimated apparent digestibility of 18:0, 18:1, 18:2, and 18:3 to be 74.0, 79.1, 71.7, and 70.2% respectively (Glasser et al., 2008). The digestibility was irrespective of the LCFA intake, except for 18:0, showing saturation at high duodenal flows (Glasser et al., 2008), which has been observed earlier (Palmquist, 1991). Intestinal digestibility of 20:5n-3 and 22:6n-3 have been estimated at 90.5 and 82.9% respectively (Loor et al., 2005).



**Figure 4.** Intestinal digestion and absorption of microbial phospholipids (PL) and free fatty acids (FA) to the lymph and blood circulation. Main lipoproteins in lymph are chylomicrons (CM) and very low density lipoprotein (VLDL) whereas in the blood low density lipoproteins (LDL) and multiple high density lipoproteins (HDL) are present as well: nascent (Pre-β HDL), light (HDL<sub>L</sub>) and heavy (HDL<sub>H</sub>) HDL. The proportion of the different lipid fractions triglycerides (TG), free cholesterol (C), cholesterol esters (CE), phospholipids (PL) and protein fraction (PR) are shown in different colours (Adapted from Davis, 1990 and Bauchart, 1993).

Once absorbed, ruminants seem to be able to conserve essential FA within the body by means of different mechanisms. The first one is the lower rate of oxidation compared to the more abundant saturated LCFA as demonstrated in sheep (Lindsay and Leat, 1977), which might be mediated via the low affinity of mitochondrial dehydrogenases for essential FA (Reid and Husbands, 1985). Of particular interest for the dairy cow is the high affinity of PL esterification enzymes for incorporation of essential FA (Lindsay and Leat, 1977), the slow turnover of the PL and cholesterol ester (CE) pools in plasma (Palmquist, 1976), and the preferential affinity of the lecithin-cholesterol acyl transferase (LCAT) for 18:2n-6 (Noble et al., 1972). This will lead to a very low transfer of PUFA into milk fat as the plasma PL and CE fraction are poor substrates for the mammary gland lipoprotein lipase (Offer et al., 2001). The mammary gland furthermore limits the excretion of PUFA as its lipoprotein lipase discriminates against TAG that contain PUFA (Melin et al., 1991), and its acyltransferase enzymes responsible for the incorporation of FA into milk fat TAG have a low affinity for n-3 PUFA (Hansen and Knudsen, 1987; Demeyer and Doreau, 1999).



**Figure 5.** The effect of the number of double bonds (left) and chain length (right) on intestinal digestibility (in %) of fatty acids (Adapted from (Loor et al., 2005)).

In contrast with the FA transport to the mammary gland, lipid supply to the bovine ovary has only been partially described. Typically, the basement membrane between the blood circulation and follicular fluid (FF) is impermeable to macromolecular particles > 400,000 Dalton (Shalgi et al., 1973). Due to their large molecular weight, VLDL and LDL are assumed not to transverse the intact blood-follicle barrier (Brantmeier et al., 1987; Grummer and Carroll, 1988), making HDL the

predominant lipoprotein in bovine FF. The difference in ability to transverse the blood-follicle barrier and the different FA profiles of the lipoproteins will have a profound effect on the FA composition of the FF when supplementing PUFA to dairy cows. Furthermore, it has been suggested that the ovary is capable of buffering oocytes against fluctuations in the n-6 and n-3 content of plasma (Fouladi-Nashta et al., 2009). However, data on transfer of n-6 and n-3 FA to the follicle, especially the very LCFA such as 20:4n-6 or 22:6n-3 is lacking. As many mechanism by which FA affect reproduction in dairy cows (see below) rely on an efficient transfer through the blood-follicle barrier, this necessitates further research.

### **SUPPLEMENTATION OR MOBILISATION DURING NEGATIVE ENERGY BALANCE**

Feeding supplemental FA to lactating dairy cows decreases the *de novo* synthesis of milk FA due to the incorporation of added dietary FA into the milk. This subsequently reduces the requirement for acetate, BHBA and NADPH, the latter being produced through oxidation of glucose. Secondly, dietary FA can induce insulin resistance in muscle and adipose tissue, which decreases peripheral glucose use (Chilliard and Ottou, 1995; Gaynor et al., 1996; Pires et al., 2008). The spared glucose is partitioned towards increased lactose synthesis, hence increasing milk yield (Wu and Huber, 1994; Hammon et al., 2008). Although highly variable, the milk yield response to supplemental FA in TMR diets generally is curvilinear, increasing until approximately 9% of ether extract (EE) in the total diet (Wu and Huber, 1994) or 3% of added fat (Drackley, 1999), thereafter decreasing due to the concomitant decrease in DMI and fiber digestibility (Palmquist and Jenkins, 1980; Coppock and Wilks, 1991; Allen, 2000). Moreover, cows seem to respond production wise best to additional fat at the time they reach positive energy balance (Grummer, 1995). Consequently, the effect of an increased milk yield seems to be limited within the first 5 weeks post partum (Schingoethe and Casper, 1991; Chilliard, 1993; Grummer et al., 1995). The response lag of a few weeks, documented for both cows and heifers, is another drawback when supplementing FA to dairy cows in early lactation (Grummer, 1995). In summary, these findings illustrate the limited effectiveness of post partum dietary FA in order to ameliorate the NEBAL and consequently have eliminated FA from most early lactation rations for dairy cows as they may imbalance the relative proportions of glucogenic and ketogenic metabolites

available to the cow during the periparturient period (Ingvartsen et al., 2003; Drackley et al., 2005; van Knegsel et al., 2007a; 2007b).

Kronfeld (1982) was one of the first to speculate that feeding supplemental fat would reduce FA mobilisation from adipose tissue. Therefore, prepartum FA feeding was proposed as a strategy to pre-adapt the dairy cow for body fat mobilisation (Friggens et al., 2004). Indeed, prepartum diets high in fat were found to increase NEFA during the dry period while decreasing the liver lipid content by half at calving as compared with a high starch and control diet (Grum et al., 1996). High NEFA prepartum seemed to prime the liver for the increased lipid mobilisation in early lactation (Grummer and Carroll, 1991; Chilliard, 1993; Grummer, 2008). However, the prepartum high fat diet also caused a significant reduction in dry period DMI relative to the control animals, and a 10% lower energy intake thereby possibly confounding the results. Douglas et al. (2006; 2007) subsequently tried to attribute the elevated NEFA to either the reduced intake or increased fat intake in a follow-up study. They found the effect of fat inclusion to be smaller but additive to that of the reduced intake.

This evidence suggested that increased FA provision to the liver, through dietary FA or fat mobilisation, can prime the liver to cope with circulating FA. Though, care should be taken as this cannot be seen as an incitement to promote excessive mobilisation of body reserves. Excessive lipid mobilisation predisposes for ketosis, fatty liver disease and other metabolic disorders, and should therefore be avoided (Friggens et al., 2004). Preventing excessive nutrient intake in the dry period and thereby inducing moderate mobilisation to prime the liver for increased exposure to FA, on the contrary may be beneficial. Although some concern on subsequent lower milk production has grown (especially in multiparous cows), controlled energy diets for dry cows have been the subject of recent research (Janovick and Drackley, 2010; Janovick et al., 2011; Litherland et al., 2011).

In general, elevated NEFA concentrations impair metabolic and immune cell function in humans (Mora and Pessin, 2002), and are associated with impaired metabolic health in transition cows (Herdt, 2000; Jorritsma et al., 2003; Bobe et al., 2004). However, little is known about the specific pathogenicity of FA differing in chain length or degree of unsaturation. Saturated NEFA have been associated with direct toxic effects for bovine oocytes, embryos and granulosa cells, being an important metabolic link between metabolic stress and subfertility in dairy cows (Leroy et al., 2005;



Vanholder et al., 2005; Van Hoeck et al., 2011). Immune cell function has been shown to be impaired by saturated NEFA in periparturient dairy cows (Contreras and Sordillo, 2011), whereas positive health effects have been attributed to UFA both in humans (Calder and Yaqoob, 2009) and in dairy cows (Zachut et al., 2010a). Moreover, *in vitro* cultures with bovine hepatocytes have shown that in increasing the length and the degree of unsaturation of FA generally decreases TAG accumulation (Mashek et al., 2002) and although contradictory, downregulates gene expression of genes involved in the synthesis and secretion of VLDL which export TAG from the liver (Pires et al., 2004; 2006).

Subcutaneously stored FA are known to have a more unsaturated FA profile than their intra-abdominally stored counterparts, as the level of saturation increases with increasing distance from the animal's exterior (De Smet et al., 2004). In humans, omental and mesenteric adipocytes are more metabolically active and sensitive to lipolysis than subcutaneous adipocytes (Wajchenberg, 2000; Jensen, 2007; Hajer et al., 2008). In Holstein Friesian heifers, abdominal adipocytes have been shown to possess, when not adjusted for their larger cell size, a greater lipogenic enzyme activity per cell in comparison with subcutaneous adipocytes (Eguinoa et al., 2003). Little is known whether these site-specific differences in FA profile also exist in dairy cows as preferential mobilisation would render them at a different level of pathogenicity in case of lipolysis during NEBAL. Further research on this is needed.

Next to supplementation and targeted mobilization of FA as a strategy to reduce the NEBAL early post partum, specific FA have been fed to dairy cows in order to decrease the milk fat output, hence reducing the energy expense in early lactation (Bauman et al., 2008).

## **INDUCTION OF MILK FAT DEPRESSION**

A vast number of theories have been proposed to explain dietary induced milk fat depression (MFD; Bauman and Griinari, 2003). Powell (1939) was the first to recognize that there was a positive correlation between the activities of the rumen and the composition of the milk, thereby acknowledging dietary alteration in the rumen microbial process to form the basis of MFD. The theories of an inadequate rumen production of acetate and butyrate to support milk fat synthesis or insulin-induced shortage of precursors for mammary synthesis of milk fat have been abandoned

(Bauman and Griinari, 2003). In the early 1970's, it was suggested that incomplete ruminal biohydrogenation of dietary PUFA may lead to the ruminal production of specific FA, that inhibit the mammary glands' ability to synthesize milk fat (Davis and Brown, 1970; Pennington and Davis, 1975). *Trans* isomers of 18:1 have been associated with low milk fat as their content increases during dietary induced MFD (Griinari et al., 1998; Bauman and Griinari, 2003), although a summary of published literature revealed inconsistency in these observations (Selner and Schultz, 1980; Kalscheur et al., 1997). Griinari et al. (1998; 1999) suggested that the degree of MFD depends more on the presence of specific isomers of *trans* MUFA and CLA rather than the total amount of these FA. More specifically, they proved *trans*-10 18:1 and *trans*-10, *cis*-2 CLA in milk fat to be a better indicator of dietary induced MFD. The latter was validated by Baumgard et al. (2002) demonstrating a decrease in milk fat yield by 44% by a post-rationally infusion of 10g *trans*-10, *cis*-12 CLA. Many researchers have performed dose response studies involving purified *trans*-10, *cis*-12 CLA and have found the specific isomers not being able to fully explain the dietary induced MFD. This has induced the quest for new milk fat depressing isomers such as *cis*-10, *trans*-12 CLA and *trans*-9, *cis*-11 (Saebo et al., 2005; Perfield et al., 2007). Although accompanied by an increase in *trans*-10 18:1, feeding marine oils (Offer et al., 2001; Shingfield et al., 2003) or marine algae (Boeckaert et al., 2008b) induced a MFD with little or no changes in *trans*-10, *cis*-12 CLA, suggesting the existence of other unidentified rumen biohydrogenation intermediates with milk fat depressing properties (Bauman and Griinari, 2001).

Although the mechanism and pathways of all different types of dietary induced low milk fat syndromes might not be fully revealed, it has been widely accepted that the induction of a MFD requires both an altered rumen fermentation and the presence of PUFA in the rumen (Bauman and Lock, 2006). Furthermore, Overton et al. (2006) indicated that MFD occurs as a result of several concurrent diet and management factors rather than as a result of one single factor (Table 3).

**Table 3.** Partial list of potential risk factors for milk fat depression in lactating dairy cows (Adapted from Bauman and Lock, 2006; Overton et al., 2006)

Altered Rumen Environment	Supply of PUFA <sup>1</sup>
<ul style="list-style-type: none"> <li>• Low rumen pH</li> <li>• Feed particle size</li> <li>• Fiber</li> <li>• Starch</li> <li>• Ionophores</li> <li>• Feeding pattern</li> </ul>	<ul style="list-style-type: none"> <li>• Amount</li> <li>• Availability</li> <li>• Ratio saturated to unsaturated</li> <li>• Feeding pattern</li> <li>• Variation in fat content and FA<sup>2</sup> composition of feed ingredients.</li> </ul>

<sup>1</sup>Poly Unsaturated Fatty Acids

<sup>2</sup>Fatty Acid

One of the hypotheses to diminish the NEBAL post partum is the induction of MFD in order to substantially decrease the energy output as the production of fat comes with the highest demand for energy (Jensen, 2002; Bauman et al., 2008). Even though the mammary gland seems refractory to *trans*-10, *cis*-12 CLA induced MFD during the first weeks in lactation (Bernal-Santos et al., 2003; Castaneda-Gutierrez et al., 2005), feeding larger amounts of *trans*-10, *cis*-12 CLA has been shown to effectively induce a MFD in early lactating dairy cows (Moore et al., 2004; Odens et al., 2007). Daily supplementation of *trans*-10, *cis*-12 CLA induced MFD in periparturient cows, which was associated with a significant decrease in NEFA and an increase in glucose concentrations (Odens et al., 2007), and greater peripheral levels of IGF-I (Castaneda-Gutierrez et al., 2007), indicating an improved energetic status of the supplemented cows. Until now, researchers have not been able to consistently prove an improvement of the NEBAL by inducing a MFD via the diet. This has been attributed to differences in the amount of *trans*-10, *cis*-12 CLA supplemented and the magnitude of the MFD (Odens et al., 2007), but also to differences in production responses showing no change (Castaneda-Gutierrez et al., 2005), only a slight (Bernal-Santos et al., 2003) or a more significant increase in milk production (Odens et al., 2007). When fed to dairy cows in established lactation, 22:6n-3 enriched marine algae (*Schizochytrium spp.*) induce MFD (Boeckeaert et al., 2008a). To our knowledge, there are no experiments in which long chain n-3 FA such as 22:6n-3 were examined to induce a MFD in early lactation with concomitant registration of NEBAL indicators. This strategy might be of particular interest as in the meanwhile more essential FA are offered to the dairy cow thereby positively influencing her reproductive capacity (Theurer et al., 2009; Bender et al., 2010; Palmquist, 2010).

## FEEDING FATTY ACIDS FOR FERTILITY

Beyond the aforementioned indirect effect on the NEBAL, supplemental FA affect fertility through a direct impact on the reproductive tissues in dairy cows (e.g. ovary and uterus). Supplementation of various types of FA has consistently increased the number and size of ovarian follicles (see review Staples et al., 1998), independently of the increased energy as the effect is still present in isocaloric diets (Lucy et al., 1991). Differential effects on follicle development according to the FA profile have been shown for n-3 FA on the diameter of the ovulatory follicle (Ambrose et al., 2006; Mendoza et al., 2011) and corpus luteum (Petit et al., 2002) but results are inconsistent (Bilby et al., 2006b; Heravi Moussavi et al., 2007). Feeding FA has been associated with increased plasma cholesterol (Ryan et al., 1992; Lammoglia et al., 1996) which serves as progesterone precursor (Staples and Thatcher, 2005). This might establish a more favorable steroidogenesis to support early embryonic growth. Furthermore, lower plasma and follicular oestradiol concentrations through FA supplementation (Ryan et al., 1992) might be desirable to prevent regression of the corpus luteum but would be undesirable for estrus expression. However, in contrast with the previous, decreased progesterone and increased oestradiol concentration have been reported when supplementing FA to dairy cows (Robinson et al., 2002).

Differences in the environment in which the oocyte matures may determine the developmental capacity of the embryo (Sturmey et al., 2009). The observations of a more unsaturated FA profile of FF, oocytes and granulosa cells in winter as an explanation for seasonal differences in dairy cow fertility *in vivo* (Zeron et al., 2001), and detrimental effects of saturated NEFA on oocyte maturation *in vitro* (Leroy et al., 2005; Aardema et al., 2011) have hastened the quest for the optimal FA profile of breeding diets. Although supplementation with UFA to dairy cows has been shown to improve oocyte (n-3; Zachut et al., 2010b) and embryo quality (n-6; Cerri et al., 2009), results have been inconsistent showing limited (Bilby et al., 2006a; Fouladi-Nashta et al., 2009) or even negative effects (Sklan et al., 1994).

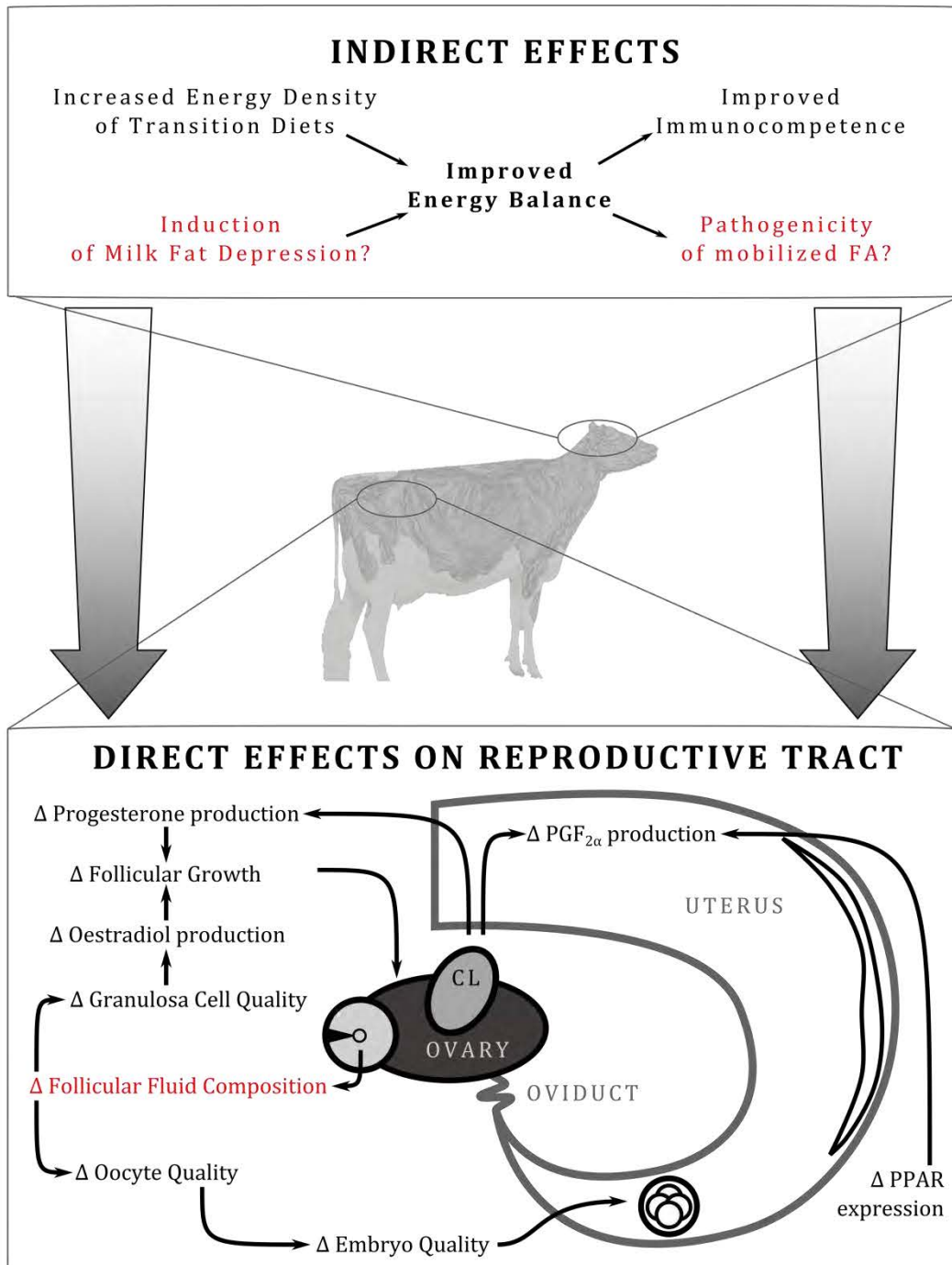
At the level of the uterus, feeding n-6 FA to dairy cows has been shown to stimulate PGF $2\alpha$  metabolism improving uterine health (Robinson et al., 2002; Petit et al., 2004). In contrast, feeding n-3 FA reduces endometrial secretion of PGF $2\alpha$ , thereby inducing antiluteolytic effects on the corpus luteum (Staples et al., 1998). Recently,

sequential and selective feeding of supplemental n-6 and n-3 FA during the transition and breeding period has been proposed as an optimal reproductive management strategy in dairy cows combining the aforementioned effects on PGF<sub>2</sub> $\alpha$  metabolism (Thatcher et al., 2011). Next to the amount and type of supplemented FA, this illustrates the effect of the FA profile of supplemental FA.

## CONCLUSION

In conclusion, FA are able to interfere at multiple levels of the productive and reproductive system in dairy cows. Different aspects of this have been discussed in a number of excellent reviews (Mattos et al., 2000; Grummer, 2008; Santos et al., 2008; Gulliver et al., 2012). Although many indirect and direct mechanisms have been elucidated, this review has demonstrated some challenges when supplementing FA to dairy cows which need further investigation. A schematic representation of the present knowledge and questions which need to be addressed in future research on FA supplementation in dairy cows is proposed in figure 6.

## THE EFFECT OF FATTY ACIDS ON DAIRY COW FERTILITY



**Figure 6.** Schematic representation of the knowledge and questions (in red) about the effect of fatty acids on reproduction in dairy cows at the onset of the study.

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AIMS





The main hypothesis of the present thesis is that fatty acids (FA) play a major role in the metabolic adaptations during the negative energy balance (NEBAL) in high yielding dairy cows and can re-establish their reproductive capacity when implemented in their nutrition. Therefore it is important to elucidate the effect of the NEBAL in early lactation on the cows performance and on predominant FA released into the blood, which was established by specifying the following aims in Chapter 4:

1. To determine the effect of periparturient diseases associated with the NEBAL on the lactation curve which allows for a better estimation of short and long term effects on milk production (Chapter 4.1).
2. To determine the FA profile of the plasma NEFA fraction, and of the abdominal and subcutaneous fat depots in dairy cows during an episode of severe NEBAL in the postpartum period in order to get a better insight in the process of lipolysis in early lactating dairy cows (Chapter 4.2).

Both attempts to minimize the FA mobilisation or to dilute NEFA by dietary supplementation of beneficial FA might be strategies to alleviate the detrimental effect of mobilised FA. Therefore, the following aims were specified in Chapter 5:

3. To determine the effect of a milk fat depression through marine algae when used as a strategy to decrease the NEBAL early post partum (Chapter 5.1).
4. To determine how sequential feeding of n-6 and n-3 FA at levels which do not induce milk fat depression, would affect the FA profiles of blood plasma, follicular fluid and milk fat in early lactation (Chapter 5.2).

Finally, the knowledge acquired in Chapter 4 and 5 was used to interpret associations between unsaturated FA in bulk milk and fertility related key-performance-indicators as observed in a larger field trial (Chapter 6).







HEALTH CHALLENGES IN HIGH YIELDING  
DAIRY COWS DURING THE TRANSITION  
PERIOD



ON FARM EVALUATION OF THE EFFECT OF  
METABOLIC DISEASES ON THE SHAPE OF THE  
LACTATION CURVE IN DAIRY COWS THROUGH THE  
MILKBOT LACTATION MODEL

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## SUMMARY

The effects of metabolic diseases (MD) occurring during the transition period on milk production of dairy cows have been evaluated in many different ways, often with conflicting conclusions. The present study uses a fitted lactation model to analyze specific aspects of lactation curve shape and magnitude in cows that avoided culling or death in the first 120 DIM. Production and health records of 1,946 lactations in a one year follow up study design were collected from a transition management facility in Germany in order to evaluate both short and long term effects of MD on milk production. Milk production data were fitted with the nonlinear MilkBot lactation model while health records were used to classify cows as healthy (H), affected by one MD (MD), or by multiple MD (MD+).

The final dataset contained 1,071 H, 348 MD and 136 MD+ cows with distinct incidences of 3.7 % twinning, 4.8 % milk fever, 3.6 % retained placenta, 15.4 % metritis, 8.3 % ketosis, 2.0 % displaced abomasum and 3.7% mastitis in the first 30 DIM. The model containing all healthy and diseased cows showed that lactations classified as H have a milk production that rises faster (lower ramp) and also declines faster (lower persistence) in comparison with cows which encounter one or more metabolic problems. The level of production (scale) was only lowered in MD+ cows compared to H and MD cows. Though the shape of the lactation curve is changed when cows encounter uncomplicated (single MD) or complicated MD (more than one MD), a slower rise to a lower peak seems to be compensated for by higher persistency resulting in the overall 305-d milk production only being lowered in MD+ cows. In the individual disease models, specific changes in the shape of the lactation curve were found for all MD except twinning. Milk fever, retained placenta, ketosis and mastitis mainly affected the lactation curve when accompanied with another MD whereas metritis and displaced abomasum affected the lactation curve equally with or without another MD. Overall, the 305-d milk production was decreased in complicated metritis ( $10,603 \pm 50$  kg vs.  $10,114 \pm 172$  kg).

Though care should be taken in generalizing conclusions from a highly specialized transition management facility, the current study demonstrates that lactation curve analysis may contribute substantially to the evaluation of both short and long term effects of MD on milk production by detecting changes in the distribution of production that are not apparent when only totals are analyzed.



## INTRODUCTION

The transition period generally defined as the time span from 3 weeks before until 3 weeks after calving, has drawn a lot of attention of applied research during the last decades (Drackley, 1999; Leblanc, 2010). As most metabolic diseases (MD) occur in this stage of lactation, long term effects on milk production and subsequent profitability can be influenced tremendously during this short period. New feeding and management practices have focused on finding the optimal ration and management strategies to achieve maximal production with the lowest incidence of MD, however with variable success. One of the strategies includes the development of special high-needs barns for dry and fresh cow management, the so called transition management facilities (TMF). In these barns special efforts have been made on housing, processing and monitoring of dry and fresh cows in order to optimally transfer the cows to the milking barn when freshened for approximately 2 weeks, having a stable milk production level and being free from clinical illness (Fetrow et al., 2004). Besides the specifically adapted facilities, most of the TMF are equipped with an extensive software package generating a vast amount of high quality data on the transition period, creating opportunities for researchers to investigate the within herd effect of e.g. MD on milk production.

Short and long term effects of MD on the lactation curve have been thoroughly described by Fourichon et al. (1999) but overall, studies have yielded conflicting results showing both a negative as well as a lack of an effect for the same disease (Fourichon et al., 1999). This might be complicated by the very diverse approaches to the summarization of milk production. Short term effects on milk production have been evaluated by comparing daily milk production before and after the clinical diagnosis (Detilleux et al., 1997), within the first 60 (M60; Sheldon et al., 2004) or 100 DIM (van Werven et al., 1992), while long term effects have been described by analyzing the 305-d milk production (M305; Dubuc et al., 2011) or overall lactation performance (Bigras-Poulin et al., 1990). One other reason for conflicting outcomes might be the use of different statistical methods which has a major determinant impact on the interpretation of the data. Fourichon et al. (1999) discussed the difficulties and pitfalls of comparing diseased versus non-diseased animals. The higher culling rate in lower producing diseased animals generally results in a loss of data and possible underestimation of the disease effect (Bartlett et al., 1997), while the definition, severity

and interrelationships of transition diseases necessitate to control for bias when estimating the effect on milk production (Rajala and Grohn, 1998).

Lactation curve models have been used to model residuals between predicted and observed milk yield in healthy and diseased animals (Lucey et al., 1986; Rowlands and Lucey, 1986). Ehrlich (2011) described a novel nonlinear lactation model which can be fitted to milk production data to summarize an individual lactation as a set of 4 fitted parameter values, each of which corresponds to a specific aspect of lactation curve shape or magnitude. It is hypothesized that fitted parameter values may be more sensitive to the effects of MD than raw production data because, like M305, parameters are attributes of the lactation as a whole, but each parameter relates to a specific aspect of the curve.

As overall herd profitability is mainly driven by the quantity of milk produced by the proportion of cows that stay in the herd (Ducrocq et al., 1988), the aim of the present study was to use a fitted lactation curve model summarizing all milk recording data in the lactation allowing for an estimation of the short and long term effects in both uncomplicated (without concurrent other MD) and complicated MD cows that avoided culling or death in the first 120 DIM.

## MATERIALS AND METHODS

### **ANIMALS AND HOUSING**

Production and health records covering all calvings during a one year period (April 2009 - April 2010) from a 2,450 cow dairy herd in Mecklenburg-Vorpommern (Germany) were recorded using the on-farm computer system Dairy Comp 305 (DC305, Valley Ag Software, Tulare, USA). In 2009, the average milk production per lactation was 11,085 kg per cow (3.64 % milk fat, 3.32 % milk protein). Transition cows were housed in a newly designed Transition Management Facility (TMF) modelled after a prototype at the College of Veterinary Medicine of the University of Minnesota which was described by Fetrow et al. (2004). This is the first such facility in Europe, designed especially for cows in the transition period. Pens to house a maximum of 32 animals per group include sand-bedded freestalls and one headlock per stall. Heifers were transferred to the TMF 40 d before the expected parturition date, and cows at dry-off to

achieve a dry period of 42 d. Cows and heifers were housed separately within the TMF until moved to the milking barns typically between 10 and 21 DIM. Separate far-off, close-up and fresh-cow diets were fed as a TMR, once daily with push-ups every 2 hours.

## **METABOLIC DISORDERS AND TREATMENTS**

In the TMF, all diagnoses and treatments were executed and documented according to specific protocols. Animals were monitored intensely for MD during the pre- and postpartum period. All postpartum cows were examined routinely for temperature and urinary ketosis (Ketostix, Bayer AG, Leverkusen, Germany) and by vaginal and rectal exam until cows were moved to the milking barns with stable milk production and free from signs of disease. The fresh cow protocol immediately after calving involved an oral drench of 500 mL of propylene glycol (Bernd-Dieter, Dusseldorf, Germany) in 50 L of lukewarm water. All cows with parity >1 were also given 500 mL of a 38.0% calcium borogluconate solution intravenously (Calcilift Forte, Albrecht GmbH, Aulendorf, Germany). Cases of milk fever (MF), identified by cold ears and extremities, mild muscle tremor, decreased rumen motility and/or the inability to rise, near the time of calving, were treated intravenously for 3 d with 500 mL of 38.0 % calcium borogluconate solution. Even though not a MD, twinning (TWIN) was included in the analysis as cows calving twins are at greater risk for many MD compared to cows calving singletons (Fricke, 2001). Retained placenta (RP) was diagnosed when fetal membranes were not expelled for at least 24 h post partum. Uncomplicated cases of RP were not treated medically, but closely monitored for other signs of disease in subsequent days. The diagnosis of metritis (METR) was indicated by depression and fever (>39.5°C) with a purulent or foetid vaginal discharge detected by vaginal exam or rectal palpation. Cows with METR received ceftiofur hydrochloride intramuscularly for 3 consecutive days at a dose of 2.2 mg/kg of BW (Excenel RTU, Pfizer Animal Health GmbH, Berlin, Germany) and a single intra-uterine infusion of oxytetracycline at a dose of 15 mg/kg of BW (Oxy-Sleecol 200 LA, Albrecht GmbH, Aulendorf, Germany). Recurrent, non-responsive cases of METR were retreated with the intra-uterine infusion of oxytetracycline, a single intravenous injection of flunixin meglumine at a dose of 2.2 mg/kg of BW (Finadyne, Intervet Deutschland GmbH, Unterschleißheim, Germany) and intra-muscular procaine benzyl penicillin at a dose of 10,000 IU/kg of BW combined with neomycin sulphate at a dose of 5 mg/kg of BW (Neopen, Intervet Deutschland

GmbH, Unterschleißheim, Germany) for 3 consecutive days. Ketosis (KET) was defined as a drop in milk yield accompanied with urinary ketone bodies exceeding 500 µmol/L (Ketostix, Bayer AG, Leverkusen, Germany). Animals having urinary ketone bodies between 1,500 and 4,000 µmol/L were treated with 500 mL propylene glycol for 3 consecutive days. Animals exceeding 4,000 µmol/L urinary ketones were treated intravenously with 500 mL of a 40% glucose solution (B. Braun, Melsungen, Germany), 40 mL of a vitamin b-complex (Vitamin B-Komplex, Serumwerk Bernburg, Bernburg, Germany), and an intramuscular injection of dexamethasone at a dose of 0.08 mg/kg of BW (Rapidexon, Albrecht GmbH, Aulendorf, Germany). The diagnosis of a left displacement of the abomasum (LDA) was made when depressed feed intake and milk yield were accompanied by the characteristic tympanic resonance (“ping”) during percussion of the left flank. The method of choice for correction of LDA at the farm was the ‘roll-and-toggle’ method as described by Newman et al. (2008) except for cases not eligible for roll-and-toggle (e.g. recurrent cases) in which a laparoscopic abomasopexy was executed as described by Newman et al. (2008). Right displacement of the abomasum was not included in the present study as it is a less common disease occurring throughout the entire lactation and therefore less associated with the transition period (Doll et al., 2009).

Even though not a MD itself, the increased incidence of mastitis (MAST) shortly after parturition (Koivula et al., 2005) has been associated with impaired metabolic health in transition cows (Chagunda et al., 2006; Moyes et al., 2009) which has a major impact on short and long term milk production (Wilson et al., 2008). Cows with clinical signs of MAST, such as udder inflammation and abnormal milk (IDF, 1997; Chagunda et al., 2006), were first identified by farm staff in the milk parlor and confirmed afterwards by the herd manager. All cows having a MAST event before 30 DIM were included in the analysis in order to restrict the analysis to transition period disease. Affected cows were treated according to the herd protocol. Animals having mild MAST were treated with an intramammary application of 88.8 mg cefquinome sulphate per infected quarter (Cobactan LC, Intervet Deutschland GmbH, Unterschleißheim, Germany) for 3 consecutive days. In case of a severe MAST, the previous protocol was extended with cefquinome intramuscular at a dose rate of 1 mg/kg BW (Cobactan 2.5%, Intervet Deutschland GmbH, Unterschleißheim, Germany) and a single intravenous injection of

flunixin meglumine at a dose of 2.2 mg/kg of BW (Finadyne, Intervet Deutschland GmbH, Unterschleißheim, Germany).

## DATA COLLECTION AND HANDLING

Between April 2009 and April 2010, data were collected from 1,946 calvings within the herd. Disease events were diagnosed by the trained dairy personnel. Monthly DHIA milk production data and all disease events were meticulously recorded by the herdsmen using DC305. First, second and older parity cows accounted for 27.9%, 28.5% and 43.8% respectively of the primary dataset. In order to accurately estimate the lactation curve of surviving cows and include at least 3 monthly milk recordings in the lactation model, cows with insufficient milk recording data (2.3%, N=45) and cows that died (3.6%, N=70) or were sold before 120 DIM (14.2%, N=276) were excluded from the primary dataset. This resulted in 1,555 cows in the secondary dataset. First, second and older parity cows accounted for 29.5%, 31.6% and 38.9% respectively of the secondary dataset. The average DIM and range of the first recorded MD event in this dataset are represented in Table 1.

**Table 1.** Average DIM for selected metabolic problems

Metabolic Problem	DIM			Count N <sup>2</sup>
	$\mu \pm se^1$	median	range	
Twin	1.00	0.00	1	58
Milk Fever	1.32	0.13	1	74
Retained Placenta	2.17	0.20	2	53
Metritis	7.57	0.19	8	239
Ketosis	8.29	0.64	6	129
Displaced Abomasum	13.96	1.58	11	31
Mastitis	14.77	1.11	14	71

<sup>1</sup> Mean  $\pm$  standard error

<sup>2</sup> Number of animals

Subsequently, cows were classified as being healthy (H) or suffering from one (MD) or more metabolic problems (MD+). To allow for an analysis within each transition disease, animals without any recorded event for TWIN, MF, RP, METR, KET, LDA or MAST were classified as being healthy (H). Animals with one single MD were classified in the appropriate uncomplicated MD (TWIN, MF, RP, METR, KET, LDA or MAST) while animals that suffered from one MD but which was complicated with another concomitant MD, were classified as complicated MD (MD+). For example, within the MF



analysis, all animals without any MD event were classified as H, all animals that only suffered from MF were classified as MF while animals that suffered from MF in combination with another MD were classified as MF+. Within each MD+ group, no further distinction was made between diseases. For example, animals with RP that had subsequently encountered KET and animals that subsequently encountered METR were both classified as RP+.

The MilkBot lactation model, which can be expressed functionally as

$$Y(t) = a \left( 1 - \frac{e^{\frac{c-t}{b}}}{2} \right) e^{-dt}$$

(Ehrlich, 2011), was used to summarize the magnitude and shape of each individual lactation curve.  $Y(t)$  is total daily milk production on day  $t$  of the lactation, and parameters  $a$ ,  $b$ ,  $c$  and  $d$  control the shape of the curve. Monthly DHIA milk weights were exported from DC305 and fitted to the MilkBot model using a proprietary maximum likelihood fitting algorithm (DairySight LLC, Argyle, NY). The fitted parameter values generated by this process quantify magnitude, and selected aspects of the shape of each curve. Specifically, lactation scale is measured by the  $a$  parameter in the MilkBot function. It is a simple linear scaler with equal influence at all stages of lactation. The parameter  $b$ , called the ramp parameter, measures the steepness of the post-parturient rise in production, so it is most influenced by changes in early lactation. Higher ramp values correspond to a slower rise in production. The decay parameter,  $d$ , relates to senescence and loss of productive capacity, so influenced by cumulative changes in productive capacity occurring throughout the lactation. As a first-order rate constant, decay can be re-expressed as half-life, called persistence, corresponding approximately to the time in days for production to drop by half in late lactation (Ehrlich, 2011). Finally, the MilkBot offset parameter,  $c$ , is the theoretical offset between parturition and the physiological start of lactation. Normal variability in the offset parameter is expected to be small, and practically undetectable without daily milk weights in the first days of lactation, so the offset value was fixed to 0 in the present study. Recent work (Cole et al., 2012) using the MilkBot model and the same fitting algorithm used in this study demonstrated high precision and low bias when used to project future milk production. This methodology allows scale, ramp, and persistence of individual lactations to be

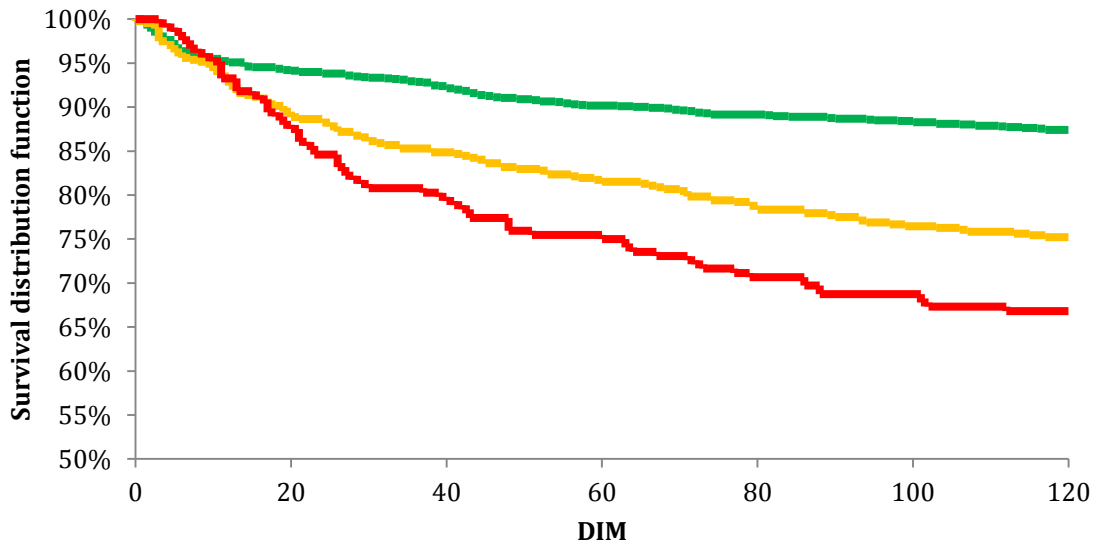
treated as independent variables in statistical models, along with the derived variables time to peak milk (TPEAK), peak milk (MPEAK), cumulative 60 d milk yield (M60) and cumulative 305 d milk yield (M305) which are easily calculated directly from MilkBot parameter values (Ehrlich, 2011).

## STATISTICAL ANALYSIS

Descriptive statistics were done using the MEANS and FREQ procedure in SAS version 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA, 2010). To evaluate the days in milk to culling or death, a Kaplan-Meier survival graph was constructed using the LIFETEST procedure in SAS version 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA, 2010). To test equality over MD strata, the Peto and Wilcoxon test was used to evaluate difference in the beginning of the survival curves, whereas the log-rank test was used to evaluate difference in the tail of the curves (Hosmer and Lemeshow, 2008). Proportion of surviving cows are reported with their 95 % confidence interval for the Kaplan-Meier estimates. To evaluate the effect of MD on the lactation curve, all MilkBot parameters and calculated production metrics were checked to approximate the Gaussian distribution. Subsequently, each variable except TPEAK was analysed using PROC MIXED from SAS version 9.2 for Windows (Institute, Inc., Cary, NC, USA, 2010). The TPEAK analysis was performed using PROC GLIMMIX with the lognormal distribution function from SAS version 9.2 for Windows (Institute, Inc., Cary, NC, USA, 2010). The statistical models contained fixed effects for MD, parity, and the interaction between MD x parity. Metabolic disease least square means and contrasts were computed using the LSMEANS and LSMESTIMATE option. Data are reported as model least square means with standard errors (LSM  $\pm$  SE) except for the TPEAK. Results from the TPEAK model were back-transformed and reported with the 95% confidence intervals using the ILINK function. Significance and tendency were declared at  $P < 0.05$  and  $0.05 < P < 0.1$  respectively.

## RESULTS

In the first analysis, the overall proportion of cows that survived until 120 DIM was 82% (80-84%) which differed between the MD strata ( $P < 0.0001$ ). The proportion of cows that survived until 120 DIM was estimated to 87% (85-89%) for H as opposed to 75% (71-79%) and 67% (60-73%) for MD and MD+ respectively (Figure 1).



**Figure 1.** Kaplan-Meier survival distribution functions for time to a died or sold event of cows that encountered no metabolic problem during the transition period (green line), cows that encountered 1 metabolic problem during the transition period (orange line) and cows that encountered more than 1 metabolic problem during the transition period (red line).

After exclusion of the cows that died or were culled before 120 DIM, 68.9% healthy cows (N=1,071), 22.4% that encountered at least one MD (N=333) and 8.7% of cows suffered from more than 1 MD (MD+, N=136) were included in the second analysis. The overall lactation incidence was highest for METR and KET occurring in 15.4% (N=239) and 8.3% (N=129) of calvings respectively (Table 2). The lowest overall lactation incidence of 1.99% was recorded for LDA (N=31).

**Table 2.** Incidence of selected metabolic problems by parity

Metabolic Problem	Parity			All	N <sup>1</sup>
	1	2	>2		
Twin	0.65%	4.48%	5.45%	3.73%	58
Milk Fever	0.00%	1.63%	10.91%	4.76%	74
Retained Placenta	3.49%	2.85%	3.80%	3.56%	53
Metritis	29.41%	11.00%	8.26%	15.37%	239
Ketosis	8.28%	4.28%	11.57%	8.30%	129
Displaced Abomasum	1.74%	1.43%	2.64%	1.99%	31
Mastitis	3.70%	4.28%	5.45%	4.57%	71
All	37.47%	20.77%	34.71%	31.13%	

<sup>1</sup> Number of animals

Results from the model that included all MD are represented in Table 3. MilkBot ramp and decay values were different between H, MD and MD+ while the Milkbot scale

**Table 3.** The effect of metabolic problems on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		MD		MD+		P-value <sup>2</sup>		
		μ	se	μ	se	μ	se	MD	INT	PAR
Ramp <sup>3</sup>	All	23.40 <sup>4a</sup>	0.20	25.11 <sup>b</sup>	0.36	26.95 <sup>c</sup>	0.56			
	1	29.07 <sup>x</sup>	0.37	29.59 <sup>x</sup>	0.55	30.49 <sup>x</sup>	1.03	<0.0001	<0.0001	<0.0001
	2	21.41 <sup>y</sup>	0.32	21.99 <sup>y</sup>	0.77	22.87 <sup>y</sup>	1.07			
	>2	19.71 <sup>az</sup>	0.32	23.75 <sup>by</sup>	0.52	27.48 <sup>cx</sup>	0.8			
Scale <sup>3</sup>	All	52.68 <sup>a</sup>	0.25	52.22 <sup>a</sup>	0.47	48.39 <sup>b</sup>	0.72			
	1	41.66 <sup>x</sup>	0.48	41.98 <sup>x</sup>	0.71	41.25 <sup>x</sup>	1.33	<0.0001	0.0030	<0.0001
	2	56.70 <sup>ay</sup>	0.41	54.51 <sup>aby</sup>	1.00	49.62 <sup>by</sup>	1.38			
	>2	59.69 <sup>az</sup>	0.41	60.16 <sup>az</sup>	0.67	54.29 <sup>by</sup>	1.03			
Decay <sup>3</sup>	All	0.002506 <sup>a</sup>	0.000023	0.002385 <sup>b</sup>	0.000043	0.002138 <sup>c</sup>	0.000067			
	1	0.001383 <sup>x</sup>	0.000045	0.001348 <sup>x</sup>	0.000065	0.001150 <sup>x</sup>	0.000122	<0.0001	0.49	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002671 <sup>y</sup>	0.000092	0.002471 <sup>y</sup>	0.000128			
	>2	0.003262 <sup>z</sup>	0.000038	0.003135 <sup>z</sup>	0.000062	0.002795 <sup>z</sup>	0.000095			
MPEAK <sup>5</sup> , kg	All	44.14 <sup>a</sup>	0.19	43.59 <sup>a</sup>	0.35	40.62 <sup>b</sup>	0.54			
	1	36.23 <sup>x</sup>	0.36	36.58 <sup>x</sup>	0.53	36.53 <sup>x</sup>	0.99	<0.0001	0.0001	<0.0001
	2	46.94 <sup>ay</sup>	0.31	45.45 <sup>ay</sup>	0.75	41.52 <sup>by</sup>	1.03			
	>2	49.25 <sup>az</sup>	0.31	48.75 <sup>az</sup>	0.50	43.80 <sup>by</sup>	0.77			
M60 <sup>6</sup> , kg	All	2,409 <sup>a</sup>	11	2,365 <sup>a</sup>	19	2,180 <sup>b</sup>	30			
	1	1,893 <sup>x</sup>	20	1,904 <sup>x</sup>	29	1,874 <sup>x</sup>	55	<0.0001	0.0001	<0.0001
	2	2,600 <sup>ay</sup>	17	2,505 <sup>ay</sup>	42	2,277 <sup>by</sup>	58			
	>2	2,734 <sup>az</sup>	17	2,686 <sup>az</sup>	28	2,389 <sup>by</sup>	43			
M305 <sup>7</sup> , kg	All	10,603 <sup>a</sup>	50	10,659 <sup>a</sup>	92	10,226 <sup>b</sup>	143			
	1	9,800 <sup>x</sup>	95	9,921 <sup>x</sup>	139	10,075	262	0.029	0.037	<0.0001
	2	10,994 <sup>ay</sup>	82	10,868 <sup>aby</sup>	197	10,114 <sup>b</sup>	273			
	>2	11,017 <sup>y</sup>	81	11,189 <sup>y</sup>	133	10,489	203			
TPEAK <sup>8</sup> , d	All	50 <sup>a</sup>	50-51 <sup>9</sup>	54 <sup>b</sup>	53-56	60 <sup>c</sup>	58-63			
	1	75 <sup>x</sup>	73-77	77 <sup>x</sup>	74-80	85 <sup>x</sup>	79-92	<0.0001	0.0001	<0.0001
	2	46 <sup>y</sup>	45-47	48 <sup>y</sup>	46-51	51 <sup>y</sup>	47-56			
	>2	38 <sup>az</sup>	38-39	44 <sup>by</sup>	42-46	51 <sup>cy</sup>	48-55			
N <sup>10</sup>		1071		348		136				

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by one metabolic disease (MD), or by multiple metabolic disease (MD+) cows

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{b} \right) e^{-dt}$$

in function of DIM (t)

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

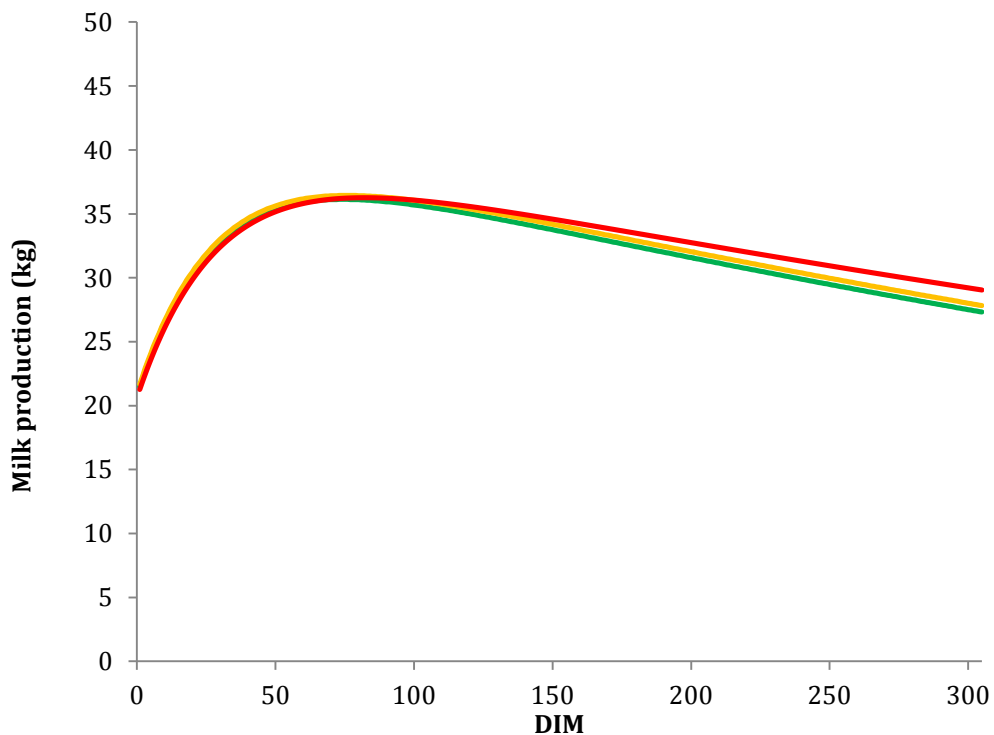
<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

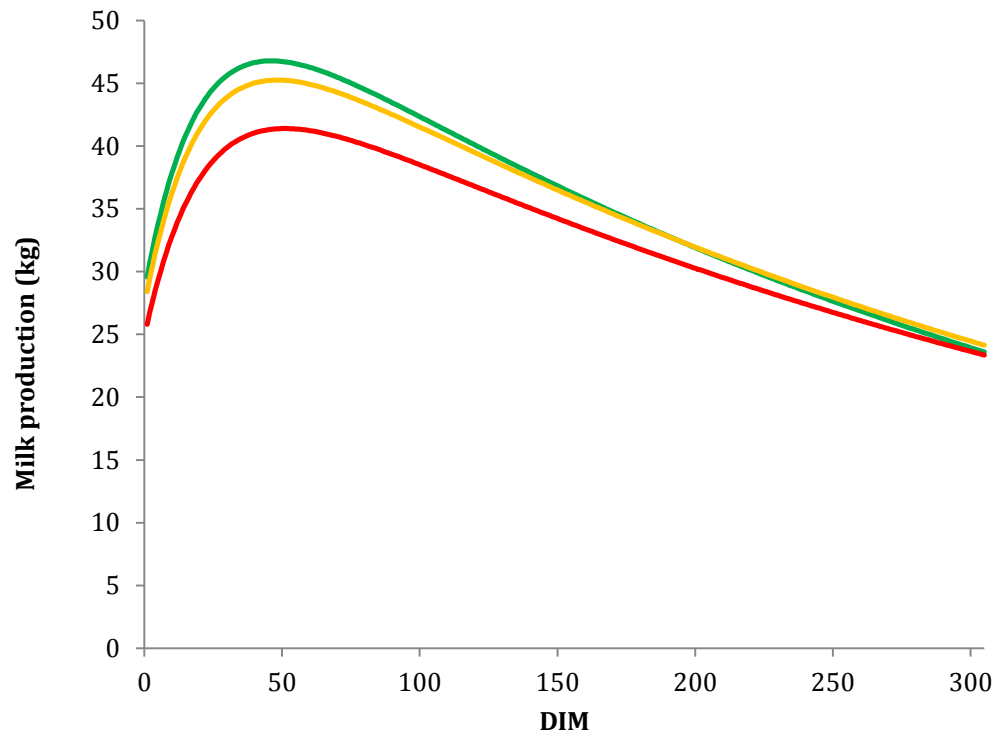
<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

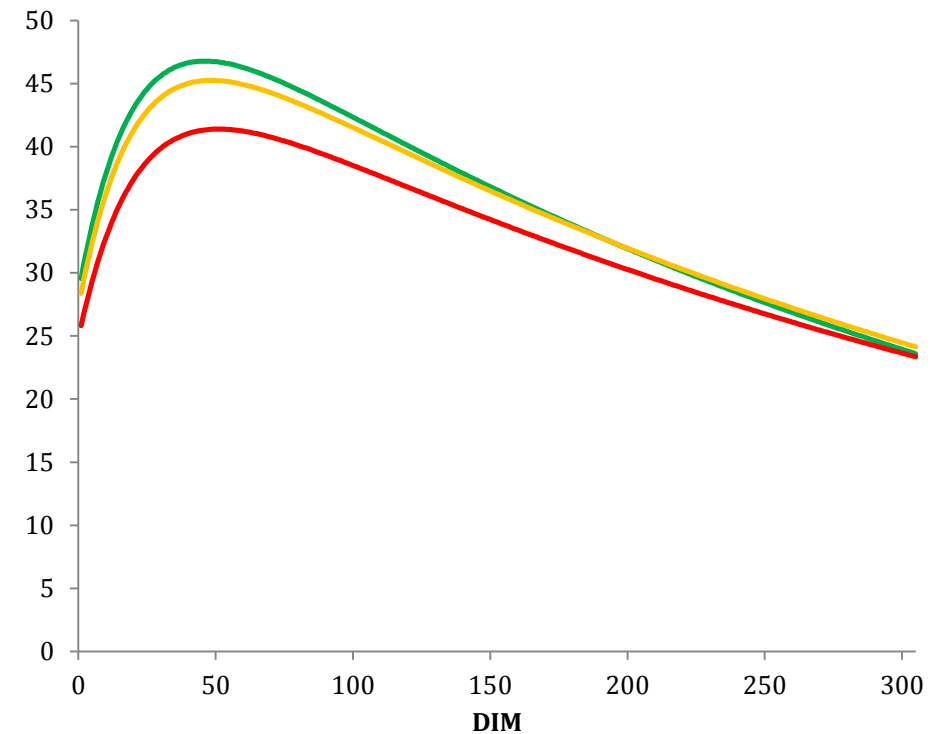
was only lowered in MD+ ( $P < 0.0001$ ). Lactations of H cows show a faster rise in production (lower ramp) and faster decline (lower persistence, higher decay,  $P < 0.0001$ ) in comparison with lactation curves of cows with one (MD) or multiple (MD+) metabolic problems. The scale of production is only diminished in MD+ cows compared to H and MD. The significant interaction term for both ramp and scale between MD and parity revealed a more severe impact of MD on the ramp in older cows (Parity  $>2$ ) next to a remarkably decreased scale in MD+ second parity cows compared to H. The lactation curves corresponding to the parameter values in Table 3 are separately shown for parity 1 (Figure 2), parity 2 (Figure 3) and parity 2+ (Figure 4). As a result of the change in the lactation curve, the TPEAK increased ( $P < 0.0001$ ) between H, MD and MD+ while both the MPEAK ( $P < 0.0001$ ) and M60 ( $P < 0.0001$ ) were only decreased when cows encountered a complicated MD+ especially in  $>1$  parity cows. The overall M305 was equal in H and MD cows but was lowered in MD+ ( $P = 0.029$ ) especially in second-parity cows ( $P = 0.037$  for the interaction term).



**Figure 2.** The effect of metabolic problems on lactation curve shape in first lactation cows: cows that encountered no metabolic problem during the transition period (green line), cows that encountered 1 metabolic problem during the transition period (orange line) and cows that encountered more than 1 metabolic problem during the transition period (red line).



**Figure 3.** The effect of metabolic problems on lactation curve shape in second lactation cows: cows that encountered no metabolic problem during the transition period (green line), cows that encountered 1 metabolic problem during the transition period (orange line) and cows that encountered more than 1 metabolic problem during the transition period (red line).



**Figure 4.** The effect of metabolic problems on lactation curve shape in third or greater lactation cows: cows that encountered no metabolic problem during the transition period (green line), cows that encountered 1 metabolic problem during the transition period (orange line) and cows that encountered more than 1 metabolic problem during the transition period (red line).

Complicated twinning (TWIN+) only tended to effect the scale ( $P = 0.061$ ) which resulted in TWIN+ animals to have a decreased MPEAK ( $P = 0.027$ ) and M60 ( $P = 0.012$ ) compared to H animals (Table 4). TPEAK was delayed in older parity TWIN+ cows compared to H cows (42-51 vs. 38-39d 95% CI).

**Table 4.** The effect of twinning on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		TWIN		TWIN+		P-value <sup>2</sup>		
		$\mu$	se	$\mu$	se	$\mu$	se	MD	INT	PAR
Ramp <sup>3</sup>	All	23.40	0.19	24.96	2.26	26.29	1.64			
	1	29.07 <sup>x</sup>	0.37	30.80 <sup>x</sup>	6.22	30.55 <sup>x</sup>	4.40	0.47	0.17	0.011
	2	21.41 <sup>y</sup>	0.32	21.87 <sup>y</sup>	2.07	23.14 <sup>y</sup>	1.72			
	>2	19.71 <sup>y</sup>	0.31	22.21 <sup>y</sup>	1.72	25.17 <sup>y</sup>	1.39			
All	52.68	0.26	49.24	3.04	48.04	2.21				
Scale <sup>3</sup>	1	41.66 <sup>x</sup>	0.49	33.62 <sup>x</sup>	8.35	40.08 <sup>x</sup>	5.91	0.061	0.47	<0.0001
	2	56.70 <sup>y</sup>	0.42	54.81 <sup>y</sup>	2.78	48.23 <sup>y</sup>	2.32			
	>2	59.69 <sup>z</sup>	0.42	59.30 <sup>z</sup>	2.32	55.82 <sup>z</sup>	1.87			
	All	0.002506	0.000023	0.002169	0.000274	0.002296	0.000199			
Decay <sup>3</sup>	1	0.001383 <sup>x</sup>	0.000044	0.000350 <sup>x</sup>	0.000754	0.001255 <sup>x</sup>	0.000533	0.28	0.55	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002736 <sup>y</sup>	0.000251	0.002543 <sup>y</sup>	0.000209			
	>2	0.003262 <sup>z</sup>	0.000038	0.003422 <sup>z</sup>	0.000209	0.003088 <sup>z</sup>	0.000169			
	All	44.14 <sup>4a</sup>	0.19	41.75 <sup>ab</sup>	2.24	40.07 <sup>b</sup>	1.63			
MPEAK <sup>5</sup> , kg	1	36.23 <sup>x</sup>	0.36	31.92 <sup>x</sup>	6.18	35.01 <sup>x</sup>	4.37	0.027	0.69	<0.0001
	2	46.94 <sup>y</sup>	0.31	45.65 <sup>y</sup>	2.06	40.20 <sup>y</sup>	1.71			
	>2	49.25 <sup>z</sup>	0.31	47.68 <sup>z</sup>	1.71	45.01 <sup>z</sup>	1.38			
	All	2,409 <sup>a</sup>	11	2,242 <sup>ab</sup>	125	2,163 <sup>b</sup>	91			
M60 <sup>6</sup> , kg	1	1,893 <sup>x</sup>	20	1,563 <sup>x</sup>	344	1,812 <sup>x</sup>	244	0.012	0.66	<0.0001
	2	2,600 <sup>y</sup>	17	2,518 <sup>y</sup>	115	2,206 <sup>y</sup>	96			
	>2	2,734 <sup>z</sup>	17	2,646 <sup>z</sup>	96	2,470 <sup>z</sup>	77			
	All	10,603	50	10,211	586	9,916	426			
M305 <sup>7</sup> , kg	1	9,800	95	9,222	1613	9,585	1140	0.22	0.79	0.27
	2	10,994	82	10,874	538	9,766	447			
	>2	11,017	81	10,537	447	10,398	361			
	All	50	50-51 <sup>9</sup>	59	51-68	57	51-64			
TPEAK <sup>8</sup> , d	1	75 <sup>x</sup>	73-77	118 <sup>x</sup>	77-190	80 <sup>x</sup>	59-111	0.33	0.027	<0.0001
	2	46 <sup>y</sup>	45-47	47 <sup>y</sup>	41-55	51 <sup>xy</sup>	45-57			
	>2	38 <sup>az</sup>	38-39	39 <sup>aby</sup>	34-44	46 <sup>by</sup>	42-51			
	All									
N <sup>10</sup>		1071		23		35				

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by twinning (TWIN), or twinning accompanied by any other metabolic disease (TWIN+) cows

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{b} \right) e^{-dt}$$

in function of DIM (t)

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

The incidence of MD per parity in the primary dataset revealed that no first parity cow suffered a MF event, so first parity animals were excluded from the MF model in order to have the statistical model converge. Due to the low number of second parity MF cows, 2nd and 3th parity cows were grouped to avoid loss of information. The MF model included 784 H, 49 MF and 25 MF+ animals with parity > 1 (Table 5). Milk fever altered the lactation curve mainly when accompanied by another MD, i.e. in MF+ cows. The ramp parameter increased (slower rise in milk production) in MF+ versus H and MF ( $P < 0.0001$ ). Scale is decreased ( $P = 0.0015$ ) and persistency increased ( $P = 0.0005$ ) for MF+ versus H and MF cows. Finally, TPEAK was delayed ( $P < 0.0001$ ) and milk production variables MPEAK ( $P = 0.0005$ ) and M60 ( $P < 0.0001$ ) were lower in MF+ compared to H and MF cows while M305 was not affected ( $P = 0.23$ ). The increased scale in parity 2-3 MF cows tended to be significant ( $P = 0.080$ ) which was reflected in both MPEAK and M60.

In the RP model, the ramp parameter was not altered ( $P = 0.33$ ). The scale parameter decreased in RP+ versus H cows ( $P = 0.0054$ ) especially in second parity RP+ cows ( $P = 0.020$ ). TPEAK was delayed in RP+ compared to H cows ( $P = 0.0028$ ), while MPEAK ( $P = 0.0055$ ) and M60 ( $P = 0.0015$ ) were decreased in RP+ compared to both RP and H cows (Table 6). Persistency was higher for RP+ cows compared to the H cows ( $P = 0.0039$ ). M305 tended to differ between RP ( $11,553 \pm 477$  kg) and RP+ ( $10,265 \pm 268$  kg) animals ( $P = 0.063$ ).

Metritis influenced all MilkBot parameters. Ramp differed between H versus METR and METR+ cows ( $P < 0.0001$ ) resulting in the steepest rise in milk production in H cows. The H cows had a higher scale compared to METR+ cows ( $P < 0.0001$ ). This resulted in a delayed TPEAK ( $P < 0.0001$ ), decreased MPEAK ( $P < 0.0001$ ) and M60 ( $P < 0.0001$ ) as cows suffered from METR or METR+ when compared to H. As can be seen in Table 7, cows experiencing uncomplicated (METR) or complicated metritis (METR+) were more persistent in their lactation compared to H cows ( $P < 0.0001$ ). Despite this persistency compensation, the M305 was decreased with 489.6 kg (95%CI: 138.0-841.1 kg) in METR+ cows compared to H. The interaction term in ramp ( $P < 0.0001$ ), scale ( $P = 0.0096$ ) and TPEAK ( $P = 0.0012$ ), MPEAK ( $P = 0.0002$ ), M60 ( $P < 0.0001$ ) and M305 ( $P = 0.044$ ) revealed a lack of an effect of METR on the shape of the lactation curve and milk production estimates in first parity cows.



**Table 5.** The effect of milk fever on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		MF		MF+		P-value <sup>2</sup>		
		$\mu$	se	$\mu$	se	$\mu$	se	MD	INT	PAR
Ramp <sup>3</sup>	All	20.55 <sup>4a</sup>	0.37	22.20 <sup>a</sup>	1.03	27.69 <sup>b</sup>	1.52	<0.0001	0.40	0.071
	2-3	20.56	0.29	22.06	1.90	25.68	1.98			
	4	20.76	0.76	23.80	1.64	32.13	2.91			
	>4	20.31	0.77	20.74	1.78	25.27	2.91			
Scale <sup>3</sup>	All	58.88 <sup>a</sup>	0.47	61.82 <sup>a</sup>	1.30	53.43 <sup>b</sup>	1.93	0.0015	0.080	0.017
	2-3	57.87 <sup>xy</sup>	0.37	65.47 <sup>xy</sup>	2.41	52.11 <sup>xy</sup>	2.50			
	4	60.99 <sup>x</sup>	0.97	64.51 <sup>x</sup>	2.07	56.78 <sup>x</sup>	3.69			
	>4	57.79 <sup>y</sup>	0.98	55.49 <sup>y</sup>	2.26	51.40 <sup>y</sup>	3.69			
Decay <sup>3</sup>	All	0.003247 <sup>a</sup>	0.000043	0.003298 <sup>a</sup>	0.000118	0.002548 <sup>b</sup>	0.000176	0.0005	0.13	0.31
	2-3	0.002982	0.000033	0.003304	0.000220	0.002763	0.000228			
	4	0.003440	0.000088	0.003405	0.000189	0.002702	0.000336			
	>4	0.003320	0.000089	0.003185	0.000206	0.002178	0.000336			
MPEAK <sup>5</sup> , kg	All	48.32 <sup>a</sup>	0.35	50.09 <sup>a</sup>	0.95	43.41 <sup>b</sup>	1.41	0.0005	0.087	0.11
	2-3	47.99	0.27	53.28	1.77	42.40	1.84			
	4	49.64	0.71	51.38	1.52	44.35	2.70			
	>4	47.34	0.72	45.61	1.65	43.49	2.70			
M60 <sup>6</sup> , kg	All	2,682 <sup>a</sup>	20	2,776 <sup>a</sup>	54	2,360 <sup>b</sup>	80	<0.0001	0.10	0.11
	2-3	2,660	15	2,948	100	2,319	104			
	4	2,754	40	2,853	86	2,403	152			
	>4	2,631	40	2,527	93	2,357	152			
M305 <sup>7</sup> , kg	All	10,869	88	11,259	240	10,624	356	0.23	0.19	0.25
	2-3	11,071	67	11,971	446	10,166	462			
	4	10,979	179	11,518	382	10,877	681			
	>4	10,557	181	10,288	417	10,829	681			
TPEAK <sup>8</sup> , d	All	40 <sup>a</sup>	39-41 <sup>9</sup>	42 <sup>a</sup>	39-45	54 <sup>b</sup>	49-60	<0.0001	0.22	0.73
	2-3	43	42-44	41	36-47	51	44-59			
	4	39	37-41	43	38-48	59	48-73			
	>4	39	37-41	41	36-47	53	43-66			
N <sup>10</sup>		784		49		25				

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by milk fever only (MF), or milk fever accompanied by any other metabolic disease (MF+) cows

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{2} \right) e^{-dt} \text{ in function of DIM (t)}$$

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

**Table 6.** The effect of retained placenta on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		RP		RP+		P-value <sup>2</sup>		
		μ	se	μ	se	μ	se	MD	INT	PAR
Ramp <sup>3</sup>	All	23.40	0.19	23.94	1.80	24.91	1.01			
	1	29.07 <sup>x</sup>	0.36	28.75 <sup>x</sup>	2.48	30.40 <sup>x</sup>	1.92	0.33	0.77	<0.0001
	2	21.41 <sup>y</sup>	0.31	20.45 <sup>y</sup>	4.30	22.11 <sup>y</sup>	1.75			
	>2	19.71 <sup>y</sup>	0.31	22.63 <sup>y</sup>	2.15	22.23 <sup>y</sup>	1.57			
All	52.68 <sup>4a</sup>	0.25	54.24 <sup>ab</sup>	2.45	48.26 <sup>b</sup>	1.38				
Scale <sup>3</sup>	All	52.68 <sup>4a</sup>	0.25	54.24 <sup>ab</sup>	2.45	48.26 <sup>b</sup>	1.38			
	1	41.66 <sup>x</sup>	0.49	46.65	3.37	41.88 <sup>x</sup>	2.61	0.0054	0.020	<0.0001
	2	56.70 <sup>ay</sup>	0.42	61.80 <sup>ab</sup>	5.83	48.46 <sup>bxy</sup>	2.38			
	>2	59.69 <sup>z</sup>	0.42	54.28	2.92	54.43 <sup>y</sup>	2.13			
All	0.002506 <sup>a</sup>	0.000023	0.002138 <sup>ab</sup>	0.000223	0.002132 <sup>b</sup>	0.000125				
Decay <sup>3</sup>	All	0.002506 <sup>a</sup>	0.000023	0.002138 <sup>ab</sup>	0.000223	0.002132 <sup>b</sup>	0.000125			
	1	0.001383 <sup>x</sup>	0.000044	0.001165 <sup>x</sup>	0.000306	0.001333 <sup>x</sup>	0.000237	0.0039	0.35	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002630 <sup>y</sup>	0.000531	0.002240 <sup>y</sup>	0.000217			
	>2	0.003262 <sup>y</sup>	0.000038	0.002620 <sup>y</sup>	0.000265	0.002824 <sup>y</sup>	0.000194			
All	44.14 <sup>a</sup>	0.19	46.21 <sup>a</sup>	1.82	41.00 <sup>b</sup>	1.03				
MPEAK <sup>5</sup> , kg	All	44.14 <sup>a</sup>	0.19	46.21 <sup>a</sup>	1.82	41.00 <sup>b</sup>	1.03			
	1	36.23 <sup>x</sup>	0.36	41.22	2.51	36.71 <sup>x</sup>	1.95	0.0055	0.0081	<0.0001
	2	46.94 <sup>ay</sup>	0.31	52.07 <sup>ab</sup>	4.35	41.20 <sup>bxy</sup>	1.78			
	>2	49.25 <sup>z</sup>	0.31	45.34	2.17	45.10 <sup>y</sup>	1.59			
All	2,409 <sup>a</sup>	11	2,505 <sup>a</sup>	102	2,207 <sup>b</sup>	57				
M60 <sup>6</sup> , kg	All	2,409 <sup>a</sup>	11	2,505 <sup>a</sup>	102	2,207 <sup>b</sup>	57			
	1	1,893 <sup>x</sup>	20	2,140	140	1,894 <sup>x</sup>	108	0.0015	0.0072	<0.0001
	2	2,600 <sup>ay</sup>	17	2,884 <sup>ab</sup>	242	2,253 <sup>bxy</sup>	99			
	>2	2,734 <sup>z</sup>	17	2,490	121	2,474 <sup>y</sup>	88			
All	10,603	50	11,553	478	10,265	268				
M305 <sup>7</sup> , kg	All	10,603	50	11,553	478	10,265	268			
	1	9,800	95	11,342	657	10,027	509	0.063	0.18	0.22
	2	10,994	82	12,455	1138	10,220	465			
	>2	11,017	81	10,862	569	10,548	416			
All	50 <sup>a</sup>	50-51 <sup>9</sup>	55 <sup>ab</sup>	48-63	57 <sup>b</sup>	53-62				
TPEAK <sup>8</sup> , d	All	50 <sup>a</sup>	50-51 <sup>9</sup>	55 <sup>ab</sup>	48-63	57 <sup>b</sup>	53-62			
	1	75 <sup>x</sup>	73-77	78 <sup>x</sup>	65-94	80 <sup>x</sup>	69-92	0.0028	0.44	<0.0001
	2	46 <sup>y</sup>	45-47	46 <sup>y</sup>	34-64	53 <sup>y</sup>	47-61			
	>2	38 <sup>y</sup>	38-39	47 <sup>y</sup>	40-56	46 <sup>y</sup>	41-51			
N <sup>10</sup>	1071		16		37					

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by retained placenta only (RP), or retained placenta accompanied by any other metabolic disease (RP+) cows

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{2} \right) e^{-dt}$$

in function of DIM (t)

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

**Table 7.** The effect of metritis on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		METR		METR+		P-value <sup>2</sup>		
		μ	se	μ	se	μ	se	MD	INT	PAR
Ramp <sup>3</sup>	All	23.40 <sup>4a</sup>	0.18	25.28 <sup>b</sup>	0.62	27.15 <sup>b</sup>	0.64			
	1	29.07 <sup>x</sup>	0.35	29.32 <sup>x</sup>	0.59	30.36 <sup>x</sup>	1.02	<0.0001	<0.0001	<0.0001
	2	21.41 <sup>y</sup>	0.30	22.34 <sup>y</sup>	1.09	22.75 <sup>y</sup>	1.22			
	>2	19.71 <sup>az</sup>	0.30	24.17 <sup>by</sup>	1.37	28.35 <sup>bx</sup>	1.07			
All	52.68 <sup>a</sup>	0.25	50.84 <sup>ab</sup>	0.84	48.26 <sup>b</sup>	0.87				
Scale <sup>3</sup>	All	52.68 <sup>a</sup>	0.25	50.84 <sup>ab</sup>	0.84	48.26 <sup>b</sup>	0.87			
	1	41.66 <sup>x</sup>	0.48	41.66 <sup>x</sup>	0.81	41.20 <sup>x</sup>	1.40	<0.0001	0.0096	<0.0001
	2	56.70 <sup>ay</sup>	0.41	52.92 <sup>aby</sup>	1.49	50.22 <sup>by</sup>	1.67			
	>2	59.69 <sup>az</sup>	0.41	57.94 <sup>aby</sup>	1.87	53.35 <sup>by</sup>	1.47			
All	0.002506 <sup>a</sup>	0.000023	0.002310 <sup>b</sup>	0.000077	0.002195 <sup>b</sup>	0.000080				
Decay <sup>3</sup>	All	0.002506 <sup>a</sup>	0.000023	0.002310 <sup>b</sup>	0.000077	0.002195 <sup>b</sup>	0.000080			
	1	0.001383 <sup>x</sup>	0.000044	0.001402 <sup>x</sup>	0.000074	0.001166 <sup>x</sup>	0.000128	<0.0001	0.21	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002594 <sup>y</sup>	0.000136	0.002572 <sup>y</sup>	0.000152			
	>2	0.003262 <sup>z</sup>	0.000037	0.002935 <sup>z</sup>	0.000171	0.002846 <sup>z</sup>	0.000134			
All	44.14 <sup>a</sup>	0.19	42.56 <sup>b</sup>	0.63	40.36 <sup>c</sup>	0.66				
MPEAK <sup>5</sup> , kg	All	44.14 <sup>a</sup>	0.19	42.56 <sup>b</sup>	0.63	40.36 <sup>c</sup>	0.66			
	1	36.23 <sup>x</sup>	0.36	36.19 <sup>x</sup>	0.61	36.47 <sup>x</sup>	1.05	<0.0001	0.0002	<0.0001
	2	46.94 <sup>ay</sup>	0.31	44.13 <sup>aby</sup>	1.12	41.83 <sup>by</sup>	1.25			
	>2	49.25 <sup>az</sup>	0.31	47.36 <sup>aby</sup>	1.41	42.79 <sup>by</sup>	1.10			
All	2,409 <sup>a</sup>	11	2,307 <sup>b</sup>	35	2,168 <sup>c</sup>	36				
M60 <sup>6</sup> , kg	All	2,409 <sup>a</sup>	11	2,307 <sup>b</sup>	35	2,168 <sup>c</sup>	36			
	1	1,893 <sup>x</sup>	20	1,890 <sup>x</sup>	34	1,873 <sup>x</sup>	58	<0.0001	<0.0001	<0.0001
	2	2,600 <sup>ay</sup>	17	2,429 <sup>aby</sup>	62	2,298 <sup>by</sup>	69			
	>2	2,734 <sup>az</sup>	17	2,603 <sup>aby</sup>	78	2,332 <sup>by</sup>	61			
All	10,603 <sup>a</sup>	50	10,479 <sup>ab</sup>	166	10,114 <sup>b</sup>	172				
M305 <sup>7</sup> , kg	All	10,603 <sup>a</sup>	50	10,479 <sup>ab</sup>	166	10,114 <sup>b</sup>	172			
	1	9,800 <sup>x</sup>	95	9,774 <sup>x</sup>	160	10,046	276	0.021	0.044	<0.0001
	2	10,994 <sup>y</sup>	81	10,608 <sup>xy</sup>	293	10,093	328			
	>2	11,017 <sup>y</sup>	81	11,054 <sup>y</sup>	369	10,202	289			
All	50 <sup>a</sup>	50-51 <sup>9</sup>	55 <sup>b</sup>	52-57	60 <sup>c</sup>	57-63				
TPEAK <sup>8</sup> , d	All	50 <sup>a</sup>	50-51 <sup>9</sup>	55 <sup>b</sup>	52-57	60 <sup>c</sup>	57-63			
	1	75 <sup>x</sup>	73-77	75 <sup>x</sup>	72-79	84 <sup>x</sup>	78-91	<0.0001	0.0012	<0.0001
	2	46 <sup>y</sup>	45-47	49 <sup>y</sup>	45-54	51 <sup>y</sup>	46-55			
	>2	38 <sup>az</sup>	38-39	45 <sup>aby</sup>	40-50	52 <sup>by</sup>	48-56			
All										
N <sup>10</sup>		1071		150		89				

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by a metritis (METR), or a metritis accompanied by any other metabolic disease (METR+) cows

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{2} \right) e^{-dt} \text{ in function of DIM (t)}$$

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

Affected cows had a delayed TPEAK both in KET (53-61d 95% CI) and KET+ (59-66d 95% CI) when compared to H cows (50-51d 95% CI) as a result of a slower milk production inclination ( $P < 0.0001$ ) especially in the older cows ( $P < 0.0001$ ). Scale of the lactation was higher in H compared to KET+ cows ( $P = 0.014$ ) resulting in similar differences for MPEAK ( $P = 0.0027$ ) and M60 ( $P < 0.0001$ ). Older KET cows had a higher lactation scale ( $P = 0.033$ ) and concomitant increased MPEAK ( $P = 0.0002$ ) and M60 ( $P = 0.0003$ ) when compared to older KET+ cows. KET+ cows had a higher persistency compared to H cows ( $P = 0.0003$ ). Overall M305 was not altered by KET ( $P = 0.64$ , Table 8).

Because of the low number of LDA (N= 31), first and second parity cows were grouped to avoid loss of information (Table 9). A slower increase in milk production ( $P < 0.0001$ ) and longer persistency in LDA+ cows compared to their healthy counterparts combined with a lower scale ( $P = 0.037$ ) results in a decreased MPEAK ( $P = 0.0041$ ) and M60 ( $P = 0.0007$ ) but overall equal M305 ( $P = 0.77$ ). The interaction term shows that older LDA cows (>2 parity) also have a slower milk increase compared to H ( $P = 0.0008$ ). Overall TPEAK was delayed in both LDA (49-82d 95% CI) and LDA+ (57-76d 95% CI) cows when compared to H cows (43-45d 95% CI,  $P < 0.0001$ ).

Mastitis during early lactation (<30 DIM) decreased the scale in MAST+ cows compared to H and MAST cows ( $P = 0.012$ ). Both MPEAK ( $P = 0.013$ ) and M60 ( $P = 0.0081$ ) were similarly decreased in MAST+ cows. As a result of a decreased decay in MAST+ cows compared to H ( $P = 0.031$ ), no overall differences in M305 could be found ( $P = 0.18$ ). The TPEAK was delayed 6 days in MAST+ cows compared to H ( $P = 0.041$ ). As shown in Table 10, no interaction of MAST with parity could be found for any of the outcome variables.

**Table 8.** The effect of ketosis on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		KET		KET+		P-value <sup>2</sup>		
		$\mu$	se	$\mu$	se	$\mu$	se	MD	INT	PAR
Ramp <sup>3</sup>	All	23.40 <sup>4a</sup>	0.19	26.59 <sup>b</sup>	0.96	28.27 <sup>b</sup>	0.80			
	1	29.07 <sup>x</sup>	0.36	30.94 <sup>x</sup>	1.54	31.10 <sup>x</sup>	1.32	<0.0001	<0.0001	<0.0001
	2	21.41 <sup>y</sup>	0.31	22.05 <sup>y</sup>	2.18	24.06 <sup>y</sup>	1.71			
	>2	19.71 <sup>az</sup>	0.31	26.78 <sup>bxy</sup>	1.06	29.64 <sup>bxy</sup>	1.03			
Scale <sup>3</sup>	All	52.68 <sup>a</sup>	0.25	52.95 <sup>ab</sup>	1.27	49.52 <sup>b</sup>	1.06			
	1	41.66 <sup>x</sup>	0.48	41.65 <sup>x</sup>	2.05	42.95 <sup>x</sup>	1.75	0.014	0.033	<0.0001
	2	56.70 <sup>y</sup>	0.42	55.91 <sup>y</sup>	2.90	51.41 <sup>xy</sup>	2.27			
	>2	59.69 <sup>az</sup>	0.41	61.30 <sup>ay</sup>	1.41	54.19 <sup>by</sup>	1.37			
Decay <sup>3</sup>	All	0.002506 <sup>a</sup>	0.000023	0.002386 <sup>ab</sup>	0.000117	0.002111 <sup>b</sup>	0.000097			
	1	0.001383 <sup>x</sup>	0.000044	0.001160 <sup>x</sup>	0.000188	0.001054 <sup>x</sup>	0.000161	0.0003	0.91	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002881 <sup>y</sup>	0.000266	0.002492 <sup>y</sup>	0.000209			
	>2	0.003262 <sup>z</sup>	0.000038	0.003116 <sup>z</sup>	0.000129	0.002786 <sup>z</sup>	0.000126			
MPEAK <sup>5</sup> , kg	All	44.14 <sup>a</sup>	0.19	43.85 <sup>ab</sup>	0.95	41.35 <sup>b</sup>	0.79			
	1	36.23 <sup>x</sup>	0.36	36.65 <sup>x</sup>	1.52	38.16 <sup>x</sup>	1.30	0.0027	0.0002	<0.0001
	2	46.94 <sup>y</sup>	0.31	46.15 <sup>y</sup>	2.16	42.68 <sup>xy</sup>	1.69			
	>2	49.25 <sup>az</sup>	0.31	48.76 <sup>ay</sup>	1.05	43.19 <sup>by</sup>	1.02			
M60 <sup>6</sup> , kg	All	2,409 <sup>a</sup>	10	2,371 <sup>ab</sup>	53	2,212 <sup>b</sup>	44			
	1	1,893 <sup>x</sup>	20	1,884 <sup>x</sup>	85	1,949 <sup>x</sup>	72	<0.0001	0.0003	<0.0001
	2	2,600 <sup>y</sup>	17	2,550 <sup>y</sup>	120	2,336 <sup>y</sup>	94			
	>2	2,734 <sup>az</sup>	17	2,677 <sup>ay</sup>	58	2,349 <sup>by</sup>	57			
M305 <sup>7</sup> , kg	All	10,603	50	10,782	250	10,477	208			
	1	9,800 <sup>x</sup>	95	10,071	402	10,589	343	0.64	0.027	0.0015
	2	10,994 <sup>y</sup>	82	10,868	569	10,408	446			
	>2	11,017 <sup>y</sup>	81	11,408	276	10,433	268			
TPEAK <sup>8</sup> , d	All	50 <sup>a</sup>	50-51 <sup>9</sup>	57 <sup>b</sup>	53-61	63 <sup>b</sup>	59-66			
	1	75 <sup>ax</sup>	73-77	84 <sup>abx</sup>	76-95	88 <sup>bx</sup>	80-97	<0.0001	0.0023	<0.0001
	2	46 <sup>y</sup>	45-47	47 <sup>y</sup>	40-55	52 <sup>y</sup>	46-60			
	>2	38 <sup>az</sup>	38-39	48 <sup>by</sup>	45-52	54 <sup>by</sup>	50-58			
N <sup>10</sup>		1071		58		71				

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by a ketosis (KET), or a ketosis accompanied by any other metabolic disease (KET+)

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{b} \right) e^{-dt} \text{ in function of DIM (t)}$$

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

**Table 9.** The effect of abomasal displacement on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		LDA		LDA+		P-value <sup>2</sup>		
		μ	se	μ	se	μ	se	MD	INT	PAR
Ramp <sup>3</sup>	All	21.42 <sup>4a</sup>	0.24	28.28 <sup>ab</sup>	2.97	32.17 <sup>b</sup>	1.64			
	1-2	24.66 <sup>x</sup>	0.26	28.45	4.76	27.39	1.87	<0.0001	0.0008	0.94
	3	19.06 <sup>ay</sup>	0.45	24.20 <sup>ab</sup>	6.74	39.00 <sup>b</sup>	3.89			
	>3	20.54 <sup>ay</sup>	0.51	32.20 <sup>b</sup>	3.37	30.11 <sup>b</sup>	2.38			
All	56.54 <sup>a</sup>	0.36	50.80 <sup>ab</sup>	4.45	50.96 <sup>b</sup>	2.46				
Scale <sup>3</sup>	All	56.54 <sup>a</sup>	0.36	50.80 <sup>ab</sup>	4.45	50.96 <sup>b</sup>	2.46			
	1-2	50.31 <sup>x</sup>	0.39	48.43 <sup>x</sup>	7.13	44.99 <sup>x</sup>	2.80	0.037	0.41	0.0026
	3	59.91 <sup>y</sup>	0.68	45.32 <sup>y</sup>	10.08	48.92 <sup>y</sup>	5.82			
	>3	59.40 <sup>y</sup>	0.77	58.65 <sup>y</sup>	5.04	58.98 <sup>z</sup>	3.56			
All	0.002931 <sup>a</sup>	0.000034	0.002113 <sup>ab</sup>	0.000417	0.002191 <sup>b</sup>	0.000231				
Decay <sup>3</sup>	All	0.002931 <sup>a</sup>	0.000034	0.002113 <sup>ab</sup>	0.000417	0.002191 <sup>b</sup>	0.000231			
	1-2	0.002241 <sup>x</sup>	0.000036	0.001045 <sup>x</sup>	0.000669	0.001625 <sup>x</sup>	0.000262	0.0011	0.81	<0.0001
	3	0.003170 <sup>xy</sup>	0.000063	0.002200 <sup>xy</sup>	0.000946	0.002310 <sup>xy</sup>	0.000546			
	>3	0.003381 <sup>y</sup>	0.000072	0.003095 <sup>y</sup>	0.000473	0.002636 <sup>y</sup>	0.000335			
All	46.91 <sup>a</sup>	0.27	42.36 <sup>ab</sup>	3.25	41.40 <sup>b</sup>	1.80				
MPEAK <sup>5</sup> , kg	All	46.91 <sup>a</sup>	0.27	42.36 <sup>ab</sup>	3.25	41.40 <sup>b</sup>	1.80			
	1-2	42.39	0.28	43.45	5.21	38.95	2.04	0.0041	0.19	0.051
	3	49.83	0.49	38.09	7.37	38.19	4.25			
	>3	48.50	0.56	45.54	3.68	47.05	2.61			
All	2,586 <sup>a</sup>	16	2,264 <sup>ab</sup>	193	2,211 <sup>b</sup>	107				
M60 <sup>6</sup> , kg	All	2,586 <sup>a</sup>	16	2,264 <sup>ab</sup>	193	2,211 <sup>b</sup>	107			
	1-2	2,300 <sup>x</sup>	17	2,253 <sup>x</sup>	309	2,056 <sup>x</sup>	121	0.0007	0.24	0.027
	3	2,766 <sup>xy</sup>	29	2,065 <sup>xy</sup>	437	2,031 <sup>xy</sup>	252			
	>3	2,693 <sup>y</sup>	33	2,474 <sup>y</sup>	218	2,547 <sup>y</sup>	154			
All	10,821	60	10,779	731	10,526	404				
M305 <sup>7</sup> , kg	All	10,821	60	10,779	731	10,526	404			
	1-2	10,487	64	11,975	1172	10,209	460	0.77	0.16	0.50
	3	11,207	111	9,549	1657	9,827	957			
	>3	10,771	126	10,813	829	11,542	586			
All	44 <sup>a</sup>	43-45 <sup>9</sup>	62 <sup>b</sup>	49-82	66 <sup>b</sup>	57-76				
TPEAK <sup>8</sup> , d	All	44 <sup>a</sup>	43-45 <sup>9</sup>	62 <sup>b</sup>	49-82	66 <sup>b</sup>	57-76			
	1-2	56 <sup>x</sup>	55-58	80 <sup>x</sup>	54-124	71 <sup>x</sup>	61-84	<0.0001	0.36	0.0049
	3	38 <sup>xy</sup>	37-40	55 <sup>xy</sup>	32-105	70 <sup>xy</sup>	51-100			
	>3	39 <sup>y</sup>	37-41	56 <sup>y</sup>	42-76	58 <sup>y</sup>	47-72			
N <sup>10</sup>	1071		7		24					

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by an abomasal displacement (LDA), or an abomasal displacement accompanied by any other metabolic disease (LDA+)

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{2} \right) e^{-dt} \text{ in function of DIM (t)}$$

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

**Table 10.** The effect of mastitis within 30 DIM on MilkBot parameter values and calculated metrics for fitted individual lactation curves

	Parity	H <sup>1</sup>		MAST		MAST+		P-value <sup>2</sup>		
		$\mu$	se	$\mu$	se	$\mu$	se	MD	Int	Parity
Ramp <sup>3</sup>	All	23.40	0.19	24.35	0.98	24.69	1.26			
	1	29.07 <sup>x</sup>	0.36	30.04 <sup>x</sup>	2.05	29.39 <sup>x</sup>	2.18	0.39	0.89	<0.0001
	2	21.41 <sup>y</sup>	0.31	21.67 <sup>y</sup>	1.59	22.32 <sup>y</sup>	2.52			
	>2	19.71 <sup>y</sup>	0.31	21.33 <sup>y</sup>	1.34	22.38 <sup>y</sup>	1.78			
All	52.68 <sup>4a</sup>	0.25	53.10 <sup>a</sup>	1.30	47.65 <sup>b</sup>	1.68				
Scale <sup>3</sup>	All	52.68 <sup>4a</sup>	0.25	53.10 <sup>a</sup>	1.30	47.65 <sup>b</sup>	1.68			
	1	41.66 <sup>x</sup>	0.48	43.74 <sup>x</sup>	2.74	40.17 <sup>x</sup>	2.90	0.012	0.46	<0.0001
	2	56.70 <sup>y</sup>	0.42	55.87 <sup>y</sup>	2.12	51.16 <sup>y</sup>	3.35			
	>2	59.69 <sup>y</sup>	0.41	59.68 <sup>y</sup>	1.79	51.62 <sup>y</sup>	2.37			
All	0.002506 <sup>a</sup>	0.000023	0.002366 <sup>ab</sup>	0.000119	0.002131 <sup>b</sup>	0.000154				
Decay <sup>3</sup>	All	0.002506 <sup>a</sup>	0.000023	0.002366 <sup>ab</sup>	0.000119	0.002131 <sup>b</sup>	0.000154			
	1	0.001383 <sup>x</sup>	0.000045	0.001363 <sup>x</sup>	0.000251	0.001340 <sup>x</sup>	0.000267	0.031	0.52	<0.0001
	2	0.002874 <sup>y</sup>	0.000038	0.002716 <sup>y</sup>	0.000195	0.002417 <sup>y</sup>	0.000308			
	>2	0.003262 <sup>y</sup>	0.000038	0.003020 <sup>y</sup>	0.000165	0.002638 <sup>y</sup>	0.000218			
All	44.14 <sup>a</sup>	0.19	44.78 <sup>a</sup>	0.97	40.52 <sup>b</sup>	1.25				
MPEAK <sup>5</sup> , kg	All	44.14 <sup>a</sup>	0.19	44.78 <sup>a</sup>	0.97	40.52 <sup>b</sup>	1.25			
	1	36.23 <sup>x</sup>	0.36	38.00 <sup>x</sup>	2.04	35.25 <sup>x</sup>	2.17	0.013	0.45	<0.0001
	2	46.94 <sup>y</sup>	0.31	46.64 <sup>y</sup>	1.58	43.08 <sup>y</sup>	2.50			
	>2	49.25 <sup>y</sup>	0.31	49.69 <sup>y</sup>	1.34	43.24 <sup>y</sup>	1.77			
All	2,409 <sup>a</sup>	11	2,423 <sup>a</sup>	54	2,191 <sup>b</sup>	70				
M60 <sup>6</sup> , kg	All	2,409 <sup>a</sup>	11	2,423 <sup>a</sup>	54	2,191 <sup>b</sup>	70			
	1	1,893 <sup>x</sup>	20	1,974 <sup>x</sup>	114	1,829 <sup>x</sup>	121	0.0081	0.41	<0.0001
	2	2,600 <sup>y</sup>	17	2,570 <sup>y</sup>	88	2,367 <sup>y</sup>	139			
	>2	2,734 <sup>y</sup>	17	2,726 <sup>y</sup>	74	2,378 <sup>y</sup>	98			
All	10,603	50	10,941	256	10,179	331				
M305 <sup>7</sup> , kg	All	10,603	50	10,941	256	10,179	331			
	1	9,800 <sup>x</sup>	96	10,281 <sup>x</sup>	540	9,618 <sup>x</sup>	573	0.18	0.95	0.0042
	2	10,994 <sup>y</sup>	82	11,119 <sup>y</sup>	418	10,551 <sup>y</sup>	661			
	>2	11,017 <sup>y</sup>	81	11,423 <sup>y</sup>	353	10,367 <sup>y</sup>	468			
All	50 <sup>a</sup>	50-51 <sup>9</sup>	53 <sup>ab</sup>	50-57	56 <sup>b</sup>	51-61				
TPEAK <sup>8</sup> , d	All	50 <sup>a</sup>	50-51 <sup>9</sup>	53 <sup>ab</sup>	50-57	56 <sup>b</sup>	51-61			
	1	75 <sup>x</sup>	73-77	78 <sup>x</sup>	68-92	77 <sup>x</sup>	66-91	0.041	0.60	<0.0001
	2	46 <sup>y</sup>	45-47	47 <sup>y</sup>	42-53	50 <sup>y</sup>	42-61			
	>2	38 <sup>y</sup>	38-39	42 <sup>y</sup>	38-46	46 <sup>y</sup>	41-53			
N <sup>10</sup>	1071		45		26					

<sup>1</sup> Values are model least square means with standard error for healthy (H), affected by a mastitis within 30 DIM (MAST), or a mastitis within 30 DIM accompanied by any other metabolic disease (MAST+)

<sup>2</sup> Effect of MD, parity (PAR), and their interaction (INT)

<sup>3</sup> The scale (a), ramp (b), offset (c=0) and decay (d) parameters from the fitted Milkbot model which can be expressed as

$$Y(t) = a \left( 1 - \frac{e^{-ct}}{b} \right) e^{-dt} \text{ in function of DIM (t)}$$

<sup>4</sup> Values differing in superscript significantly differ within the row<sup>abc</sup> or column<sup>xyz</sup>

<sup>5</sup> Peak milk yield

<sup>6</sup> Cumulative 60 DIM milk yield

<sup>7</sup> Cumulative 305 DIM milk yield

<sup>8</sup> Time to peak milk yield

<sup>9</sup> Back-transformed 95% confidence interval

<sup>10</sup> Number of animals

## DISCUSSION

Wallace et al. (1996) were the first to report an overall daily milk production decrease related to all transition health disorders, of 7.2 kg during the first 20 d post partum. The objective of the current study was to describe the effect of multiple MD on the shape of the entire lactation curve for cows that avoided culling or death during the first 120 DIM. Though the maximum likelihood algorithm used for fitting can exploit a priori information to achieve a solution even from a single data point, in that case the solution will be influenced by population means more than the data point. As a practical matter, to achieve reasonable estimates of the 3 parameters (scale, ramp, and decay) for individual lactations, at least three monthly test days were needed. This required censoring lactations from the main data set in which the cow died or was culled before 120 DIM. This limitation reflects the actual on farm situation where herd profitability is driven by the quantity of milk produced by the surviving proportion of cows in the herd (Ducrocq et al., 1988) while negatively influenced by culling (Essl, 1998; De Vries, 2006). The surviving proportion at 60 DIM in this study (13.6 %) was higher when compared to data from Dechow and Goodling (2008) who found an average proportion of animals culled at 60 DIM of  $6.8 \pm 4.6$  % in 2,574 dairy herds. A more recent study analyzing 107 large dairy farms revealed a proportion of culled animals at 60 DIM of 10.5 % which is more comparable to our data (Scheifers et al., 2010). Although the observation of an increased culling rate in larger herds has been described earlier (Hadley et al., 2006), differences in categorisation of cull events (e.g. inclusion or exclusion of dairy sales) and the method of recording might explain differences between studies as well (Fetrow et al., 2006).

Though the shape of the curve is changed with uncomplicated or complicated MD, a slower rise to a lower peak seems to be compensated for by better persistency so that the change in total production (M305) is less marked than the change in the distribution of production within the lactation. Metabolic problems have been described to negatively affect fertility in dairy cows since the early 90's (Staples et al., 1990). As cows are less likely to conceive when they contract one or more MD (Walsh et al., 2007; Sheldon et al., 2009), the absence of a pregnancy may be related to the higher persistency in both MD and MD+ cows (Svennersten-Sjaunja and Olsson, 2005).



In most studies a positive association between TWIN and milk production in the lactation carrying twins has been described, as comprehensively discussed by Fricke (2001). The results in the present study showing a decreased M60, MPEAK and M305 in the lactation subsequent to complicated TWIN compared to H cows confirm the conclusions made by Nielen et al. (1989) and Mostafa (2009). Both the latter studies however did not account for all other diseases which might explain the lack of an effect in our study for the uncomplicated TWIN cows. As TWIN and RP are both risk factors for METR, the lactation curve might be influenced severely by the METR cases (see further).

The incidence of MF in the current study is similar to that reported by DeGaris and Lean (2009) and Fourichon et al. (1999). As in the present study, cows that fail to recover after treatment are often culled before recording of milk yields thereby biasing the estimation of the effect of MF on milk production (Fourichon et al., 1999). Uncomplicated MF has been found to have no effect on milk production variables in early lactation (Lucey et al., 1986; Deluyker et al., 1991) except for the studies by Rajala-Schultz et al. (1999a) that showed decreased milk production in the first 4-6 weeks and Heuer et al. (1999) that found a 1.3 kg higher first test milk production in MF cows. Rajala-Schultz et al. (1999a) showed an increased milk production in late lactation of 1.1 to 1.7 kg/d. One of the only studies reporting long term effect (Bigras-Poulin et al., 1990) found no difference in M305 suggesting a difference in persistency which could be confirmed by our study. Milk fever has been linked with many other MD as a gateway disease facilitating the occurrence of other disorders (Houe et al., 2001; Goff, 2008). Our data strongly supports this finding as only complicated MF has a significant impact on milk production variables.

Short term effects of RP on milk production have been described as from 5 DIM by Deluyker et al. (1991), up to the first 4 weeks (Lucey et al., 1986), 60 DIM (Sheldon et al., 2004) and 100 DIM (van Werven et al., 1992). Conclusions are in accordance with our results for RP+ but not for RP cows. Differences might be explained by the statistical methodology used by other authors that did not always adjust for concomitant periparturient diseases in the aforementioned studies as discussed by Fourichon et al. (1999). Animals contracted with RP or RP+ in our data had equal persistency but different MPEAK resulting in a tendency for a higher M305 in RP versus RP+ cows. Increased M305 in cows affected by RP had been demonstrated as early as 1974 by

Muller and Owens (1974) and Martin et al. (1986), but these results had not been repeated since (Rajala and Grohn, 1998; Fourichon et al., 1999).

Studies reporting the effect of METR that were published before the end of the 20th century, generally produced conflicting results as both a negative (Deluyker et al., 1991; Rajala and Grohn, 1998) as well as a lack of an effect (Fourichon et al., 1999) on short term milk production had been shown. Our data accord with reports of Markusfeld (2003) showing a decreased peak yield and later occurrence of peak. In contrast with Dubuc et al. (2011) and Wittrock et al. (2011) we were able to find a decreased M305 in METR+ cows independent of parity. Markusfeld (2003) concluded that primary METR lowered persistency which was defined as peak yield / 180-DIM yield \* 100, in both primiparous and multiparous cows. We calculated persistency differently (Ehrlich, 2011) but final conclusions are similar: METR and METR+ cows decrease milk production at a slower rate compared to H cows. Uterine infection after calving has been demonstrated to compromise fertility in dairy cows (Opsomer et al., 2000; Melendez et al., 2004), extending the interval calving to conception which might explain this higher persistency in METR and METR+ cows (Svennersten-Sjaunja and Olsson, 2005).

From both the review of Fourichon et al. (1999) and our results, it can be concluded that short term effects of KET are relevant. The impact in early lactation differed according to the diagnosis being either based on clinical signs (Deluyker et al., 1991), or on elevated levels of milk (Dohoo and Martin, 1984b) or blood ketone bodies (Duffield et al., 2009). The latter study showed an altered impact when using different threshold levels at week 1 or week 2 after calving for blood ketone bodies (Duffield et al., 2009). The greatest loss in milk yield occurred at blood ketone bodies of 1,400  $\mu\text{mol/L}$  (-1.88 kg/d) and 2,000  $\mu\text{mol/L}$  (-3.3 kg/d) in the first and second week post partum, respectively. When confirmed by milk (Dohoo and Martin, 1984a) or blood ketone bodies (Duffield et al., 2009), KET seems to result in a milk yield loss of 1 to 2 kg/d at first DHI test. Reported long term effects have been more conflicting going from decreased (Gustafsson et al., 1993), no difference (Dohoo and Martin, 1984a) or even an increased M305 (Detilleux et al., 1994). A recent study contributed to the explanation for these observations by reporting a positive and negative relationship between blood BHBA levels and the projected 305-d mature equivalent (ME305) milk yield in heifers and multiparous cows respectively (Ospina et al., 2010). In addition, Duffield et al. (2009)

reported a positive and negative association between blood BHBA levels and the projected ME305 in week 1 and week 2 after calving respectively. As in our study, Gustafsson et al. (1993) and Gustafsson and Emanuelson (1996) have reported a flattened lactation curve in ketotic cows (confirmed by milk ketone bodies) which is similar to our results for KET+ cows. A different effect of KET on persistency between primiparous and multiparous cows as found by Markusfeld (2003) could not be confirmed by our results.

The calculated decrease in milk production in early lactation (M60) of 375 kg in LDA+ cows is smaller when compared with the loss reported by Detilleux et al. (1997), probably due to a difference in timeframe during which milk production was monitored. We found a higher concurrent disease incidence in the current study (77%) when compared to the data of Detilleux et al. (54%; 1997). The latter might be caused by the very intense monitoring of cows in the TMF. Milk loss due to LDA has been reported to occur as soon as 10 d prior to diagnosis (Van Winden et al., 2003), which might explain the 30% of M305 losses occurring before diagnosis as found by Detilleux et al. (1997). M305 milk losses up to 1,016 kg per lactation have been reported after surgical correction of LDA cases (Hamann et al., 2004). Raizman and Santos (2002) did find a decreased milk production per day and a tendency for a 320 and 544 kg lower ME305 in primiparous and multiparous cows, respectively. Our results are the first that strengthen the findings of Jorritsma et al. (2008), reporting unchanged M305 production in dairy cows suffering from LDA. Bartlett et al. (1997) and Ehrlich (1995) observed that milk production of cows suffering from LDA catches up to their herdmates by 5 months after appropriate therapy. Thereafter, LDA cows as in our study, produced more milk than their herdmates. Bias as low producing LDA cows are being culled at a faster rate might have contributed to our and others finding of a higher persistency in LDA+ cows (Bartlett et al., 1997). Both LDA and KET nicely demonstrate that the different shape of the lactation curve in uncomplicated and complicated MD with a slower rise to a lower peak being compensated for by better persistency in cows that avoid culling during the first 120 DIM.

The effect of MAST has been described and reviewed intensively by Hortet and Seegers (1998) and Seegers et al. (2003). Many studies have focused on the effect of MAST on short term effects around the moment of diagnosis (Rajala-Schultz et al., 1999b;

Wilson et al., 2004) but reports studying the effect of MAST in early lactation on the shape of the lactation curve have been limited. Andersen et al. (2011) reported differences between lactations with records of veterinary clinical MAST treatment or without records of veterinary treatments. Considering that most MAST cases in the aforementioned study occurred in early lactation, our results confirm that MPEAK and TPEAK are not altered in uncomplicated MAST. In contrast with the results of Andersen et al. (2011), we were not able to find differences in the slope before peak milk production in second and older parity MAST cows. A higher persistency in MAST cows as reported by Appuhamy et al. (2007) could be confirmed in our study for MAST+ cows but not by others (Andersen et al., 2011). Overall, short term effects on milk yield were limited to MAST+ cows which together with the increased persistency resulted in an overall neutral effect on M305. This is in contrast with studies reporting M305 losses up to about 10% (Hortet and Seegers, 1998; Wilson et al., 2004; Hagnestam et al., 2007). Milk losses have been reported to be largest when MAST occurs around the moment of peak milk yield in primiparous and multiparous cows (Hagnestam et al., 2007). In this transition period study, time to peak milk yield was substantially higher compared to the upper limit of inclusion of MAST (30 DIM) which might have resulted in the observed lack of an effect on M305 compared to previous studies. This implies that in cows that avoid culling up to 120 DIM, long term effects on M305 due to MAST in the first month of lactation might be limited.

## CONCLUSIONS

The results of the current study demonstrate that lactation curve analysis might contribute substantially to the evaluation of both short and long term effects of MD during the transition period of dairy cows. Milk fever, retained placenta, ketosis and mastitis mainly affected the lactation curve when accompanied with another MD whereas metritis and displaced abomasum affected the lactation curve equally with or without another MD. Overall, analysis of long term effects of MD under the circumstances of this TMF study suggest a compensatory increase in persistency may partially counteract milk production losses at the time of disease. Further research should focus on the analysis of survival and life time production to document in more detail the effect of MD in dairy cows as well as possible interaction with the effects of pregnancy.

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# THE FATTY ACID PROFILE OF SUBCUTANEOUS AND ABDOMINAL FAT IN DAIRY COWS WITH LEFT DISPLACEMENT OF THE ABOMASUM

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## SUMMARY

The objective of this study was to determine the fatty acid (FA) profile and assess desaturase indices of non esterified fatty acids (NEFA) in the blood as well as in the abdominal (ABD) and subcutaneous (SUBC) fat stores in dairy cows with left displacement of the abomasum (LDA). Blood, ABD and SUBC samples were taken from 50 Holstein cows offered for surgery to correct LDA. The FA profile of the 3 compartments was determined by gas chromatography after lipid extraction, methylation and, in the case of blood plasma, separation of lipid classes.

The most abundant FA in all three compartments were 16:0, 18:0 and 18:1 *cis*-9, with a total proportion of 82.5, 68.0 and 74.1 g/100 g FA in ABD, NEFA and SUBC, respectively. A principal component analysis (PCA) was performed on the entire FA profile as well as on the  $\Delta$ 9-desaturase indices (14:1 *cis*-9/14:0, 16:1 *cis*-9/16:0, 18:1 *cis*-9/18:0). The PCA extracted 2 principal components (PC) representing 51.6 % (PC1) and 21.1 % (PC2) of the total variance in FA composition of the three compartments. The loading plot for the regression factors revealed a strong positive correlation between PC1 and the  $\Delta$ 9-desaturase indices, the proportions of 14:1 *cis*-9 and 16:1 *cis*-9, while negatively correlated with the proportion of 18:0 and saturated FA. The correlation with PC2 was positive for the proportion of unsaturated FA, 18:2n-6 and 18:3n-3 and negative for the proportion of 14:0, 16:0 and saturated FA. The SUBC could be distinguished from the NEFA and ABD by a positive score for PC1 while differentiation among the latter 2 compartments could be made by a positive (NEFA) or negative (ABD) score for PC2. The  $\Delta$ 9-desaturase indices for C14 and C16 differed between all compartments but were numerically closer for NEFA and ABD versus NEFA and SUBC. The desaturase indices of the main FA (18:1 *cis*-9 and 18:0) did not differ between NEFA and ABD.

In conclusion, these results suggest the existence of a different FA composition in ABD versus SUBC. The findings of a greater similarity between the FA profiles of ABD and NEFA compared to SUBC and NEFA and the closer desaturase indices of ABD and NEFA, support the hypothesis of a preferential mobilization of ABD fat in dairy cows with LDA.



## INTRODUCTION

The onset of lactation in dairy cows is typically characterised by a number of vast metabolic adaptations. An imbalanced energy input-output switches the metabolism of the dairy cow from an anabolic to a catabolic state, commonly called the negative energy balance (NEBAL). Homeorhetic control mechanisms assure that body reserves, mainly the adipose tissues, will be mobilized to meet this temporary energy deficit (Bauman and Currie, 1980). The body fat mobilization results in an increase in blood NEFA concentrations around parturition. Elevated NEFA concentrations impair metabolic and immune cell function in humans (Mora and Pessin, 2002), and are associated with impaired metabolic health in transition cows (Herdt, 2000; Jorritsma et al., 2003; Bobe et al., 2004). Especially the saturated NEFA have been associated with direct toxic effects for bovine oocytes, embryos and granulosa cells, being an important metabolic link between metabolic pressure and subfertility in dairy cows (Leroy et al., 2005; Vanholder et al., 2005; Van Hoeck et al., 2011). The chain length, degree of saturation and position of the double bonds in fatty acids (FA) determine their function in different biological processes (Mattos et al., 2000). Immune cell function has been shown to be impaired by saturated NEFA in humans (Haversen et al., 2009) and periparturient dairy cows (Contreras and Sordillo, 2011), whereas positive health effects have been attributed to unsaturated FA both in humans (Calder, 2009) and in dairy cows (Aardema et al., 2011). Moreover, lipomobilization in early lactation releases large amounts of FA (up to 3.2 kg/d; Drackley et al., 2001) mainly from the subcutaneously (SUBC) and abdominally (ABD) stored fat depots. SUBC is known to have a more unsaturated FA profile than ABD, as the level of saturation increases with increasing distance from the animal's exterior (De Smet et al., 2004). In humans, omental and mesenteric adipocytes are more metabolically active and sensitive to lipolysis than SUBC adipocytes (Wajchenberg, 2000; Jensen, 2007; Hajer et al., 2008). In Holstein Friesian heifers, ABD adipocytes have been shown to possess, when not adjusted for their larger cell size, a greater lipogenic enzyme activity per cell in comparison with SUBC adipocytes (Eguinoa et al., 2003). We hypothesized that ABD and SUBC fat tissues do have a different FA profile rendering them at a different level of pathogenicity in case of lipolysis. Therefore, the objective of this study was to determine the FA profile of the plasma NEFA fraction, and the ABD and

SUBC fat depots in dairy cows during an episode of severe NEBAL in the postpartum period.

## MATERIALS AND METHODS

### COWS AND EXPERIMENTAL DESIGN

Between July 2008 and January 2009, seven veterinarians took samples from dairy cows offered for surgery to correct a left displacement of the abomasum (LDA). The average monthly temperature as registered by the Royal Meteorological Institute of Belgium (RMI, Ukkel, Belgium) in this period was 8.7°C with a range from 0.7 to 17.6 °C. An average monthly relative humidity of 84 % was reported with a range from 75 to 91 % (RMI, Ukkel). Cow specific data (breed, calving date, parity) were collected at the moment of surgery. At the same moment, each veterinarian recorded the BCS on a 1-5 scale, with 0.25 increments (Edmonson et al., 1989), together with the omental fat score (OFS) based on a 5-point scale as described by Van Eetvelde et al. (2011). In total, samples were collected from 50 cows that were black and white (83 %) and red and white Holsteins (17 %). The contribution of first parity versus older cows was 39 % and 61 % respectively. The median DIM at the time of sampling (day of LDA surgery) was 11 (range: 1 – 113 DIM). No individual feed intakes were available as samples were taken from cows on commercial dairy herds. Typically for the part of Belgium where samples were taken, postpartum rations were based on corn- and grass-silage supplemented with sugar beet pulp, next to concentrate and a protein supplement.

### SAMPLING

Prior to surgery, blood samples were taken from the coccygeal vein (sample was discarded when arterial blood was observed) into a tube with ethylene diamine tetra acetic acid as anticoagulant (Venoject, Autosep, Terumo Europe, Leuven, Belgium). Blood samples were taken before surgery to avoid elevated NEFA concentrations due to stress (Leroy et al., 2011). Within 2 hours after collection, blood samples were centrifuged (1,500 x *g*, 20 min) and the plasma was stored at -20°C until analysis. The animals were prepared for left paralumbar fossa abomasopexy or right paralumbar fossa omentopexy as described by Turner et al. (1989). After incision of the skin, a subcutaneous fat sample (1 g) was harvested. After removal of most blood with blotter

paper, the sample was deposited into a 10 mL tube containing phosphate buffered saline (PBS) solution to remove the rest of the blood. After opening the peritoneal cavity, omental fat (1 g) was dissected. The latter fat sample was treated similarly as the subcutaneous sample. The fat samples were subsequently moved into tubes without PBS – solution and stored at – 20°C until further analysis.

## **CHEMICAL ANALYSIS**

The blood plasma analysis for aspartate aminotransferase (AST), BHBA, gamma-glutamyltransferase (GGT) and NEFA were carried out by means of an automated colorimetric analyser (Konelab™ 20 XTi Clinical chemistry analyzer, Thermo Electron Cooperation, Vantaa, Finland) at The Flemish Veterinary Health Service (Dierengezondheidszorg-Vlaanderen, Torhout, Belgium).

## **LIPID EXTRACTION**

Lipids in ABD and SUBC fat were extracted with chloroform/methanol (C/M: 2/1, vol/vol) as described by Lourenço et al. (2007). Lipids in blood plasma (0.5 mL) were extracted overnight with chloroform/methanol (10 mL; C/M: 2/1, vol/vol) and butylated hydroxytoluene (BHT) in chloroform (0.5 mL; 0.1% w/vol) as described by Raes et al. (2001). Samples were filtered (Fiorini Filtres, Ingré, France) and the filtrate collected in extraction tubes containing 15 mL of distilled water. The original tubes (two times) and filter were washed with 5 mL C/M (2/1, vol/vol). After shaking and centrifugation (1821 x *g*, 10 min), the aqueous layer was removed. The remaining liquid in the extraction tube was further allowed to separate in a separatory funnel. The extracts were evaporated in a water bath at 40°C using a rotary evaporator and subsequently dissolved in 10 mL C/M (2/1, vol/vol). Extracts were stored at -20°C. Lipids in plasma were fractionated in neutral (triacylglycerol and cholesterylesters), NEFA and phospholipids using an aminopropyl silica column (Bond Elut-NH<sub>2</sub>, Varian Medical Systems Belgium, Diegem, Belgium). After washing the column with hexane (2 × 1 mL), lipids were loaded in 1 mL chloroform containing an internal standard (17:0). Neutral lipids were eluted with chloroform (6 × 0.5 mL). The NEFA fraction was eluted with 2% acetic acid in diethyl ether (6 × 0.5 mL). Finally, the polar fraction was eluted with C/M (6/1, vol/vol, 3 × 0.5 mL) followed by sodium-acetate (0.05M) in C/M (6/1, vol/vol, 3 × 0.5 mL).

## METHYLATION AND GC ANALYSIS

Fatty acids in lipid extracts and lipid fractions were methylated with NaOH/MeOH (0.5 M) followed by HCl/MeOH (1:1; v/v) as described by Raes et al. (2001). After extraction of the fatty acid methyl esters (FAME) with hexane and evaporation under a stream of nitrogen, FAME were finally dissolved in hexane, 1 mL for ABD and SUBC fat and 250  $\mu$ L for the NEFA fraction in plasma lipids, respectively. FAME were stored at -18°C until further analysis.

The FAME analysis was performed with a gas chromatograph (GC; Hewlett-Packard 6890 gas chromatograph, Hewlett-Packard, Brussels, Belgium) equipped with a SolGel-wax column (30 m x 0.25 mm x 0.25  $\mu$ m; SGE Analytical Science, Victoria, Australia). The temperature program was 150 °C for 2 min, increasing at 3°C/min until 250°C. The injection temperature was set at 250°C, the detector temperature fixed at 280°C.

## DATA HANDLING AND STATISTICAL ANALYSIS

All statistical analyses were performed using SAS software (Release 9.2, SAS Institute, Cary, NC). Descriptive statistics mean, SD, median and range were done using the MEANS procedure. The Pearson correlation was calculated between BCS and OFS with the CORR procedure after standardization by subtracting the mean average veterinarian BCS and OFS to account for the effect of the different veterinarians that participated in the study. Fatty acids were grouped according to the degree of saturation in SFA (14:0, 16:0, 18:0), MUFA (14:1 *cis*-9, 16:1 *cis*-9, 18:1 *cis*-9, 18:1 *trans*-11) and PUFA (18:2 *cis*-9, *trans*-11, 18:2n-6, 18:3n-3). The  $\Delta$ 9-desaturase indices were calculated as the ratio between the product and its precursor for 14:1 *cis*-9/14:0 (C14-ratio), 16:1 *cis*-9/16:0 (C16-ratio) and 18:1 *cis*-9 /18:0 (C18-ratio). The MIXED procedure was used to analyze the effect of the compartment (plasma NEFA, ABD and SUBC) on the FA composition. The model included compartment and cow as respectively fixed and random effect. Data are reported as model least square means with standard errors unless indicated otherwise. Significance and tendency were declared at  $P < 0.05$  and  $0.05 < P < 0.1$  respectively.

A principal component analysis (PCA), using the PRINCOMP procedure was conducted after standardisation of the data. The objective of the PCA is to synthesize the

overall information in a large set of variables into a smaller number of linear combinations of orthogonal variables called principles components (PC). The analysis sequentially minimizes the remaining variation in the multivariate data. As a result, the PCA condenses the information into loadings that show the relative importance of the original variables in accounting for the variability in the observed data, which is shown in a loading plot. Finally, the distribution of the observed data across the PC is shown by the scores in a score plot (Desnoyers et al., 2011). Each object (cow x compartment) was analysed to be a data vector of 16 variables (14:0, 14:1 *cis*-9, 16:0, 16:1 *cis*-9, 18:0, 18:1 *cis*-9, 18:1 *trans*-11, 18:2 *cis*-9, *trans*-11, 18:2n-6, 18:3n-3, SFA, MUFA, PUFA, C14-ratio, C16-ratio, C18-ratio). Compartment effects are evaluated for the individual PC scores for PC1 and PC2 with the same MIXED procedure as reported for the individual FA.

## RESULTS

### CHEMICAL COMPOSITION OF BLOOD PLASMA

The descriptive statistics of the blood plasma AST, BHBA, GGT and NEFA concentration is represented in Table 1.

**Table 1.** Blood plasma metabolite concentrations in dairy cows suffering from a left displacement of the abomasum

	Mean $\pm$ SD	Median	Range
NEFA, mmol/L	1.28 $\pm$ 0.61	1.16	0.21 – 2.93
BHBA, mmol/L	2.48 $\pm$ 1.94	2.01	0.34 – 8.40
GGT, IU/L <sup>1</sup>	205 $\pm$ 92	187	66 – 557
AST, IU/L <sup>2</sup>	39 $\pm$ 46	30	6 – 295

<sup>1</sup> Gamma-glutamyltransferase

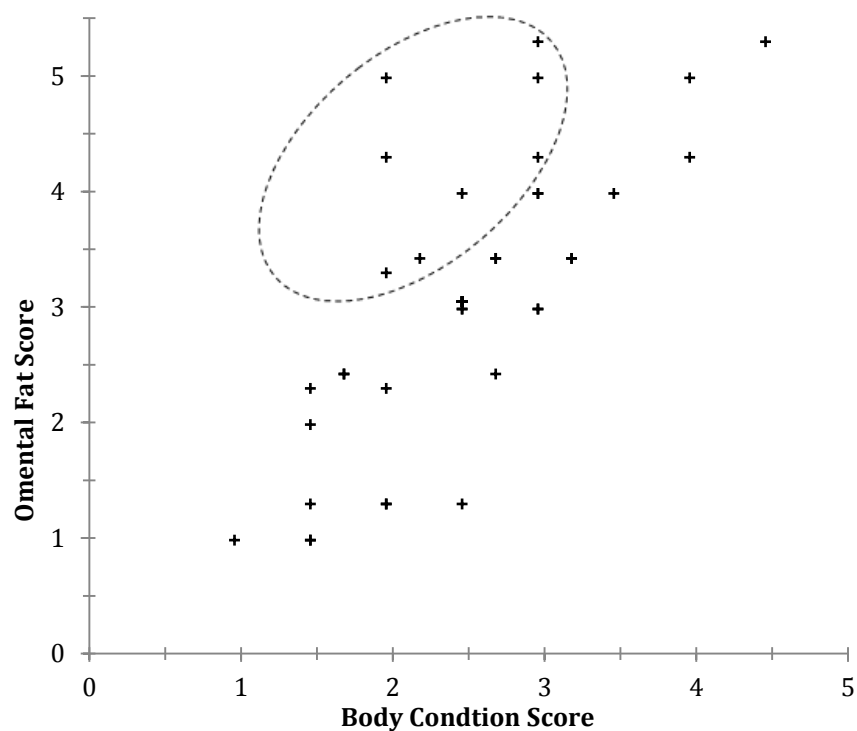
<sup>2</sup> Aspartate aminotransferase

The NEFA concentration in blood plasma averaged 1.28  $\pm$  0.61 mmol/L (mean  $\pm$  SD) with a range from 0.21 to 2.93 mmol/L. The BHBA concentration in blood plasma was 2.48  $\pm$  1.94 mmol/L (mean  $\pm$  SD) with a range from 0.34 to 8.40 mmol/L.



## CORRELATION BETWEEN THE BODY CONDITION SCORE AND THE OMENTAL FAT SCORE

Four cows had missing data for BCS and OFS and were not included in the correlation analysis. The mean standardized BCS and OFS were  $2.46 \pm 1.18$  and  $2.46 \pm 0.70$  respectively. A positive Pearson correlation coefficient of 0.73 was calculated between both scores ( $P < 0.0001$ ) and is represented in Figure 1. Nevertheless, some observations showed a high OFS and normal BCS, which is represented by a circle in Figure 1.



**Figure 1.** Scatterplot presenting the correlation between the body condition score and omental fat score at the moment of correction for a left abomasal displacement (n=46). The circle represents cows with a high omental fat score and normal body condition score.

## FATTY ACID COMPOSITION OF PLASMA NEFA, ABDOMINAL AND SUBCUTANEOUS FAT

Least square means of the FA proportion by weight of total identified FA in ABD, NEFA and SUBC in dairy cows are shown in Table 2 and Figure 2. The 3 most abundant FA in all compartments 16:0, 18:0 and 18:1 *cis*-9 represented 82.5, 68.0 and 74.1 % of the total FA in ABD, NEFA and SUBC, respectively. The highest level of saturation (SFA) was found in ABD followed by NEFA and SUBC ( $P < 0.0001$ ). The proportion of MUFA

was significantly higher in SUBC than in ABD and NEFA ( $P < 0.0001$ ). The PUFA level was higher in NEFA compared with ABD and SUBC ( $P < 0.0001$ ). The  $\Delta 9$ -desaturase indices for C14 and C16 were lowest in ABD and highest in SUBC ( $P < 0.0001$ ). The C18-ratio was lower in ABD and NEFA compared with SUBC ( $P < 0.0001$ ; Figure 3).

**Table 2.** Fatty acid composition (g/100 g FA) of the 3 main fat compartments in dairy cows suffering from a left displacement of the abomasum

Fatty acid	Compartment			SEM	P
	Abdominal <sup>1</sup>	NEFA	Subcutaneous		
	(g/100 g)				
14:0	2.52 <sup>a2</sup>	1.80 <sup>b</sup>	3.03 <sup>c</sup>	0.098	<0.0001
14:1 <i>cis</i> -9	0.22 <sup>a</sup>	0.30 <sup>a</sup>	1.57 <sup>b</sup>	0.090	<0.0001
16:0	24.7 <sup>a</sup>	22.0 <sup>b</sup>	25.2 <sup>a</sup>	0.48	<0.0001
16:1 <i>cis</i> -9	1.30 <sup>a</sup>	2.02 <sup>a</sup>	6.07 <sup>b</sup>	0.255	<0.0001
18:0	26.6 <sup>a</sup>	20.1 <sup>b</sup>	11.0 <sup>c</sup>	0.61	<0.0001
18:1 <i>cis</i> -9	31.1 <sup>a</sup>	26.2 <sup>b</sup>	37.9 <sup>c</sup>	0.79	<0.0001
18:1 <i>trans</i> -11	1.97 <sup>a</sup>	2.14 <sup>ab</sup>	2.31 <sup>b</sup>	0.100	<0.0001
18:2 <i>cis</i> -9, <i>trans</i> -11	0.272 <sup>a</sup>	0.288 <sup>a</sup>	0.644 <sup>b</sup>	0.0459	<0.0001
18:2n-6	0.94 <sup>a</sup>	3.02 <sup>b</sup>	0.87 <sup>a</sup>	0.107	<0.0001
18:3n-3	0.31 <sup>a</sup>	0.72 <sup>b</sup>	0.27 <sup>a</sup>	0.022	<0.0001
Summation					
SFA <sup>3</sup>	53.9 <sup>a</sup>	44.0 <sup>b</sup>	39.3 <sup>c</sup>	0.84	<0.0001
MUFA <sup>4</sup>	34.6 <sup>a</sup>	30.7 <sup>b</sup>	47.9 <sup>c</sup>	0.97	<0.0001
PUFA <sup>5</sup>	1.52 <sup>a</sup>	4.02 <sup>b</sup>	1.79 <sup>a</sup>	0.117	<0.0001
Delta-9 desaturase indices					
C14-ratio <sup>6</sup>	0.088 <sup>a</sup>	0.167 <sup>b</sup>	0.505 <sup>c</sup>	0.0274	<0.0001
C16-ratio <sup>7</sup>	0.054 <sup>a</sup>	0.091 <sup>b</sup>	0.242 <sup>c</sup>	0.0107	<0.0001
C18-ratio <sup>8</sup>	1.22 <sup>a</sup>	1.37 <sup>a</sup>	4.17 <sup>b</sup>	0.172	<0.0001
Total fatty acids content	56.9		48.4	2.99	0.0065

<sup>1</sup>Values are model least square means

<sup>2</sup>Values with different subscripts in the same row significantly differ ( $P < 0.05$ )

<sup>3</sup>Saturated fatty acids (14:0 + 16:0 + 18:0)

<sup>4</sup>Mono unsaturated fatty acids (14:1 *cis*-9 + 16:1 *cis*-9 + 18:1 *cis*-9 + 18:1 *trans*-11)

<sup>5</sup>Poly unsaturated fatty acids (18:2 *cis*-9, *trans*-11 + 18:2n-6 + 18:3n-3)

<sup>6</sup>14:1 *cis*-9/14:0

<sup>7</sup>16:1 *cis*-9/16:0

<sup>8</sup>18:1 *cis*-9/18:0

The PCA extracted 16 principal components of which the first two represented 51.6 % (PC1) and 21.1 % (PC2) of the total variance in FA composition and desaturase indices in the three compartments. The loading plot for the regression factors (Figure 4)

revealed a strong positive correlation between PC1 and the  $\Delta 9$ -desaturase indices, the proportion in fat of 16:1 *cis*-9 and 14:1 *cis*-9 while negatively correlated with the proportion of 18:0 and SFA. The loading plot for the regression factors (Figure 4) revealed a strong positive correlation between PC1 and the  $\Delta 9$ -desaturase indices, the proportion in fat of 16:1 *cis*-9 and 14:1 *cis*-9 while negatively correlated with the proportion of 18:0 and SFA. The correlation with PC2 was positive for the proportion of PUFA, 18:2n-6 and 18:3n-3 and negative for the proportion of 14:0, 16:0 and SFA.

The PCA score plot (Figure 5) proves a differentiation between the 3 compartments based on both PC1 and PC2. The SUBC could be distinguished from the NEFA and ABD by a positive score for PC1. The differentiation between the 2 compartments with a negative score for PC1 could be made by a positive (NEFA) or negative (ABD) score for PC2 which is illustrated by the mean PC1 scores for SUBC that differ from the ABD and NEFA ( $P < 0.0001$ ). The individual PC2 scores differ between all classes ( $P < 0.0001$ , Table 3).

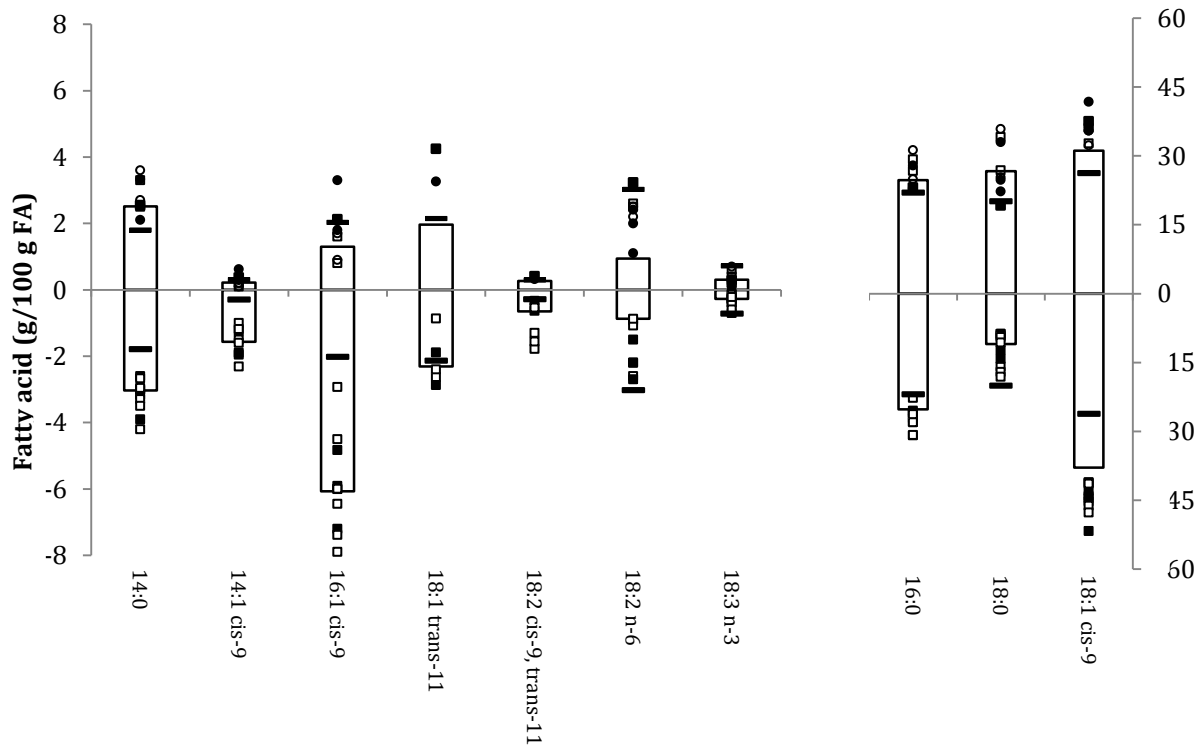
**Table 3.** Principal components for the fatty acid profile of the 3 main fat compartments in dairy cows suffering from a left displacement of the abomasum

Principal component	Proportion <sup>1</sup> (%)	Compartment			SEM	<i>P</i>
		Abdominal <sup>2</sup>	NEFA	Subcutaneous		
PC1	51.6	-1.86 <sup>a3</sup>	-2.03 <sup>a</sup>	2.91 <sup>b</sup>	0.296	<0.0001
PC2	21.1	-1.37 <sup>a</sup>	2.16 <sup>b</sup>	-0.19 <sup>c</sup>	0.180	<0.0001

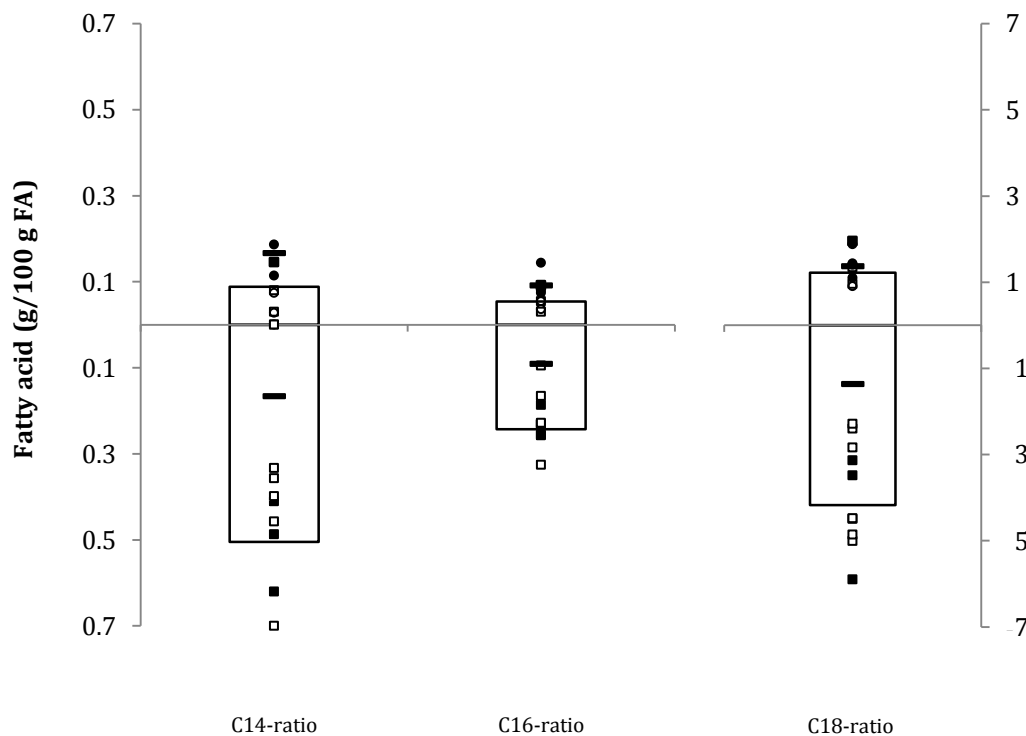
<sup>1</sup>Proportion of variance explained by the principal component

<sup>2</sup>Values are model least square means

<sup>3</sup>Values with different subscripts in the same row significantly differ ( $P < 0.05$ )



**Figure 2.** Bar chart combining the individual fatty acid composition of abdominal fat (upward bars), subcutaneous fat (downward bars) and NEFA (—) in dairy cows suffering from a left displacement of the abomasum. The cited literature is indicated for dairy (open symbols: Rukkamsuk et al., 2000; Sato and Inoue, 2006; Douglas et al., 2007; Zachut et al., 2010a) or beef (black symbols: Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002; Siebert et al., 2003) cattle. The abdominal depot is presented as mesenterical (square symbol) or perirenal (round symbol) fat. To better allow comparison with both adipose tissues, blood plasma NEFA proportions as measured in the current study were presented both in the upward and downward bars.



**Figure 3.** Bar chart combining columns for  $\Delta 9$ -desaturase indices of abdominal fat (upward bars), subcutaneous fat (downward bars) and NEFA (—) in dairy cows suffering from a left displacement of the abomasum. The cited literature is indicated for dairy (open symbols: Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002; Siebert et al., 2003) or beef (black symbols: Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002; Siebert et al., 2003) cattle. The abdominal depot is presented as mesenterical (square symbol) or perirenal (round symbol) fat. To better allow comparison with both adipose tissues, blood plasma NEFA  $\Delta 9$ -desaturase indices as measured in the current study were presented both in the upward and downward bars.

## DISCUSSION

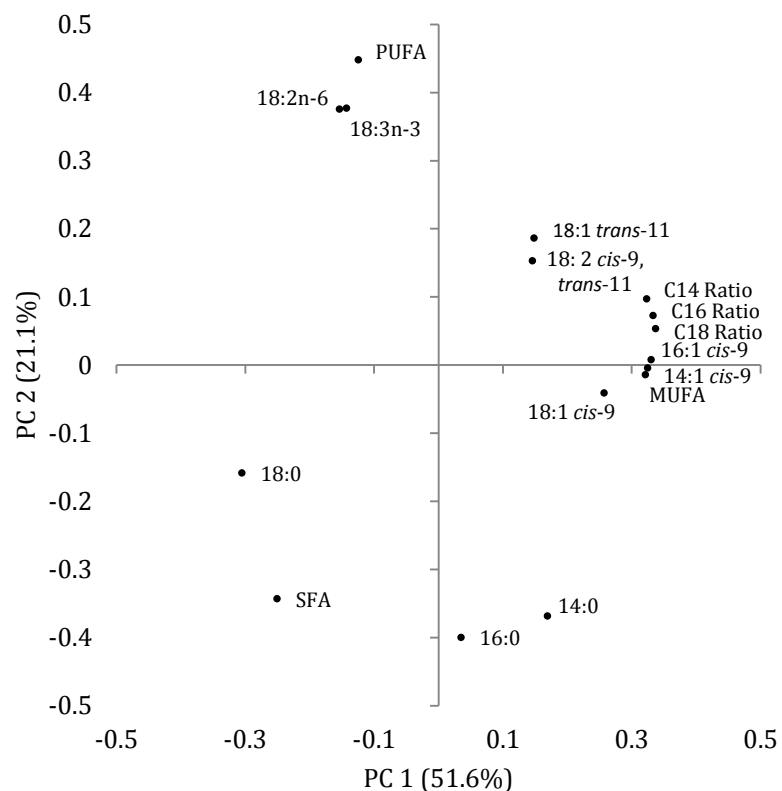
Many studies have focused on the deleterious effects of lipolysis in dairy cattle. The objective of the current study was to investigate the FA profile of plasma NEFA, and ABD and SUBC fat stores in dairy cows during NEBAL. First, the metabolic blood profile from the animals in this experiment, especially the high NEFA and high BHBA support the working hypothesis of sampling animals in severe NEBAL when suffering from LDA (Leblanc et al., 2005; Chapinal et al., 2011).

We hypothesized that ABD and SUBC fat tissues do have a different FA profile rendering them at a different level of pathogenicity in case of lipolysis, as it is known that lipotoxicity of FA increases with their level of saturation (Leroy et al., 2005; Vanholder et al., 2005; Van Hoeck et al., 2011). As the amount of data is limited in this research area, we compared our results for the individual FA (Figure 2) and the  $\Delta 9$ -desaturase indices (Figure 3) with data from both dairy and beef cattle. In general, the SUBC FA profile was in agreement with those reported in dairy (Rukkwamsuk et al., 2000; Sato and Inoue, 2006; Douglas et al., 2007; Zachut et al., 2010a) as well as in beef cattle (Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002; Siebert et al., 2003). Omental FA profiles are seldom reported in both dairy (Sato and Inoue, 2006) and beef cattle (Beaulieu et al., 2002) but seem consistent with perirenal FA profiles (dairy: Sato and Inoue, 2006; beef: Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002). In our study, the FA with the highest contribution to the ABD and SUBC FA was 18:1 *cis*-9, which is in agreement with previous dairy (Rukkwamsuk et al., 2000; Sato and Inoue, 2006; Douglas et al., 2007; Zachut et al., 2010a) and beef cattle studies (Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002; Siebert et al., 2003), although concentrations mentioned in previous studies were higher (Figure 2). In the present study, 16:0 represented the second largest proportion of the SUBC FA followed by 18:0, which is in agreement with all aforementioned beef and dairy cattle studies. The higher contribution of 18:0 compared to 16:0 in ABD was not found in all dairy (Sato and Inoue, 2006) and beef cattle studies (Beaulieu et al., 2002; Oka et al., 2002).

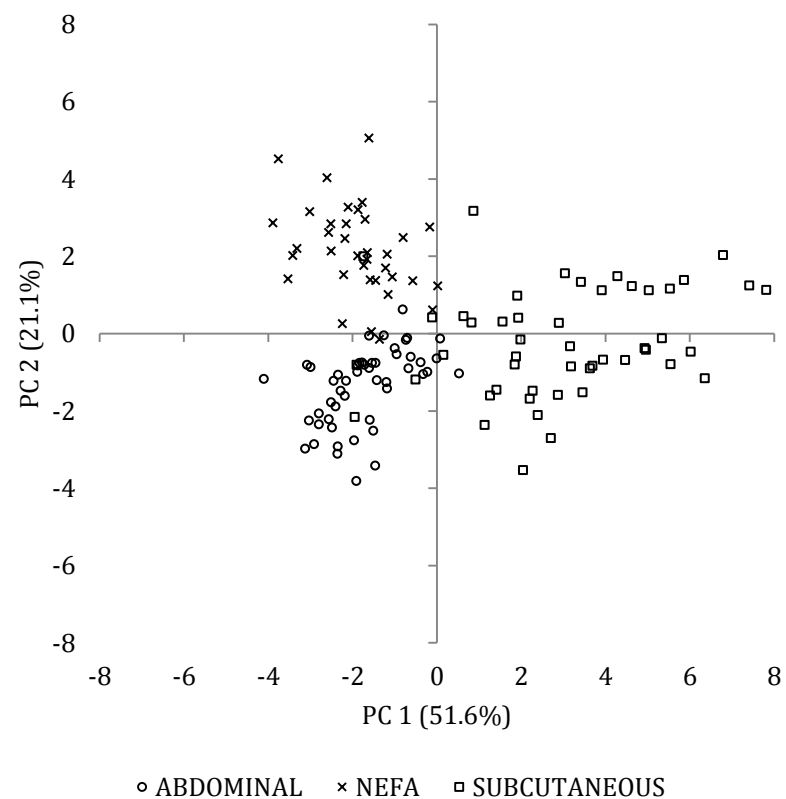
Furthermore, we aimed to find out which of the two fat stores is preferentially broken down in lactating dairy cows when suffering from LDA. The latter was done by comparing the FA profile of these two fat stores with the profile of NEFA circulating in the peripheral circulation. To enhance the interpretation of the FA profile in circulating

NEFA in comparison to ABD and SUBC, we included the NEFA composition from the current study in Figures 2 and 3. Until now, dairy cattle experiments that compared the FA composition of different adipose compartments with the blood mainly reported the total lipid content of plasma which includes the dietary FA, making the interpretation of the FA contribution by the different body fat depots to circulating NEFA indulging (Douglas et al., 2007; Zachut et al., 2010a). Three experiments (Moallem et al., 1999; Leroy et al., 2005; Contreras et al., 2010) involving healthy dairy cows showed a major contribution of 16:0, 18:0 and 18:1 *cis*-9 to the circulating NEFA as in our study. In comparison to our results, Moallem et al. (1999) and Contreras et al. (2010) showed a remarkably higher 16:0 and 18:0 and lower 18:1 *cis*-9 content. This higher level of saturated FA in NEFA has been linked to an altered leukocyte function early post partum enhancing susceptibility to transition diseases (Contreras et al., 2011). Therefore, from our study, cows with LDA would be experiencing a less severe proinflammatory environment. However, a higher contribution of 18:1 *cis*-9 has been observed in healthy dairy cows early post partum as well (d16; Leroy et al., 2005). Future research on the composition of NEFA in early lactating dairy cows is therefore needed to further distinguish physiological (stage of lactation, diet, breed) and pathophysiological effects.

As a result of the reported differences in FA profile, the ratios of the products and their precursors (14:1 *cis*-9/14:0, 16:1 *cis*-9/16:0 and 18:1 *cis*-9/18:0) were greater in the SUBC compared to NEFA and ABD. Similar differences between SUBC and ABD were observed in other dairy (Sato and Inoue, 2006) and beef cattle studies (Beaulieu et al., 2002; Oka et al., 2002). The largest range was found for the SUBC C18-ratio ranging from 2.29 (Zachut et al., 2010b) to 5.90 (Oka et al., 2002) followed by the SUBC C14-ratio ranging from 0.33 (Sato and Inoue, 2006) to 0.62 (Siebert et al., 2003). The ABD C14 and C16-ratios but not the C18-ratio mentioned in beef studies (Eichhorn et al., 1986; Beaulieu et al., 2002; Oka et al., 2002), were generally greater when compared with our and other dairy cattle experiments (Sato and Inoue, 2006; Figure 3). Stearoyl-CoA desaturase (SCD) responsible for the conversion of 14:0 to 14:1 *cis*-9, 16:0 to 16:1 *cis*-9 and 18:0 to 18:1 *cis*-9 was found in bovine liver (St. John et al., 1991) and subcutaneous fat stores (Chang et al., 1992) but not in the NEFA fraction of plasma, as SCD expression is regulated at the cellular transcriptional level (Nakamura and Nara, 2004).



**Figure 4.** Loading plot presenting the relationship among fatty acids derived from a principal component analysis based on proportions (% of total fatty acids) of C14 tot C18 fatty acids, proportions of saturated, mono unsaturated and poly unsaturated fatty acids and the  $\Delta^9$ -desaturase indices in the NEFA fraction of plasma, abdominal and subcutaneous fat (n=150) in dairy cows suffering from a left displacement of the abomasum.



**Figure 5.** Score plot of the individual samples (from the principal components analysis in Figure 4) represented according to the compartment of origin (abdominal fat (○), NEFA (x), subcutaneous fat (□)) in dairy cows suffering from a left displacement of the abomasum.

Therefore the  $\Delta 9$ -desaturase indices did not change in the blood (NEFA) and hence could reflect the main site of lipolysis. The desaturase index of the proportionally main FA (18:1 *cis*-9 and 18:0) was not different between NEFA and ABD. Moreover, even though significantly different, the  $\Delta 9$ -desaturase indices for the C14-ratio and C16-ratio in NEFA were closer to ABD than SUBC. Finally, loading plots of PCA appear to offer an interesting way to indicate mutual metabolic relationships between milk FA and their origin (Fievez et al., 2003). Therefore, we used a similar approach to find clusters in the FA patterns originating from the different lipid compartments. We were able to show that the ABD and NEFA have a more similar FA profile as suggested by the PC1, that does not differ between the ABD and NEFA compared to SUBC. These findings further strengthen our conclusion that the FA profile of NEFA is closer to the FA profile of ABD versus SUBC.

The significant difference in contribution to the overall lipomobilization between the different fat depots is important in relation to the development of metabolic disorders in dairy cows as suggested by Mukesh et al. (2010). Indeed, specific FA profiles in circulating NEFA during early lactation may potentially affect inflammatory responses in transition dairy cows (Contreras et al., 2010). In ruminants, as in humans (Arner, 1998), the visceral but not the SUBC tissue is drained by the portal venous system, thus being in direct contact with the liver (Emery et al., 1992). Considering that the latter organ serves as the principal actor in nutrient metabolization during the periparturient period of dairy cows, a temporary overflow by visceral FA may render the animal more susceptible to several kinds of metabolic diseases in the most crucial period of the lactation cycle (Drackley et al., 2001). Two mechanisms might cause the observed differences in FA metabolism in the fat compartments. Firstly, in ruminants the blood flow to carcass fat depots is lower than that to abdominal fat depots, especially in the immediate postpartum period (Vernon, 1980; Barnes et al., 1983; Gregory et al., 1986), leading to a temporary decreased nutrient flow to and from the SUBC. The second mechanism might be related to site-specific differences in lipogenic and lipolytic rates. Previous reports in beef cattle have reported a greater lipolytic (Etherton et al., 1977) and lipogenic (Baldwin et al., 2007) rate in SUBC when compared to perirenal fat. This difference in metabolic activity between the different fat depots had previously been brought forward by Eguinoa et al. (2003) as a compensatory mechanism for the decreased nutrient flow in the carcass. The latter authors investigated differences in



lipogenic rate between different depots in non-lactating Holstein heifers and concluded that the greater lipogenic rate seen in SUBC has to be adjusted for adipocyte size, resulting in a higher net lipogenic rate in abdominal fat. In non-lactating heifers, a larger adipocyte size was found in ABD compared to SUBC (Eguinoa et al., 2003), which was recently confirmed for the retroperitoneal but not for the omental fat in lactating dairy cows (Akter et al., 2011). The latter authors concluded from their study that retroperitoneal fat was preferentially mobilized during peak lactation and reacts most sensitive as DIM increase. Recently, Nikkhah et al. (2008) reported that the internal fat mass in dairy cows during the dry period increased over 70 % without a concomitant increase in BCS, when cows were moderately overfed during an 8-week period. This also suggests an increased ABD FA metabolism during an anabolic period. Interestingly, in our study in which cows were offered for a correction of LDA, we observed animals with a moderately low BCS but with a concomitant high OFS (indicated by the diagram in Figure 1). The latter seems contrasting with the higher catabolic activity of ABD and provides interesting evidence to question the change in BCS as an accurate estimator of the lipolysis rate in early lactating dairy cows.

## CONCLUSIONS

The current study provides evidence for a different FA profile in ABD versus SUBC fat stores in dairy cows suffering from LDA. The results of the multivariate approach, which included the desaturase indices along with the entire FA profile of ABD, SUBC and NEFA, support the hypothesis of a preferential mobilization of ABD in dairy cows suffering from LDA.

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THE USE OF DIETARY FATTY ACIDS DURING  
THE TRANSITION PERIOD AS AN  
OPTIMIZATION STRATEGY



# THE EFFECT OF DHA ENRICHED MARINE ALGAE IN THE RATION OF HIGH YIELDING DAIRY COWS DURING TRANSITION ON METABOLIC PARAMETERS IN SERUM AND FOLLICULAR FLUID AROUND PARTURITION

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## SUMMARY

Sixteen Holstein cows were assigned to 2 groups to evaluate the caloric and metabolic effect of feeding marine algae (ALG) from 3 weeks pre partum until 12 weeks post partum. Milk production characteristics and the profiles of hormones and metabolites in the serum were monitored from -7 to 46 days in milk (DIM) and follicular fluid (FF) from 14 to 46 DIM. All cows received a corn- and grass silage based partially mixed ration supplemented with concentrate and protein supplement. In the diet of the ALG group, 2 kg of the concentrates were replaced by a concentrate containing ALG (44 g/d docosahexaenoic acid). Diets were isocaloric (net energy basis) and equal in intestinal digestible protein.

ALG increased milk yield (41.2 vs. 38.2 kg/d), whereas milk fat yield (1.181 vs. 1.493 kg/d) and milk fat content (31.6 vs. 40.7 g/kg) decreased. Protein yield (1.336 vs. 1.301 kg/d) was not affected but a tendency towards reduced milk protein content (32.8 vs. 34.7 g/kg) was observed. Marine algae supplementation increased the  $\beta$ -hydroxy butyric acid (BHBA) concentration in the FF of the ALG compared to the control (0.992 vs. 0.718 mmol/L). The total protein concentration in FF was decreased in the ALG (62.9 vs. 67.6 g/L). The plasma and serum metabolites did not significantly differ between treatments except for a tendency towards a lower urea concentration in the serum of the control versus the ALG (4.69 vs. 5.13 mmol/L). Based on metabolizable energy (ME) calculations, a daily energy sparing effect of 3.48 MCal was obtained due to the milk fat depression (MFD).

The concomitant milk yield increase suggests that at least part of this spared energy is used to stimulate milk production. Theoretically, 3.48 MCal of ME could lead to an increase in milk yield of 7.43 kg/d which is higher than the observed 3 kg/d. However, when evaluating nutrient requirements during MFD in early lactation, we calculated that increased milk production is caused by a propionate saving effect of 2.71 mol in the udder when milk fat is depressed. Concurrent increased BHBA concentration in the FF in the ALG cannot be attributed to a worsened energy status of the animals as all other indicators contradict any change in energy balance (EBAL), indicating that BHBA might not be an appropriate metabolic parameter to estimate the EBAL in early lactating dairy cows during MFD.

## INTRODUCTION

Milk production of high yielding dairy cows has increased over the last 40 years while fertility has decreased (Lucy, 2001). In Belgium, milk production in dairy cattle has increased from 6,000 kg milk per lactation in 1991 to 9,000 kg milk per lactation in 2007 while simultaneously the calving interval has increased 28 d (Flemish Cattle Breeding Association, 2008). This fertility 'drop' has been shown to be associated with the negative energy balance (NEBAL) high yielding dairy cows encounter during the transition period (Dobson et al., 2007, Grummer, 2007, van Knegsel et al., 2005). In this period, substrate availability for gluconeogenesis is limiting due to both a decreased DMI and the adaptation of the rumen. A simultaneous increasing demand for milk lactose synthesis pushes the cows' metabolism to switch to depletion of body reserves, a phenomenon which is commonly known as NEBAL (Drackley et al., 2005). Leroy et al. (2004) showed a significant correlation between the concentrations of biochemical indicators of NEBAL (BHBA, glucose, NEFA) in the serum and follicular fluid (FF) of high yielding dairy cows early post partum. Afterwards, the same authors (Leroy et al., 2005, 2006) mimicked the *in vivo* encountered follicular environment in an *in vitro* maturation model and observed a marked deleterious effect on the developmental capacity of bovine oocytes. These findings substantiate the suggestions made by others (Horan et al., 2005, O'Callaghan and Boland, 1999) that the decline in fertility is mainly caused by an inferior oocyte and embryo quality. Britt (1991) suggested a carry over effect of metabolic conditions in times of energy deficit early post partum, on pre-ovulatory follicles 2 to 3 months later. Therefore, the effect of the FF, the environment in which the oocyte matures before ovulation, cannot be neglected when evaluating the effect of the NEBAL in high yielding dairy cows. This explains why many efforts have been made to alleviate the NEBAL of high yielding dairy cows early post partum and consequently improve the concentration of biochemical markers in both serum and FF of high yielding dairy cows in order to ameliorate reproductive capacity. Additionally, the fatty acid composition of the FF might be of importance, with 16:0 and 18:0 having a detrimental effect on oocyte maturation (Leroy et al., 2005).

One of the hypotheses to diminish the NEBAL post partum is the induction of milk fat depression (MFD) in order to substantially decrease the energy output as the production of fat comes with the highest demand for energy (Jensen, 2002). Daily

supplementation of 19 g of the *trans*-10, *cis*-12 isomer of conjugated linoleic acid (CLA) induced MFD in periparturient cows, which was associated with a significant decrease in NEFA and an increase in glucose concentrations, indicating an improved energetic status of the supplemented cows (Odens et al., 2007).

When fed to dairy cows in established lactation, 22:6n-3 (docosahexaenoic acid; DHA) enriched marine algae (*Schizochytrium* spp.) induce MFD (Boeckaert et al., 2008). To our knowledge, there are no experiments in which long chain n-3 fatty acids (FA) such as 22:6n-3 were examined to induce a MFD in early lactation with concomitant registration of NEBAL indicators. Our objectives were to induce MFD in early lactation by feeding 22:6n-3 and to determine the dietary effect on milk production, milk components and metabolic status as measured in the serum and FF early post partum in high yielding dairy cows.

## MATERIALS AND METHODS

### EXPERIMENTAL DESIGN

All procedures and protocols were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, N°: EC 2007/063). Sixteen healthy primiparous (n=6; PRIM) and multiparous (n=10; MULT) Holstein cows participated in this study. The study was conducted at the experimental research farm of the Ghent University (Biocentrum Agrivet, Melle, Belgium). On this farm the average 305d milk yield per cow was 9,934 kg milk (38.6 g/kg fat and 34.7 g/kg protein). During the experimental period (August 2007 until March 2008) all cows were housed in a loose stable with cubicles. Cows were milked by means of an automated voluntary milking system (VMS, Delaval, Sweden).

The cows were blocked for parity, expected calving week, estimated milk fat production and genetic origin (cows with same ancestors were assigned to different treatment groups) and assigned to 2 different treatment groups in a randomized complete block design. MULT cows were dried off in their previous lactation to achieve an average dry period length of 55 d, during which they were offered wheat straw (ad libitum), 7 kg DM of corn silage, and 1.4 kg DM of soybean meal (Aveve, Merksem, Belgium), 25 g of magnesium oxide (Nutreco, Ghent, Belgium) and 200 g/d of a dry



period mineral mixture (Runergeen, P5, Nutreco, Ghent, Belgium) until d 20 ( $\pm$  3.5 SD) before the calving date. For these last 20 d, the animals were transferred to the lactating group in order to adapt to the partially mixed ration (PMR) offered to the cows after calving. Similarly, PRIM cows were transferred to the lactating group 19 d ( $\pm$  3 SD) before their actual calving date. The pen fed PMR offered to all close up and lactating cows consisted of high quality roughages corn- and grass silage, soybean meal, corn cob mix, sugar beet pulp, hay grass and minerals (531, 241, 100, 57, 45, 22 and 4 g/kg DM basis respectively) besides the specific concentrates to which they were assigned.

### **DIETARY TREATMENTS**

In addition to the PMR, the control group (CON) was offered 2 kg of concentrate (Glucolac 21, AVEVE Group, Merksem, Belgium; Table 1) from 14 d prior to parturition. This concentrate allowed for equal fat- and protein corrected milk (FPCM) production from the energy (based on NEL; Van Es, 1978) and protein (based on the Dutch DVE system; Tamminga et al., 1994) content. After parturition, the amount of concentrate increased over 20 d leading to a maximum of 3 kg for PRIM cows and 6 kg for MULT cows. As from parturition a protein supplement (Aminolac 38 Extra, AVEVE Group, Merksem, Belgium; Table 1) was offered at a constant amount of 2 kg to all animals until the end of the trial in addition to the concentrate. The concentrate and protein supplement were individually offered via computerised feeders (DeLaval feeding station standard, Delaval, Sweden).

The cows in the marine algae group (ALG) were fed according to the same protocol as the CON. The algae concentrate included 22:6n-3 enriched marine algae (*Schizochytrium* sp.) at a final concentration of 110 g/kg DHA-Gold (Martek DHA gold, Martek Biosciences Corp., Colombia, MD; Table 1). The amount of marine algae concentrate supplied to the cows of the treatment group remained 2 kg (224 g of DHA-Gold, 44 g of 22:6n-3) throughout the entire postpartum period (46 d), whereas the prepartum supply was fixed at 1.8 kg DHA concentrate (202 g of DHA-Gold) from 20 d prior to parturition.

The pre and postpartum supply of the concentrate (Glucolac 21) and protein supplement (Aminolac 38 Extra) was calculated in order to provide the same energy (based on NEL; Van Es, 1978) and protein (based on the Dutch DVE system; Tamminga

et al., 1994) supply in the total ration as calculated for the CON. For PRIM and MULT cows this was calculated to be respectively 0.81 kg Glucolac 21, 1.17 kg Aminolac 38 Extra®, 2 kg marine algae and 2.81 kg Glucolac 21, 1.17 kg Aminolac 38 Extra and 2 kg marine algae at 21 d.

**Table 1.** Ingredient composition of concentrates used in the experiment

	ALG	AMINOLAC <sup>4</sup>	GLUCOLAC <sup>5</sup>
<b>Chemical composition, g/kg of DM</b>			
Dry matter	890.0	890.0	890.0
Crude ash	46.0	110.0	70.0
Crude protein	198.0	390.5	217.5
Crude fat	82.0	42.0	42.5
VEM <sup>1</sup> (/kg of DM)	1,148.00	930.00	960.00
DVE <sup>2</sup>	139.00	200.00	115.00
<b>Ingredient, g/kg of product</b>			
Rapeseed meal	96.0	150.0	300.0
Rapeseed hulls		150.0	
Soybean hulls <sup>3</sup>			210.0
Corn middlings			110.0
Corn gluten feed	96.0		
Wheat middlings			110.0
Beet vinasse		20.0	90.0
Wheat	192.0		80.0
Toasted soybean expeller	55.0	600.0	61.0
Citrus pulp			30.0
Formaldehyde treated soybean meal	53.0		
Sunflower expeller		40.0	
Palmseed hulls			8.0
Palm oil		2.0	4.0
Beet pulp	288.0		
Beet molasse	67.0		
Alfalfa pellets			
DHA Gold <sup>4</sup>	110.0		
<b>Other components</b>	<b>43.0</b>	<b>38.0</b>	

<sup>1</sup> Voeder Eenheid Melk (Van Es, 1978)

<sup>2</sup> Darm Verteerbaar Eiwit (Tamminga et al., 1994)

<sup>3</sup> Martek DHA gold, Martek Biosciences Corp., Colombia, MD

<sup>4</sup> Aminolac 38 Extra, AVEVE Group, Merksem, Belgium

<sup>5</sup> Glucolac 21, AVEVE Group, Merksem, Belgium

## BODY CONDITION SCORING

Body condition scores were recorded weekly by the same person using a score on a 1-5 scale, with 0.25 increments (Edmonson et al., 1989) from the week before the expected calving day until week 12 after parturition.

**SAMPLING**

The planned day of prepartum blood samples on d 10 before parturition (d-10) differed from the actual collection day (d-13  $\pm$  4.5 SD) due to unpredictable differences between the expected and actual calving date of the cows. Postpartum samples were taken on d 0, d 11, d 14, d 20, d 26, d 33, d 40 and d 46 following parturition. Blood samples were taken from the coccygeal vein (sample was discarded when arterial blood was observed) into 3 unheparinised, silicone coated tubes (Venoject, Autosep, Gel + Clot. Act.; Terumo Europe N.V., Leuven, Belgium), 2 tubes with ethylene diamine tetraacetic acid (EDTA; Venoject, Autosep, Terumo Europe N.V., Leuven, Belgium), and 1 tube with sodium fluoride (NaF, glycolytic inhibitor; Venoject, Autosep, Terumo Europe N.V., Leuven, Belgium). Follicular fluid was collected on d 14, d 20, d 26, d 33, d 40 and d 46 by means of transvaginal aspiration following the protocol described by Leroy et al. (2004). The follicles with a diameter greater than 8 mm were aspirated in order to minimize size effects on FF composition (Leroy et al., 2008). Attention was paid to prevent blood contamination. Follicular fluid samples with obvious blood contamination were omitted from further processing. All blood and follicular samples were transported to the laboratory at 4 °C. Within 1.5 hours after collection, samples were centrifuged (20 min at 1,500 x *g*). Serum and plasma samples were stored at -20 °C, FF samples at -80 °C until biochemical analysis.

Milk samples were taken weekly starting on d 5 after parturition during 12 consecutive weeks. After proportionally pooling all milk samples from one day according to milk yield, subsamples were mixed with a preservative (8 mg bronopol and 0.3 mg natamycine) and sent refrigerated (4 °C) to the Milk Control Centre of Flanders (Melk Controle Centrum Vlaanderen, Lier, Belgium) for milk fat and protein analysis. A second milk sample was stored frozen prior to milk fat extraction for milk FA determination.

**CHEMICAL ANALYSES**

Analysis of chemical composition of concentrates consisted of determination of DM (European Economic Community 1971a), crude ash by incineration (550°C, 2h; European Economic Community 1971b), crude protein according to the Kjeldahl method

(European Community 1993) and crude fat with the Soxhlet method according to ISO 6492-1999 (ISO, 1999).

The serum and FF analysis for albumin, aspartate aminotransferase (AST), BHBA, glucose, gamma-glutamyltransferase (GGT), L-lactate, NEFA, urea, total cholesterol, and total protein were carried out by means of an automated colorimetric analyser (Konelab™ 20 XTi Clinical chemistry analyzer, Thermo Electron Cooperation, Vantaa, Finland) at The Flemish Veterinary Health Service (Dierengezondheidszorg-Vlaanderen, Torhout, Belgium). Complementary to the analyser, commercial kits were used for the determination of AST (Cat N°: 981363/981771) and urea (Cat N°: 981818/981820) from Konelab™/DPC T Series (Thermo Electron Cooperation, Vantaa, Finland), for albumin (Cat N°: 981358/981767), glucose (Cat N°: 981304/981779), GGT (Cat N°: 981377/981778), NEFA (Cat N°: FA 115), total cholesterol (Cat N°: 981812/981813) and total protein (Cat N°: 981387/981785) from Konelab™ (Thermo Electron Cooperation, Vantaa, Finland), for BHBA (Ranbut, Cat N°: RB1007/RB1008) and L-lactate (Cat N°: LC 2389) from Randox (Randox Laboratories Ltd, Antrim, Crumlin, United Kingdom). Determination of plasma insulin, growth hormone (GH) and IGF-I was performed by radioimmunoassay according to the methods described by Istasse et al. (1990) and Renaville et al. (1993) respectively. All blood analyses were carried out on serum derived from the unheparinised, silicone coated tubes except the ones for insulin, GH, IGF-I and glucose. The insulin, GH and IGF-I analyses were performed on plasma derived from EDTA tubes while the glucose assay was performed on plasma derived from the NaF tubes.

Oestrogen (E2) and progesterone (P4) concentration of FF samples was determined via radioimmunoassay using commercial kits from Diagnostic Systems Laboratories, Inc. for E2 (Estradiol Double Antibody RIA-kit) and P4 (Progesterone Double Antibody RIA-kit). Follicles determined as being atretic according to the classification described by Hendriksen et al. (2003), were omitted from further analysis.

Milk fat and protein content were analyzed by mid infrared spectrophotometry (MilkoScan 4000/FT6000, Foss, Amersfoort, The Netherlands) in the milk subsample containing the preservative. The FPCM was calculated as  $[(0.337 + 0.116 \times \text{milk fat \%} + 0.06 \times \text{milk protein \%}) \times \text{kg of milk}]$  (CVB, 2007). Milk fat was extracted as described by

Vlaeminck et al. (2005) and fatty acid methyl esters (FAME) were prepared by base-catalyzed transmethylation according to Christie (1982) with modifications by Chouinard et al. (1999). FAME were quantified using a gas chromatograph (HP 6890, Brussels, Belgium) equipped with a CP-Sil88 column for FAME (100 m × 250 μm × 0.2 μm, Chrompack, Middelburg, The Netherlands). Conditions for FAME analysis in milk fat were as described by Vlaeminck et al. (2005). FAME were identified using external standards (S37, Supelco, Poole, Dorset, UK; CLA c9t11, CLA t10c12, odd- and branched-chain FA, Larodan Fine Chemicals AB, Malmö, Sweden) and quantified using the internal standard. Milk FA were grouped according to their chain length into short chain fatty acids (SCFA =  $\sum$  [4:0, 5:0, 6:0, 7:0, 8:0, 9:0]), mid chain fatty acids (MCFA =  $\sum$  [10:0, 10:1, 11:0, 12:0, 12:1, 13:0, 14:0, 14:1, 15:0, iso 13:0, anteiso 13:0, iso 14:0, iso 15:0, anteiso 15:0]) and long chain fatty acids (LCFA =  $\sum$  [17:0, 17:1, 18:0, 18:1 *trans*-4, 18:1 *trans*-5, 18:1 *trans*-6, 18:1 *trans*-9, 18:1 *trans*-10, 18:1 *trans*-11, 18:1 *trans*-12, 18:1 *cis*-9, 18:1 *cis*-11, 18:1 *cis*-12, 18:1 *cis*-13, 18:1 *cis*-14, 18:1 *cis*-15, 18:2 *trans*-11, *cis*-15, 18:2, 20:0, 18:2n-6, 18:3n-3, 18:2 *cis*-9, *trans*-11, 18:2 *trans*-9, *cis*-11, 18:2 *trans*-10, *cis*-12, 22:6n-3]) and according to the level of saturation into saturated fatty acids (SFA =  $\sum$  [4:0, 5:0, 6:0, 7:0, 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, iso 13:0, iso 14:0, iso 15:0, anteiso 15:0, iso 16:0, iso 17:0, iso 18:0]), mono unsaturated fatty acids (MUFA =  $\sum$  [10:1, 12:1, 14:1, 16:1 *cis*-9, 16:1 *trans*-9, 17:1, 18:1 *trans*-4, 18:1 *trans*-5, 18:1 *trans*-6, 18:1 *trans*-9, 18:1 *trans*-10, 18:1 *trans*-11, 18:1 *trans*-12, 18:1 *cis*-9, 18:1 *cis*-11, 18:1 *cis*-12, 18:1 *cis*-13, 18:1 *cis*-14, 18:1 *trans*-15]) and poly unsaturated fatty acids (PUFA =  $\sum$  [18:2 *trans*-11, *cis*-15, 18:2, 18:2n-6, 18:3n-3, 18:2 *cis*-9, *trans*-11, 18:2 *trans*-9, *cis*-11, 18:2 *trans*-10, *cis*-12, 22:6n-3]).

## DATA HANDLING AND STATISTICAL ANALYSIS

Descriptive statistics and tests of significance were done using PROC MEANS and PROC FREQ of SAS (Institute, Inc., Cary, NC, USA, 2010). The distribution of all variables was checked to approximate the normal Gaussian distribution. If necessary, a box-cox transformation (Box and Cox, 1964) was performed. This transformation was carried out for the serum albumin, AST, BHBA, GGT, NEFA and follicular BHBA, glucose and NEFA variables. Subsequently, the data were analysed as a randomized complete block design using the MIXED procedure of SAS (Institute, Inc., Cary, NC, USA, 2010). The statistical model included the fixed effect treatment as the main variable of interest as

well as time, parity and the interaction between time and treatment. Repeated measures (d) within the cow were taken into account by adding cow as random effect. All variables were subjected to the autoregressive order 1 (AR(1)) covariance structure as determined by the Schwarz's Bayesian criterion and Akaike's information criterion (best fit closest to 0). Significance and tendency were declared at  $P < 0.05$  and  $0.05 < P < 0.1$  respectively. Data are reported as reduced model least square means with pooled standard errors unless indicated otherwise. Pearson correlations ( $r$ ) were calculated using the CORR procedure of SAS (Institute, Inc., Cary, NC, USA, 2010) for metabolites within and between the serum and FF.

## RESULTS

The prefaced duration of prepartum concentrate feeding of 14 d eventually extended to 20 d due to unpredictable differences between the expected and actual calving date of the cows. The prepartum supplementation period did not differ between treatments (ALG: 23 d  $\pm$  4 SD, CON: 17 d  $\pm$  6 SD) and parity (PRIM: 19 d  $\pm$  9 SD, MULT: 21 d  $\pm$  4 SD;  $P > 0.05$ ).

### ANIMAL CHARACTERISTICS

The BCS did not significantly differ between the ALG (3.02  $\pm$  0.05 SD) and CON (2.98  $\pm$  0.08 SD,  $P > 0.05$ ) during the experiment. The BCS was dependent of time relative to calving decreasing from calving to its nadir in week 5 post partum ( $P < 0.001$ ). No interaction was found between treatment and week.

### MILK PRODUCTION CHARACTERISTICS

Effect of algae supplementation on weekly milk production characteristics are presented in Table 2. ALG supplementation increased milk yield ( $P = 0.054$ ), whereas milk fat yield ( $P = 0.007$ ) and milk fat content ( $P = 0.015$ ) decreased. Protein yield ( $P = 0.465$ ) and FPCM ( $P = 0.192$ ) was not affected by ALG supplementation whereas a tendency for reduced milk protein content ( $P = 0.097$ ) was observed (Table 2). The number of daily milkings per cow in the automated voluntary milking system did not differ between treatments ( $P = 0.229$ ). Dietary effects on milk production characteristics were independent of week after parturition (treatment x time,  $P > 0.1$ ; Table 2) although dietary effects were small during the first two weeks of lactation.

**Table 2.** Effect of dietary treatment on production characteristics, composition of milk in the first 12 weeks of lactation and BCS (n=16)

Item	Treatment <sup>1</sup>			P-value		
	CON	ALG	SEM <sup>2</sup>	Treatment	Tr x Ti	Time
Milk, kg/d	38.2 <sup>3</sup>	41.2	1.08	0.054	0.139	<0.001
FPCM <sup>4</sup> , kg/d	37.7	35.8	1.03	0.138	0.454	0.276
Milk fat						
g/kg	40.7	31.6	2.33	0.015	0.163	<0.001
kg/d	1.493	1.181	75.3	0.007	0.121	0.013
Milk protein						
g/kg	34.7	32.8	0.78	0.097	0.608	<0.001
kg/d	1.301	1.336	34.6	0.460	0.325	0.322
Milkings						
n/d	2.15	2.41	0.14	0.245	0.484	0.115
BCS	2.98	3.02	0.05	0.648	0.896	<0.001

<sup>1</sup> CON = Control group, ALG = Algae group<sup>2</sup> Pooled standard error of the mean<sup>3</sup> Values are reduced model least square means<sup>4</sup> Fat and protein corrected milk

## MILK FATTY ACID COMPOSITION

Treatment effects on milk fatty acid composition are presented in Table 3. Proportions of SCFA decreased ( $P < 0.05$ ) with ALG whereas 4:0 was not affected (ALG  $3.84 \pm 0.15$  vs. CON  $4.13 \pm 0.14$ ,  $P > 0.05$ ). The decreased proportions of SCFA were not observed during the first 3 weeks of lactation, resulting in a significant treatment  $\times$  time interaction ( $P < 0.05$ ). MCFA and LCFA were not significantly altered with ALG. Supplementation with marine algae resulted in marked alterations in milk C18-fatty acid composition, changes that were characterized as a reduction ( $P < 0.05$ ) in 18:0, 18:1 *cis*-9 and 18:1 *cis*-12 ( $0.22 \pm 0.019$  vs.  $0.34 \pm 0.017$ ;  $P < 0.001$ ) and an increase in 18:1 *trans* ( $11.6 \pm 0.67$  vs.  $2.72 \pm 0.60$ ,  $P < 0.001$ ), 18:2 *trans*-11, *cis*-15 ( $0.26 \pm 0.026$  vs.  $0.077 \pm 0.024$ ,  $P < 0.001$ ), 18:2 *cis*-9, *trans*-11, 18:2 *trans*-9, *cis*-11 and 18:2 *trans*-10, *cis*-12 concentrations. Dietary effects on proportion of C18-fatty acids in milk fat were generally independent of week of lactation and effects were observed from the first week of lactation. Nevertheless, differences between dietary treatments in 18:1 *trans*-10, 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-9, *cis*-11 were smaller during the first weeks of lactation (Vlaeminck et al., 2009). Milk from cows receiving ALG had greater concentrations of 22:6n-3 in milk fat (Table 3).

**Table 3.** Main effects of dietary treatment on fatty acids composition in milk fat (in g/100 g FA)

	Treatment <sup>1</sup>		SEM <sup>2</sup>	P-value		
	CON	ALG		Treatment	Tr ×Ti	Time
SCFA <sup>4</sup>	7.56 <sup>3</sup>	6.66	0.27	0.038	0.012	0.442
MCFA <sup>5</sup>	18.23	16.96	0.71	0.232	0.109	<0.001
LCFA <sup>6</sup>	43.7	44.5	1.2	0.635	0.002	<0.001
SFA <sup>7</sup>	63.8	55.5	1.2	<0.001	0.002	<0.001
MUFA <sup>8</sup>	31.2	37.1	1.1	0.002	0.008	<0.001
PUFA <sup>9</sup>	2.93	4.22	0.16	<0.001	<0.001	<0.001
C16 <sup>10</sup>	28.39	28.33	0.48	0.923	0.874	<0.001
18:0	11.4	5.70	0.51	<0.001	0.752	0.035
18:1 <i>cis</i> -9	23.1	18.6	1.0	0.009	0.953	<0.001
18:1 <i>trans</i> -10	0.42	4.28	0.51	<0.001	0.163	0.063
18:1 <i>trans</i> -11	1.25	4.54	0.21	<0.001	0.458	0.318
18:1 <i>trans</i> -12	0.51	1.42	0.08	<0.001	0.572	0.084
18:3n-6	0.0184	0.0139	0.0015	0.048	0.266	0.718
18:3n-3	0.496	0.457	0.021	0.395	0.373	0.530
18:2 <i>cis</i> -9, <i>trans</i> -11	0.487	1.360	0.099	<0.001	0.016	0.005
18:2 <i>trans</i> -9, <i>cis</i> -11	0.0105	0.0729	0.0080	<0.001	0.052	0.031
18:2 <i>trans</i> -10, <i>cis</i> -12	0.0032	0.0097	0.0012	0.002	0.394	0.442
22:6n-3	0.013	0.196	0.016	<0.001	0.933	0.695

<sup>1</sup> CON = Control group, ALG = Marine algae group

<sup>2</sup> Pooled standard error of the mean

<sup>3</sup> Values are reduced model least square means

<sup>4</sup> Short chain fatty acids (See materials and methods for details on fatty acids)

<sup>5</sup> Medium chain fatty acids

<sup>6</sup> Long chain fatty acids

<sup>7</sup> Saturated fatty acids

<sup>8</sup> Mono unsaturated fatty acids

<sup>9</sup> Poly unsaturated fatty acids

<sup>10</sup> 16:0 + 16:1 *cis*-9

## BLOOD SERUM AND PLASMA METABOLITES

The plasma and serum metabolites did not differ between treatments except for a tendency to a higher urea concentration in the serum of ALG fed cows ( $P = 0.075$ ). None of the blood parameters showed a significant treatment x time interaction (Table 4). A significant time effect was found for all serum and plasma metabolites except urea ( $P = 0.09$ ) and BHBA and albumin ( $P > 0.1$ ). Serum NEFA, AST and GH peaked around parturition only to decrease after calving whereas cholesterol, glucose, IGF-1 and insulin followed an opposite change ( $P < 0.05$ ). The concentration of total protein and GGT gradually increased over time from the week before parturition.



**Table 4.** Effect of dietary treatment on serum or plasma metabolite concentrations during the first 6 weeks of lactation (n=16)

	Treatment <sup>1</sup>			P-value		
	CON	ALG	SEM <sup>2</sup>	Treatment	Tr x Ti	Time
Total Cholesterol, mmol/L	3.21 <sup>3</sup>	3.33	0.19	0.703	0.768	<0.001
Urea, mmol/L	4.69	5.13	0.17	0.075	0.786	0.091
Glucose, mmol/L	3.42	3.22	0.09	0.104	0.251	0.036
Total Protein, g/L	81.2	77.5	1.9	0.131	0.936	<0.001
NEFA, mmol/L	0.309	0.323	0.054	0.915	0.444	<0.001
BHBA, mmol/L	0.732	0.880	0.082	0.251	0.548	0.201
GGT, IU/L <sup>4</sup>	27.0	36.8	4.3	0.124	0.182	0.002
AST, IU/L <sup>5</sup>	78.8	85.7	6.7	0.375	0.169	<0.001
Albumin, g/L	29.72	30.45	0.51	0.478	0.914	0.513
GH, ng/mL <sup>6</sup>	5.53	4.57	0.33	0.182	0.749	<0.001
IGF-1, ng/mL	70.1	77.2	7.9	0.724	0.461	<0.001
Insulin, IU/mL	1.68	1.57	0.14	0.675	0.891	0.002

<sup>1</sup> CON = control group, ALG = algae group<sup>2</sup> Pooled standard error of the mean<sup>3</sup> Values are reduced model least square means<sup>4</sup> Gamma-glutamyltransferase<sup>5</sup> Aspartate aminotransferase<sup>6</sup> Growth hormone

## FOLLICULAR FLUID METABOLITES

On average  $1.10 \pm 0.33$  follicles were aspirated per cow and per session. Due to atresia, based on the E2 and P4 concentration in the FF, or because of blood contamination, 14 % of FF samples were excluded from further analysis. The number of excluded FF samples did not differ between treatments.

Marine algae supplementation increased the BHBA concentration in the FF of the ALG compared to the CON ( $P = 0.04$ ; Figure 1). The BHBA concentration peaked in both groups around d 33. Total protein concentration was decreased in the FF of the ALG but increased over time after calving. The FF cholesterol concentration also increased over time ( $P < 0.001$ ) and the significant interaction with treatment ( $P = 0.042$ ; Table 5) was caused by a decreased cholesterol concentration on d 46 for the ALG vs. CON (Figure 2). An increase over time was registered for the total protein ( $P < 0.001$ ) and IGF-1 ( $P = 0.0011$ ). NEFA tended to decrease over time ( $P = 0.063$ ; Figure 3).

**Table 5.** Effect of dietary treatment on follicular fluid metabolite concentrations during the first 6 weeks of lactation (n= 16)

Item	Treatment <sup>1</sup>			P-value		
	CON	ALG	SEM <sup>2</sup>	Treatment	Tr x Ti	Time
Total cholesterol, mmol/L	1.548 <sup>3</sup>	1.339	0.081	0.189	0.042	<0.001
Urea, mmol/L	4.63	4.88	0.18	0.601	0.797	0.426
Glucose, mmol/L	3.414	3.273	0.099	0.395	0.534	<0.001
Total Protein, g/L	67.6	62.9	1.7	0.053	0.330	0.007
NEFA, mmol/L	0.218	0.193	0.035	0.842	0.231	0.063
BHBA, mmol/L	0.718	0.992	0.088	0.043	0.399	0.072
Albumin, g/L	28.56	28.59	0.50	0.891	0.304	0.311
IGF-1, ng/mL	70.1	67.0	4.6	0.498	0.352	0.001
Insulin, IU/mL	1.74	1.33	0.22	0.159	0.534	0.167

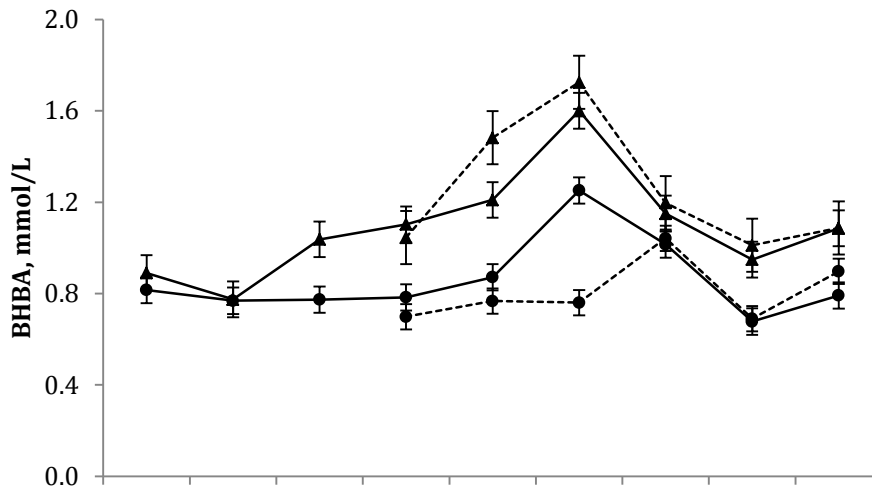
<sup>1</sup> CON = control group, ALG = algae group

<sup>2</sup> Pooled standard error of the mean

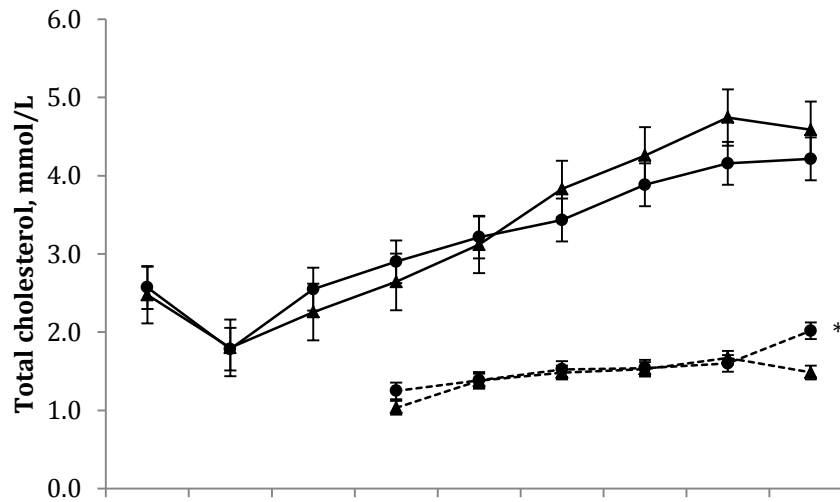
<sup>3</sup> Values are reduced model least square means

## CORRELATIONS BETWEEN BLOOD AND FOLLICULAR FLUID METABOLITES

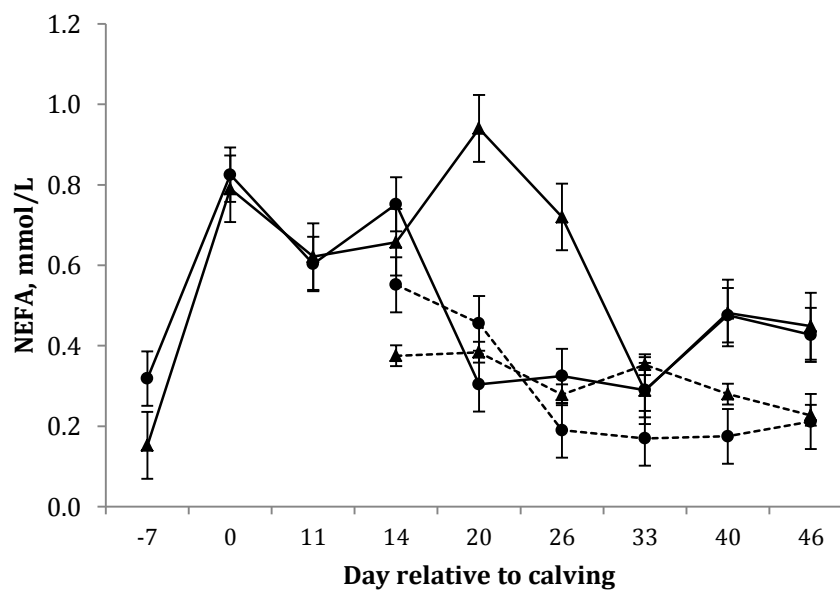
Strong correlations between the serum and FF were calculated for BHBA ( $r = 0.78$ ,  $P < 0.001$ ), glucose ( $r = 0.76$ ,  $P < 0.001$ ) and urea ( $r = 0.81$ ,  $P < 0.001$ ). Lower Pearson's correlation between these fluids were apparent for albumin ( $r = 0.64$ ,  $P < 0.001$ ), total protein ( $r = 0.69$ ,  $P < 0.001$ ) and total cholesterol ( $r = 0.57$ ,  $P < 0.001$ ). When comparing different metabolites within the serum or FF, BHBA and glucose in both serum ( $r = -0.64$ ,  $P < 0.001$ ) and FF ( $r = -0.63$ ,  $P < 0.001$ ) were negatively correlated. A similar negative correlation existed between GH and total cholesterol in the serum ( $r = -0.51$ ,  $P < 0.001$ ) while in the FF IGF-1 and glucose were positively correlated ( $r = 0.58$ ,  $P < 0.001$ ).



**Figure 1.** Profiles of serum (continuous lines) and follicular (dotted lines)  $\beta$ -hydroxy butyric acid (BHBA) concentrations of periparturient cows fed the control diet (●) or marine algae diet (▲).



**Figure 2.** Profiles of plasma (continuous lines) and follicular (dotted lines) total cholesterol concentrations of periparturient cows fed the control diet (●) or marine algae diet (▲).



**Figure 3.** Profiles of plasma (continuous lines) and follicular (dotted lines) non esterified fatty acids (NEFA) concentrations of periparturient cows fed the control diet (●) or marine algae diet (▲).

## DISCUSSION

### **EFFECT OF MARINE ALGAE SUPPLEMENTATION ON MILK PRODUCTION PARAMETERS IN EARLY LACTATION**

In this experiment, MFD was induced by a daily intake of 44 g 22:6n-3 from marine algae (species *Schizochytrium*) as described earlier by Franklin et al. (1999) and Boeckaert et al. (2008) feeding respectively 37.6 and 43.7 g/d of 22:6n-3 to mid-lactating dairy cows. The reduced milk fat yield and content are consistent with Boeckaert et al. (2008; 47.9 vs. 22.5 g/kg and 1.27 vs. 0.52 kg/d) and Franklin et al. (1999; 37.0 vs. 29.5 g/kg and 0.84 vs. 0.69 kg/d). Studies which included marine products such as fish oil (FO; Donovan et al., 2000) and fish meal (FM; Abu-Ghazaleh et al., 2004), reported similar findings of reduced milk fat yield. As in the study of Franklin et al. (1999) which was the first one using ALG in dairy cows, the present study demonstrated a tendency for reduced milk protein content in the ALG group (34.7 vs. 32.8 g/kg). Franklin et al. (1999) did not increase milk yield nor the FPCM by feeding the ALG supplement whereas milk yield increased by 8.1 % in the current study. Mattos et al. (2004) fed 200 g FO (72 g/d 20:5n-3 (eicosapentaenoic acid; EPA) and 56 g/d 22:6n-3) to early lactating dairy cows compared to an isoenergetic, isonitrogenous and isolipidic ration containing olive oil (OO). They reported a decreased milk fat yield (1.27 vs. 0.81 kg/d) for the FO supplemented group. Milk production was recorded the first 3 weeks after parturition and did not differ between groups which is similar to the limited effect of treatment on milk production in the first 2 weeks in the present study. In the study of Mattos et al., (2004), although there were large numerical differences in DMI and milk yield recorded these were not significant. Hence, responses of milk yield on energy sparing through ALG induced MFD differed considerably between studies which is most probably caused by the difference in lactation stage of cows in our experiment compared with others (Franklin et al., 1999; Boeckaert et al., 2008) as comprehensively postulated by Friggens et al. (2010).

**MILK FAT DEPRESSION IN RELATION TO BIOCHEMICAL PARAMETERS OF THE ENERGY BALANCE IN BLOOD AND FOLLICULAR FLUID**

The induction of MFD has been proposed (Shingfield et al., 2004; Kay et al., 2006) and proven (Odens et al., 2007) to reduce the NEBAL in dairy cows early post partum. All researchers mainly focused on the reduced energy loss via the energetically most expensive component synthesized in milk (50 % of total milk energy), the milk fat which happens to be the milk component most easily controlled by dietary management (Odens et al., 2007). Feeding *trans*-10, *cis*-12 CLA which is known to be a potent milk fat depressing isomer of CLA (Baumgard et al., 2002) in early lactation improved the calculated energy balance (EBAL) in early lactation in the study of Kay et al. (2006). This could not be confirmed by any of the reported biochemical markers in the blood plasma to estimate the EBAL (BHBA, glucose, insulin, NEFA). So far, we are aware of only one study (Odens et al., 2007) showing marked results on the energy balance. In that study a MFD was induced (a decrease of 26% in milk fat content and 23% in milk fat yield) as from 8 DIM using a diet containing 600 g of rumen inert CLA supplement with 29 g *trans*-10, *cis*-12 CLA. The researchers were able to show a significant decrease of 12% in NEFA and increase of 11% in glucose concentration early post partum in the CLA supplemented group indicating an improved energetic status of these cows.

Regarding marine products, Mattos et al. (2004) registered a MFD (a 36 % decrease in milk fat yield) induced by supplementing 18 g/kg DM FO post partum (72 g/d 20:5n-3 and 56 g/d 22:6n-3) compared to OO without any effect on milk yield and milk protein content or yield. Nevertheless, in that study postpartum DMI significantly decreased with 18% which obviously could interfere with EBAL parameters, leading to increasing BHBA at d 10 and d 13 and lower glucose concentrations but no effect on blood plasma NEFA. Due to the experimental setup of the present study no individual DMI were recorded and hence, dietary effects on DMI cannot be excluded. However, maintained glucose, insulin, GH and IGF-1 blood concentrations and a lack of NEFA increase despite the greater milk production does not suggest DMI impairment. BHBA increased in FF, although the increase in the serum was not significant. Nevertheless, BHBA concentrations in both compartments were strongly correlated (Figure 1). The increase in milk yield in the current experiment might have induced an enlarged need of the ALG cow's metabolism for glucose pushing her to switch the hepatic pathways converting glucogenic amino acids into glucose (Drackley et al., 2001). This could

explain the tendency for increased urea concentration measured in the serum of the ALG cows. A positive effect on blood energy parameters, in particular increased blood glucose (Ballou et al., 2009; Heravi Moussavi et al., 2007) and insulin (Heravi Moussavi et al., 2007) concentration as well as decreased BHBA (Ballou et al., 2009), was reported when supplementing 20:5n-3 + 22:6n-3. Supplementing 20:5n-3 + 22:6n-3 from fish meal (23.2 g/d, Heravi Moussavi et al., 2009) but not from fish oil (46.7 g/d, Ballou et al., 2009) was associated with increased milk yield.

### **CORRELATION BETWEEN BLOOD AND FOLLICULAR FLUID BIOCHEMICAL PARAMETERS OF THE ENERGY BALANCE**

In the current trial, 20 d after calving, NEFA peaked in the serum but not in the FF (Figure 3) which is also reflected in the smaller coefficient of variation in follicular fluid (0.69) as compared with serum (0.83). Our results are similar to those of Leroy et al. (2004) and substantiate the probable existence of a protective mechanism of the follicular wall against high blood NEFA concentrations. However, this mechanism does not hold for the smaller molecule BHBA (molar mass 104.1 g/mol). Elevated follicular BHBA concentrations as observed in the current experiment are similar to those found under (sub-) ketotic conditions during NEBAL, which were mimicked in bovine *in vitro* maturation models by Leroy et al. (2006). They proved that these elevated BHBA concentrations, in particular when combined with low follicular glucose concentrations, which is also the case in the current experiment, are detrimental to the oocyte's developmental capacity. Finally, the decrease of FF protein through ALG feeding should not be neglected. Even though the presence of protein seems necessary for normal *in vitro* fertilisation (Eckert and Niemann, 1995), changes in FF protein concentration did not affect developmental capacity of oocytes in beef heifers (Iwata et al., 2006). However, to the best of our knowledge the effect of FF protein levels on fertility of high yielding cows has not yet been reported.

**NUTRIENT SPARING AND REDIRECTION THROUGH MFD : A CALORIC AND METABOLIC APPROACH**

In the present trial, a daily ME sparing effect of 3.48 MCal was obtained due to the MFD of 313 g/d (11 MCal/kg milk fat, Dado et al., 1993). Assuming an ME requirement for lactose and protein production of 5.11 MCal/kg and 6.43 MCal/kg respectively (Dado et al., 1993), the ME sparing of 3.48 MCal theoretically could lead to an increase in milk yield of 7.43 kg/d which is higher than the 3 kg/d increase observed in our current experiment (Table 6). This most probably is due to a limiting nutrient availability in early lactation (Drackley et al., 2001). Therefore, calculation of nutrient availability (e.g. glucose) and requirement possibly is a better approach (Dado et al., 1993) to explain differences in milk yield. The two main substrates required for milk fat synthesis are acetate (62%) and propionate (26%; Dado et al., 1993). The molar mass of milk fat was calculated based on the FA profile respectively to 786 g/mol and 793 g/mol for CON and ALG. In the current trial, milk fat production is decreased by 0.411 mol of milk fat due to the MFD which saves 6.47 mol of acetate, 2.71 mol of propionate, 0.205 mol of glycerol, 0.476 mol of LCFA and 0.3 mol of BHBA (Dado et al., 1993; Table 6). The fate of excess propionate might be easy to follow as hepatic gluconeogenesis lags behind in early lactation due to a lack of substrate (Drackley et al., 2001, Overton et al., 1999). Assuming an overall turnover efficiency of propionate via glucose to lactose of 0.55 (Lemosquet et al., 2009), theoretically the extra supply of 2.71 mol of propionate could be converted into 0.414 mol of lactose. As milk volume and lactose production are linked via an osmotic pressure mechanism (Linzell and Peaker, 1971), this could lead to an increased milk yield of 2.95 kg/d (48 g lactose/kg milk) which meets the 3.00 kg/d increase in milk production as observed in the present study. Until now, research has not been able to clarify the pathway by which the unused acetate and LCFA are metabolized when milk fat is depressed. Although not quantified in early lactating dairy cows, increased lipogenesis seems the most plausible route (Harvatine et al., 2009). Even though small when compared to the contribution of propionate, glycerol has been described to contribute 0.15 to 0.20 of the glucose demand at d 4 post partum (Bell, 1995). The MFD in the current trial saved 0.205 mol of glycerol (molar mass: 92.1 g/mol) daily which equals 18.9 g/d of glycerol.

**Table 6.** Caloric and metabolic approach to milk fat depression

CALORIC MILK FAT DEPRESSION				
	ALG <sup>1</sup>		CON	
Fat content (g/kg)	31.6		40.7	
Milk yield (kg/d)	41.2		38.2	
Fat yield (g/d)	1.181	793 g/mol <sup>2</sup>	1.493	786 g/mol <sup>2</sup>
<b>Fat yield (mol/d)</b>	<b>1.49</b>	<sup>8.47</sup> MCal/mol <sup>3</sup>	<b>1.90</b>	
Energy requirement for milk fat production (MCal/d)	12.6		16.1	
<u><i>ENERGY available for increased milk production</i></u>				
= 16.1 – 12.6				
= 3.48 MCal				
= kg milk * (6.43 MCal/kg milk protein + 5.11 MCal/kg milk lactose) <sup>3</sup>				
milk protein = 34.7g/kg				
milk lactose = 48 g/kg				
= 7.43 kg milk				
METABOLIC MILK FAT DEPRESSION				
Fat yield (mol/d)	<b>1.49</b>	6.6 mol/mol <sup>3</sup>	<b>1.90</b>	
Propionate requirement for <i>de novo</i> milk fat synthesis (mol/d)	9.8		12.5	
<u><i>PROPIONATE available for increased milk production</i></u>				
= 12.5 – 9.8				
= 2.71 mol propionate (0.50 – 0.60 efficiency) <sup>4</sup>				
= 1.49 mol propionate (3.6 mol propionate/mol lactose) <sup>3</sup>				
= 0.414 mol lactose (molar mass : 342.30 g/mol)				
= 141.8 g lactose (milk lactose content 48 g/kg)				
= 2.95 kg milk				
Fat yield (mol/d)	<b>1.49</b>	0.73 mol/mol <sup>3</sup>	<b>1.90</b>	<u>RATIO</u>
BHBA requirement for <i>de novo</i> milk fat synthesis (mol/d)	1.09		1.39	0.78
BHBA <sub>SERUM</sub> (mmol/l)	0.88		0.73	1.20
BHBA <sub>FF</sub> (mmol/l)	0.99		0.72	1.38

<sup>1</sup>ALG = algae group, CON = control group

<sup>2</sup>Weighted mean molar mass based on the CON and ALG fatty acid profile

<sup>3</sup>Metabolisable Energy, Dado et al., 1993

<sup>4</sup>Lemosquet et al., 2009



However, this is minor compared with the glycerol released during extensive mobilisation of adipose tissue in early lactation (up to 433 g/d; Drackley et al., 2001). Finally, *de novo* synthesis of SCFA requires 0.5 mol of BHBA per mole of FA (Dado et al., 1993). Taking into account the milk FA profile of milk in the current trial (Table 3), ALG depressed fat synthesis by 1.23 mol/d FA saving 0.3 mol BHBA (Dado et al., 1993), which represents about 0.78 of the BHBA requirement for milk FA production in the CON. The unused BHBA surplus might be at the origin of the 1.2 to 1.4 fold BHBA concentration in blood serum ( $0.88/0.73 = 1.20$ , Table 3) and FF ( $0.99/0.72 = 1.38$ , Table 4) of ALG versus CON cows. Further, a higher butyrate production in the rumen, as suggested from rumen FA proportions of ALG fed cows (Boeckaert et al., 2008), also could have contributed to higher BHBA in blood and FF as observed in the current trial. Hence, the approach of taking both metabolic limitations and nutrient requirements in early lactation into account seems to be more capable of explaining observations seen during MFD in high yielding dairy cows.

## CONCLUSIONS

Santos et al. (2008) stated that improved reproductive performance have not consistently been observed when feeding FA to dairy cattle especially when accompanied with an increased milk yield and a loss in body weight. The results from the current study substantiate this statement as increased BHBA concentrations in the FF that have been associated with poor reproductive performance in high yielding dairy cows were detected in the PUFA supplemented group (ALG). However, when calculating nutrient requirements during MFD in early lactation, increased BHBA concentrations can be attributed to a nutrient saving effect at the udder level. In conclusion, the downregulation of *de novo* milk fat synthesis has 2 main consequences. The first is an increased milk production most likely caused by a propionate saving effect when milk fat is depressed and the second, an increased BHBA concentration in the FF that cannot be attributed to a worsened energy status of the animals as all other indicators contradict any change in EBAL, indicating that BHBA might not be an appropriate metabolic parameter to estimate the EBAL in early lactating dairy cows during MFD.

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# THE EFFECT OF FEEDING OMEGA-6 AND OMEGA-3 FATTY ACIDS IN EARLY LACTATION ON BLOOD AND FOLLICULAR FLUID FATTY ACID PROFILES

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## SUMMARY

The objective of this study was to determine the effect of feeding fat supplements, varying in saturation and length of the fatty acids (FA) during the transition and early breeding period on production variables and the FA composition of blood (BP), blood cholesterol esters (CE), nonesterified fatty acids (NEFA), phospholipids (PL) and triacylglycerols (TAG), follicular fluid (FF) and milk fat (MF). Holstein Friesian dairy cows (n=18) were assigned in a randomized complete block design to one of three isoenergetic, isonitrogenous and isolipidic diets containing either palm prills (16:0; CON), sequential 18:2n-6 and 18:3n-3 (SHORT), or sequential 20:4n-6 and 22:6n-3 (LONG). The fat supplements were fed from approximately 14d before parturition until 49 d in milk.

Production variables (dry matter intake, milk production and content, body condition score) did not differ between treatments throughout the experimental period. Metabolic parameters in BP and FF did not differ between treatments except the BP urea content which was higher in LONG versus SHORT (3.74 vs.  $4.58 \pm 0.189$  mmol/L). Feeding SHORT increased 18:2n-6 in MF compared with LONG but not CON. Effects on BP fractions were minimal except for the increased BP TAG 18:2n-6 content compared with CON prepartum. Compared to CON, feeding SHORT increased the 18:3n-3 content in BP, FF and MF 1.25, 1.46 and 1.66 times respectively which was reflected in a higher 18:3n-3 content in all BP fractions on d 46 except TAG. Feeding LONG increased the 20:4n-6 in BP compared with SHORT, and in the FF compared with SHORT and CON, but not in MF. As opposed to 18:2n-6, this was reflected in CE and PL but not NEFA and TAG. Furthermore, compared with CON a 2.6, 4.1 and 2.5-fold increase in 22:6n-3 was observed in BP, FF and MF respectively, when feeding LONG. This was consistently reflected in all BP fractions. The follicular environment and mammary gland seem to respond in a different way to supplemental SHORT and LONG n-6 and n-3, most probably due to discrimination against specific FA in the different lipid classes. We were able to show limited effects on the FA 18:2n-6 proportion in BP and FF, while substantially increasing 18:3n-3 in BP and FF. In contrast, sequential feeding of LONG n-6 and n-3 FA did increase both 20:4n-6 and 22:6n-3 in BP and FF without disturbing animal performance.



## INTRODUCTION

As pregnancy rates have dropped considerably during the last decades, dietary fat supplementation has been proposed by researchers and nutritionists as a promising approach to increase dairy cow fertility (Thatcher et al., 2011). However, results of fat feeding strategies to restore reproductive performance in dairy cattle have been conflicting (Staples et al., 1998; Santos et al., 2008). Primarily, fats have been included in transition diets to increase caloric energy intake in order to support the postpartum dairy cow in coping with the acute energy deficit (Staples et al., 1998; Drackley, 1999). However, cows often respond to this supplemental fat with an increased production (Erickson et al., 1992; Lucy et al., 1992) or decreased DMI (Son et al., 1996), resulting in a net unchanged energy balance early post partum (Santos et al., 2008).

Fatty acids (FA) are able to improve fertility beyond their ability to increase energy density of fresh cow diets (Staples et al., 1998; Staples and Thatcher, 2005), many through their specific composition and direct effects at cellular level. An increased number (Lucy et al., 1991) and size of the preovulatory follicles (Lucy et al., 1993; Moallem et al., 1999; Bilby et al., 2006a) when feeding calcium soaps of FA have been reported, and linked to earlier postpartum ovulation (Beam and Butler, 1998). Feeding n-6 FA to dairy cows has been shown to stimulate PGF $2\alpha$  metabolism improving uterine health (Robinson et al., 2002; Petit et al., 2004). In contrast, feeding n-3 FA reduced endometrial secretion of PGF $2\alpha$ , thereby induce antiluteolytic effects on the corpus luteum (Staples et al., 1998). Researchers have targeted specific alterations in FA composition of reproductive tissues such as the endometrium (Bilby et al., 2006b), the ovarian follicular fluid (FF), cumulus cells and oocytes (Adamiak et al., 2006). Recently, especially sequential and selective feeding of supplemental n-6 and n-3 FA during the transition and breeding period has been proposed as an optimal reproductive management strategy in dairy cows (Thatcher et al., 2011). Short, this strategy combines the improved uterine health around parturition through n-6 supplementation (Robinson et al., 2002; Juchem et al., 2010) with an improved embryo quality when supplementing n-3 during the breeding period (Zeron et al., 2002; Zachut et al., 2010b) by switching diets around 20 DIM. When compared to an entire pre- and postpartum period supplementation with palm oil (PO; rich in 16:0), safflower oil (rich in 18:2n-6) during the transition period followed by fish oil (FO; rich in 20:5n-3 and 22:6n-3) during the



breeding period reduced the pregnancy loss at first service and increased the pregnancy rate at the second AI but not at first AI (Silvestre et al., 2011b). Interestingly, differences in fertility between heifers and cows have partially been attributed to the higher 22:6n-3 content of FF in heifers (Bender et al., 2010) but experimental designs on sequential supplementation with FA with a chain length larger than 18 carbons, such as 20:4n-6 and 22:6n-3 to dairy cows are missing. *In vitro* results are promising as in contrast with its shorter counterpart 18:2n-6, *in vitro* maturation with 20:4n-6 did show beneficial effects during oocyte maturation on subsequent embryo quality (Marques et al., 2007).

We hypothesized that sequential dietary long chain FA with a chain length larger than 18 carbon atoms are able to alter the FA composition of blood plasma (BP), FF and milk fat (MF) as occurs when supplementing their 18 carbon length counterparts. Therefore, the objective of the current experiment was to evaluate how sequential n-6 and n-3 feeding of C18 (SHORT) and >C18 FA (LONG) during the transition period affects the FA profile of BP, FF and MF.

## MATERIALS AND METHODS

### ANIMALS, EXPERIMENTAL DESIGN AND FEEDING

All procedures and protocols were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University, N°: EC EC2009/012). The experiment was conducted at the Institute for Agricultural and Fisheries Research (ILVO-Dier, Melle, Belgium). Cows were housed in a tie-stall barn during the pre- and postpartum stages of the study, and were milked twice daily with a 12 hour interval (5 am – 5 pm).

The cows (n=18) were blocked for parity, expected calving week, estimated MF production and genetic origin (cows with same ancestors were assigned to different dietary groups) and assigned to 3 different experimental diets in a randomized complete block design approximately 2 weeks ( $15.6 \pm 6.9$  d) before the expected calving date (Figure 1). The experimental diets (Table 1) were designed in order to provide the same amount of energy (based on  $NE_L$ , Van Es, 1978), protein (based on the Dutch DVE system, Tamminga et al., 1994) and FA content in the total ration, and differed only in the FA profile of the supplemented fat.

**Table 1.** Ingredients and fatty acid composition of diets consumed in the pre- and postpartum experimental period.

Ingredient (%)	Prepartum d -14-0			Postpartum d 0-14			Postpartum d 14-46		
	CON <sup>1</sup>	SHORT	LONG	CON	SHORT	LONG	CON	SHORT	LONG
Corn silage	51.5	50.1	51.1	43.1	38.7	41.8	39.7	35.5	39.6
Gras silage	34.3	33.6	34.0	28.7	26.0	27.8	26.4	25.2	26.3
Beans							0.22	0.26	0.22
Beetpulp	1.54	1.78	1.63	4.96	6.1	5.25	5.65	6.4	5.66
Corn	0.37	0.43	0.39	1.19	1.46	1.26	1.36	1.54	1.36
Corn gluten feed	0.61	0.71	0.65	1.98	2.44	2.1	2.26	2.56	2.26
Soybean meal	2.07	2.39	2.19	5.94	7.74	6.75	6.32	7.52	6.36
Rumi-S <sup>2</sup>	0.37	0.43	0.39	3.7	4.71	3.94	3.26	3.85	3.28
Wheat	1.57	1.82	1.66	5.06	6.22	5.35	6.77	7.75	6.79
Molasses	0.87	1	0.92	1.19	1.46	1.26	1.36	1.54	1.36
Rapeseed expeller	0.49	0.57	0.52	1.59	1.95	1.68	1.81	2.05	1.81
Sunflower expeller							0.72	0.87	0.73
Min/Vit mix <sup>3</sup>	4.37	5.03	4.62	0.89	1.1	0.94	1.14	1.3	1.12
Tallow	0.09	0.11	0.1	0.3	0.37	0.31	0.34	0.38	0.34
Palm prills	1.79		1.48	1.42		1.19	0.57		0.35
Soybean oil		2.06			1.83		0.37		0.38
Vevodar <sup>3</sup>			0.41			0.33			
Linseed expeller							1.74		1.75
Linseed oil								0.06	
Linseed extruded								3.26	
DHA Gold <sup>4</sup>									0.38
Fatty acid, g/kg									
12:0	15.5	14.7	15.3	13.5	12.2	12.9	12.3	11.5	12.1
14:0	3.95	3.37	4.10	4.31	3.32	4.52	4.15	3.64	5.98
16:0	171.4	158.2	173.4	186.7	151.8	185.8	169.2	146.5	166.0
16:1 <i>cis</i> -9	3.46	3.71	3.51	3.64	3.88	3.76	4.63	4.78	4.74
18:0	52.7	27.8	54.4	70.6	32.9	69.3	50.3	31.6	43.7
18:1 <i>cis</i> -11	7.23	10.24	7.37	8.44	11.3	9.02	11.7	12.6	12.0
18:1 <i>cis</i> -9	140.7	175.1	141.1	140.7	169.1	144.5	165.0	179.4	167.0
18:2n-6	309.2	304.4	304.8	308.5	360.1	307.8	330.8	328.3	331.5
18:3n-3	213.0	204.9	211.9	188.9	182.5	181.0	174.3	214.0	173.1
20:4n-6	0.07	0.08	0.47	0.10	0.20	5.71	0.19	0.21	0.48
22:5n-3	0.77	0.77	0.75	0.69	0.66	0.76	0.64	0.62	2.86
22:6n-3	4.43	4.39	4.42	3.92	3.67	3.97	3.51	3.33	9.79

<sup>1</sup> CON = saturated fatty acids, SHORT = short chain fatty acids, LONG = long chain fatty acids

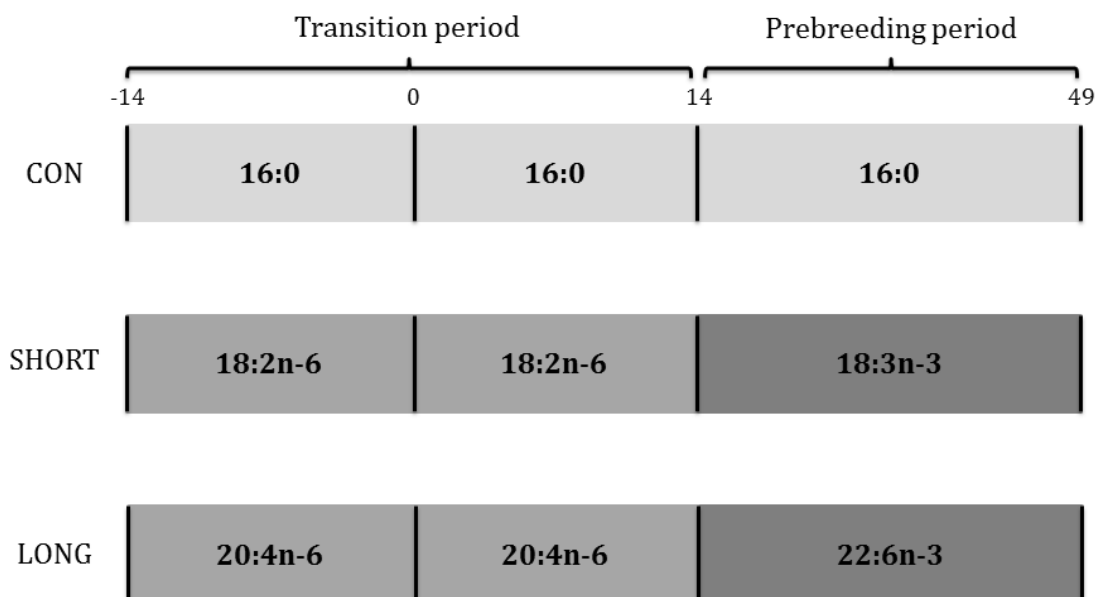
<sup>2</sup> Formaldehyde treated soybean meal (Schouten Industries, Giessen, The Netherlands)

<sup>3</sup> Mineral and vitamin mix

<sup>4</sup> DSM Nutrition Products, Deinze, Belgium

<sup>5</sup> Martek DHA gold, Martek Biosciences Corp., Colombia

More specifically, the transition diets (2 weeks before until 2 weeks post partum) were supplemented either with rumen inert PO (16:0; CON; Palmit 80, Trouw Nutrition, Ghent, Belgium), soybean oil (18:2n-6; SHORT; Berton, Moen, Belgium) or arachidonic acid oil (20:4n-6; LONG; Vevodar, DSM Nutrition Products, Deinze, Belgium). Two weeks after parturition, cows were changed to the breeding diets with either PO, extruded linseed for SHORT (18:3n-3 source; Nutex Compact, Dumoulin, Kortrijk, Belgium) or 22:6n-3 enriched marine algae for LONG (Martek DHA gold, Martek Biosciences Corp., Colombia). Concentrates were mixed at the Institute for Agricultural and Fisheries Research (ILVO-Dier, Melle, Belgium) to have an identical non-pelleted physical form. In summary, CON cows were fed PO throughout the entire experiment. Cows in SHORT were fed supplemental 18:2n-6 during the transition period to be switched to 18:3n-3 around 20 DIM whereas cows in LONG were fed supplemental 20:4n-6 during the transition period to be switched to 22:6n-3 around 20 DIM. Cows were offered their allocated concentrates twice daily next to the corn- and grass silage based diet, targeting 5% daily feed orts. Individual DMI was determined on a daily basis by subtracting the total feed offered minus the feed orts which were collected and weighed twice weekly. Weekly averages were calculated for the postpartum DMI.



**Figure 1.** Schematic representation of the experimental design. Holstein Friesian dairy cows (n=18) were assigned in a randomized complete block design to one of three isoenergetic, isonitrogenous and isolipidic diets containing either palm prills (16:0; CON), sequential 18:2n-6 and 18:3n-3 (SHORT), or sequential 20:4n-6 and 22:6n-3 (LONG). Diets were switched from n-6 to n-3 fatty acids around 14 DIM for the SHORT and LONG.

## **MILK PRODUCTION, FEED SAMPLES AND BODY CONDITION SCORE**

Milk samples were taken weekly starting at d 5 after parturition during 7 consecutive weeks. After proportionally pooling all milk samples from one day according to milk yield, two subsamples were frozen (-20°C) and stored prior to milk component analysis and MF extraction for FA determination. Feed samples from all forages and concentrates were taken at the start of the experimental period, and stored deeply frozen (-20°C) in plastic bags until FA determination. Body condition scores were recorded weekly by the same person using a score on a 1-5 scale, with 0.25 increments (Edmonson et al., 1989), from the week before the expected calving day until week 6 after parturition.

## **MILK, BLOOD AND FOLLICULAR FLUID SAMPLING**

Pre- and postpartum blood samples were taken on d-7 ( $\pm 2.3$ ), d 0, d 7, d 14, d 20, d 26, d 33, d 40 and d 46 following parturition. Blood samples were taken from the coccygeal vein (sample was discarded when arterial blood was observed) into unheparinised, silicone coated tubes (Venoject, Autosep, Gel + Clot. Act.; Terumo Europe N.V., Leuven, Belgium), tubes with ethylene diamine tetraacetic acid (EDTA, Venoject, Autosep, Terumo Europe, Leuven, Belgium), and 1 tube with a glycolytic inhibitor (NaF, Venoject, Autosep, Terumo Europe, Leuven, Belgium). The FF was simultaneously collected with the blood samples from on d 14 post partum onwards following the protocol described by Leroy et al. (2004). The follicles with a diameter greater than 8 mm were aspirated in order to minimize follicle size effects on FF composition (Leroy et al., 2004). The FF samples with obvious blood contamination were omitted from further processing. All BP and FF samples were transported to the laboratory at 4 °C. Within 1.5 hours after collection, samples were centrifuged (20 min at 1,500 x *g*). Serum and plasma samples were stored at -20 °C, FF samples at -80 °C until biochemical analysis.

## **CHEMICAL ANALYSES**

The blood and FF analyses for albumin, aspartate aminotransferase (AST), BHBA, glucose, gamma-glutamyltransferase (GGT), NEFA, urea, total cholesterol, and total protein were carried out by means of an automated colorimetric analyser (Konelab™ 20 XTi Clinical chemistry analyzer, Thermo Electron Cooperation, Vantaa, Finland) at

The Flemish Veterinary Health Service (Dierengezondheidszorg-Vlaanderen, Torhout, Belgium). All blood analyses were carried out on serum derived from the unheparinised, silicone coated tubes except the one for glucose determination (NaF tubes). Milk protein (MP) and MF content were analyzed by Fourier Transform Infrared spectrophotometry (Lactoscoop FTIR Advanced, Delta Instruments, Drachten, The Netherlands). The fat and protein corrected milk (FPCM) was calculated as  $[(0.337 + 0.116 \times \text{MF \%} + 0.06 \times \text{MP \%}) \times \text{kg of milk}]$  (CVB, 2007).

### **FATTY ACID ANALYSIS**

Fatty acids in dietary components (200 mg freeze dried material), BP (EDTA tubes) and FF (200  $\mu\text{L}$ ) were methylated by a base-catalyzed followed by an acid-catalyzed step. Toluene (2 mL) containing the internal standard (13:0) and methanolic NaOH (2 mL) was added and the mixture was incubated at 70°C (60 min) followed by 30 min at 50°C after addition of methanolic HCl (3 mL), prepared by dissolving 10 mL acetyl chloride in 50 mL methanol. Fatty acid methyl esters (FAME) were extracted with hexane. For feed samples, an aliquot of the hexane extract was directly used for injection. Extracted FAME from BP and FF were dried under  $\text{N}_2$  and redissolved in 200  $\mu\text{L}$  of hexane prior to injection. Milk lipids were extracted as described by Chouinard et al. (1997b) and FAME prepared as described by Stefanov et al. (2010).

To assess FA composition of lipid fractions in BP, plasma lipids (2 mL) of samples taken on d -7, 14 and 46 were extracted with methyl-tert-butyl-ether as described by Matyash et al. (2008). BP lipids fractions were separated using SPE-columns (Burdge et al., 2000; Pinkart et al., 1998). Total plasma lipid extracts were dissolved in chloroform (1.0 mL) and applied to an aminopropyl silica column (Pasteur pipette containing 100 mg aminopropyl silica gel) under gravity. Cholesteryl-esters (CE) and triacylglycerols (TAG) were eluted with chloroform (1.0 mL and 0.5 mL), combined, dried under  $\text{N}_2$  and dissolved in 1.0 mL hexane. NEFA were eluted with diethyl ether/acetic acid (100:2; 1.0 mL and 0.5 mL) and phospholipids (PL) with 1 mL methanol/chloroform (6:1) followed by 0.5 mL 0.05M sodium acetate in methanol/chloroform (6:1). CE and TAG were further separated on a pre-packed 100 mg aminopropyl column (Bond Elut-NH<sub>2</sub>, Varian Medical Systems Belgium, Diegem, Belgium). The CE and TAG fraction was loaded in 1 mL hexane and CE were eluted with hexane (1.0 mL and 0.5 mL). TAG were eluted with

hexane/chloroform/ethyl acetate (100:5:5; 1.0 mL and 0.5 mL). FA in PL and CE were methylated using a basic followed by an acid methylation step. FA in the TAG fraction were methylated as described by Stefanov et al. (2010) whereas NEFA were methylated by an acid methylation step only. Composition analyses of the FA were carried out with a gas chromatograph (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a 75-m SP-2560 capillary column (i.d., 0.18 mm, film thickness, 0.14  $\mu$ m; Supelco Analytical, Bellefonte, PA) and a flame ionization detector. A combination of two oven temperature programs was used according to the method of Kramer et al. (2008). In order to screen milk FA composition, a first temperature program was as follows: at the time of sample injection the column temperature was 70 °C for 2 min, then ramped at 15 °C/min to 150 °C, followed by a second increase at 1 °C/min to 165 °C and maintained for 12 min, followed by a third increase at 2 °C/min to 170 °C, maintained for 5 min, and a final increase at 5 °C/min to 215 °C, and maintained for 10 min. A second temperature program was used to separate most of the co-eluting isomers: at the time of sample injection, the column temperature was 70 °C, then ramped at 50 °C/min to 175 °C, and maintained isothermal for 13 min, followed by a second increase at 5 °C/min to 215 °C, and maintained for 10 min. For both programs, inlet and detector temperatures were 250°C and 255°C, respectively. The split ratio was 100:1. The flow rate for hydrogen carrier gas was 1 mL/min. FA peaks were identified and using quantitative mixtures of methyl ester standards (BR2 and BR3, Larodan Fine Chemicals, Malmö, Sweden; Supelco 37, Supelco Analytical, Bellefonte, PA). FA for which no standards were available commercially were identified by order of elution according to Kramer et al. (2008).

## **DATA HANDLING AND STATISTICAL ANALYSIS**

Descriptive statistics were done using PROC MEANS (SAS Institute, Inc., Cary, NC, USA, 2010). Prior to analysis, the distribution of all variables was checked to approximate the normal Gaussian distribution. If necessary, a box-cox transformation (Box and Cox, 1964) was performed. This transformation was carried out for the serum AST, BHBA, cholesterol, glucose and NEFA, and follicular BHBA, cholesterol and NEFA. Subsequently, the data were analysed as a randomized complete block design using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA, 2010). The MIXED procedure was used to analyse the effect of the diet on the production characteristics, composition

of milk and BCS and FA composition of BP, FF and MF FA. Separate models were built for prepartum (time=day) and postpartum (time=week) DMI. All models included diet as the main variable of interest next to time and the interaction between diet and time. Repeated measures within the cow were taken into account by adding cow within diet in the RANDOM statement with the unstructured covariance structure and time in the REPEATED statement with an autoregressive order 1 covariance structure. Reported least square means and contrasts were computed using the LSMEANS. Significant interaction terms between diet and time were plotted or reported after adjustment for multiple comparison using the LSMESTIMATE option with Bonferroni correction. Significance and tendency were declared at  $P < 0.05$  and  $0.05 < P < 0.1$  respectively.

## RESULTS

### **MILK PRODUCTION, COMPOSITION, DMI AND BCS**

Milk production ( $P = 0.77$ ) and milk components fat ( $P = 0.63$ ) and protein ( $P = 0.84$ ) did not differ between the three diet groups throughout the entire experimental period of 7 weeks (Table 2). Consequently, also both the fat ( $P = 0.60$ ) and protein ( $P = 0.67$ ) yield remained unaffected. Although CON numerically produced 2.2 kg more milk when compared with SHORT, when corrected for MF and protein content, no differences were found in FPCM as well ( $P = 0.66$ ). Pre- and postpartum DMI did not differ between diets. The lowest intake was observed pre- ( $11.2 \pm 0.99$  kg/d) and postpartum ( $17.5 \pm 0.82$  kg/d) with SHORT. No interaction between diet and time was found for any of the production variables ( $P > 0.1$ ). The BCS was not affected by diet and the time dependent ( $P < 0.001$ ) decrease in BCS postpartum was equal over diets ( $P = 0.31$  for diet x time).

**Table 2.** Effect of diet on production characteristics, composition of milk in the first 7 weeks of lactation and BCS (n=18)

Item	Diet <sup>1</sup>				P-value		
	CON	SHORT	LONG	SEM	Diet	Int <sup>2</sup>	Time
Milk, kg/d	37.2	35.0	36.3	2.10	0.77	0.12	<0.001
FPCM <sup>3</sup> , kg/d	39.7	37.1	37.2	2.29	0.66	0.75	<0.001
Milk fat							
g/kg	48.0	47.4	45.0	2.35	0.63	0.29	<0.001
kg/d	1.73	1.61	1.56	0.125	0.60	0.67	0.19
Milk protein							
g/kg	32.4	31.9	32.8	1.04	0.84	0.50	<0.001
kg/d	1.18	1.10	1.16	0.067	0.67	0.56	0.08
DMI, kg/d							
Prepartum	12.9	11.2	12.0	0.99	0.24	0.49	0.74
Postpartum	19.0	17.5	18.3	0.82	0.50	0.77	<0.001
BCS	2.63	2.77	2.52	0.093	0.19	0.31	<0.001

<sup>1</sup> CON = saturated fatty acids, SHORT = short chain fatty acids, LONG = long chain fatty acids

<sup>2</sup> Int = Interaction term between diet and time

<sup>3</sup> Fat and protein corrected milk

## METABOLIC PARAMETERS IN BLOOD SERUM AND FOLLICULAR FLUID

Urea was significantly different between diets ( $P = 0.021$ ) with SHORT having a lower urea concentration compared with LONG. The change in blood urea concentration was reflected in the follicular fluid by a tendency for a lower urea concentration in SHORT compared with LONG ( $P = 0.052$ ). All other BP or FF metabolic parameters did not differ between diets (Table 3). A higher serum protein on D40 and serum cholesterol on D46 was discovered in the LONG compared with SHORT. Furthermore, no significant time dependent interactions (diet x time) were found for any of the blood serum or follicular fluid metabolic parameters.



**Table 3.** Effect of diet on blood plasma and follicular fluid characteristics in early lactating dairy cows fed short and long chain n-6 and n-3 fatty acids.

Item	Diet <sup>1</sup>			SEM	P-value		
	CON	SHORT	LONG		Diet	Int <sup>2</sup>	Time
Blood plasma							
Albumin, g/L	29.6	28.4	29.7	0.63	0.36	0.33	0.37
NEFA, mmol/L	0.55	0.44	0.46	0.084	0.59	0.59	0.01
BHBA, mmol/L	1.01	0.99	1.07	0.093	0.87	0.17	<0.001
Glucose, mmol/L	3.46	3.63	3.54	0.117	0.57	0.15	<0.001
Cholesterol, mmol/L	3.42	3.60	3.88	0.229	0.34	0.03	<0.001
Protein, g/L	76.2	78.1	73.5	1.63	0.17	0.04	<0.001
Urea, mmol/L	3.97 <sup>5ab</sup>	3.74 <sup>a</sup>	4.58 <sup>b</sup>	0.189	0.017	0.15	<0.001
AST <sup>3</sup> , IU/L	102.1	94.3	92.4	10.40	0.86	0.04	<0.001
GGT <sup>4</sup> , IU/L	29.2	30.3	31.0	2.38	0.86	0.07	0.002
Follicular fluid							
Albumin, g/L	29.5	27.7	29.0	0.78	0.30	0.24	0.17
NEFA, mmol/L	0.29	0.37	0.27	0.044	0.70	0.78	0.35
BHBA, mmol/L	1.26	1.03	1.20	0.162	0.58	0.19	0.54
Glucose, mmol/L	3.27	3.52	3.09	0.135	0.12	0.22	0.90
Cholesterol, mmol/L	2.02	2.03	1.93	0.105	0.73	0.25	<0.001
Protein, g/L	69.0	69.3	64.5	1.55	0.073	0.34	0.08
Urea, mmol/L	4.60	4.22	5.25	0.272	0.052	0.17	0.38

<sup>1</sup> CON = saturated fatty acids, SHORT = short chain fatty acids, LONG = long chain fatty acids

<sup>2</sup> Int = Interaction term between diet and time

<sup>3</sup> Aspartate aminotransferase

<sup>4</sup> Gamma-glutamyltransferase

<sup>5</sup> Values differing in superscript significantly differ within the row

## BLOOD PLASMA AND PLASMA LIPID FRACTIONS, FOLLICULAR FLUID AND MILK FAT

The proportion of 18:2n-6, 18:3n-3, 20:4n-6 and 22:6n-3 in BP, FF and MF as influenced by the dietary treatment is represented in Table 4. Feeding SHORT increased 18:2n-6 in MF compared with LONG ( $P = 0.036$ ) but not CON (Figure 2). No differences between diets were found in total BP, plasma fractions and FF 18:2n-6 content when feeding SHORT except an increased BP TAG 18:2n-6 content compared with CON on d-7 (Figure 3).

Compared to the CON, the 18:3n-3 in BP, FF and MF FA were 1.25, 1.46 and 1.66 fold increased when feeding SHORT (Table 4). Furthermore, feeding SHORT increased 18:3n-3 over time in MF ( $P < 0.001$ ), FF ( $P < 0.001$ ) and BP ( $P < 0.001$ ) which was reflected in a higher 18:3n-3 content in all BP fractions on d 46 (Figure 4) except BP TAG ( $P = 0.66$ ). Nevertheless, greater, though relatively variable, BP TAG 18:3n-3 concentrations on d-7 and d14 resulted in overall tendency for increased TAG 18:3n-3 concentrations ( $P = 0.060$ ). Remarkably, the BP 18:3n-3 content on d-7 was lower in LONG ( $P = 0.027$ ) and tended to be lower in SHORT ( $P = 0.081$ ) compared with CON which was the result of a lower proportion in the CE fraction ( $P < 0.001$ ).

Feeding LONG increased the 20:4n-6 in BP compared with SHORT ( $P = 0.017$ ), in the FF compared with SHORT and CON ( $P = 0.004$ ), but not in MF ( $P = 0.14$ ). The single time dependent effect (diet x time) which could be found in MF was a tendency for an increased 20:4n-6 content in the first week for LONG compared with SHORT ( $P = 0.066$ ). Analysis of the fractions revealed an overall increased 20:4n-6 content in the CE fraction for LONG compared with CON and SHORT ( $P = 0.007$ ) and a tendency for an increased 20:4n-6 in the TAG fraction in LONG compared with CON ( $P = 0.081$ ), particularly due to an increase in those fractions on d14 (Figure 5). Additionally, the 20:4n-6 in the PL fraction was increased at d14 compared with CON ( $P = 0.006$ ). Interestingly, TAG 20:4n-6 content was increased when feeding SHORT on d-7 compared with CON ( $P = 0.0077$ ) whereas no difference between LONG and CON was observed.

Feeding LONG consistently increased the 22:6n-3 content compared with both SHORT and CON in BP ( $P < 0.001$ ), and specifically all BP fractions, i.e. CE ( $P = 0.023$ ), NEFA ( $P = 0.002$ ), PL ( $P = 0.002$ ), TAG ( $P = 0.018$ ), FF ( $<0.001$ ), as well as in MF ( $P < 0.001$ ). When compared with CON, a 2.6, 4.1 and 2.5-fold increase in 22:6n-3 was observed in BP, FF and MF respectively, when feeding LONG. A steady increase in 22:6n-3 concentrations was observed in BP and FF ( $P < 0.05$ ) whereas in MF 22:6n-3 tended to reach a maximum after 5 weeks in milk and decreased thereafter (Figure 2). Interestingly, feeding SHORT increased the 22:6n-3 content in CE on d-7 in comparison with CON ( $P = 0.027$ , Figure 6).

**Table 4.** The effect of sequential feeding of SHORT (18:2n-6 and 18:3n-3) and LONG (20:4n-6 and 22:6n-3) on the proportion of fatty acids in blood plasma, follicular fluid and milk fat (in g/100g).

	CON <sup>1</sup>	DIET SHORT	LONG	SEM <sup>2</sup>	P-value
<b>18:2n-6</b>					
Blood Plasma	29.99	30.20	29.57	0.88	0.88
CE <sup>3</sup>	47.0	47.6	47.5	1.0	0.91
NEFA <sup>4</sup>	3.83	4.09	3.65	0.26	0.50
PL <sup>5</sup>	17.8	19.0	17.5	1.1	0.60
TAG <sup>6</sup>	2.99	3.81	2.87	0.37	0.18
Follicular Fluid	35.2	34.7	34.1	0.9	0.72
Milk Fat	1.508 <sup>7ab</sup>	1.656 <sup>a</sup>	1.453 <sup>b</sup>	0.052	0.036
<b>18:3n-3</b>					
Blood Plasma	5.63 <sup>a</sup>	7.08 <sup>b</sup>	5.40 <sup>a</sup>	0.19	<0.001
CE	10.98 <sup>ab</sup>	11.72 <sup>a</sup>	10.17 <sup>b</sup>	0.38	0.027
NEFA	0.848 <sup>a</sup>	1.214 <sup>b</sup>	0.777 <sup>a</sup>	0.063	0.003
PL	2.08	2.41	2.14	0.12	0.15
TAG	0.89	1.04	0.55	0.14	0.06
Follicular Fluid	5.71 <sup>a</sup>	8.34 <sup>b</sup>	5.63 <sup>a</sup>	0.25	<0.001
Milk Fat	0.371 <sup>a</sup>	0.617 <sup>b</sup>	0.353 <sup>a</sup>	0.030	<0.001
<b>20:4n-6</b>					
Blood Plasma	2.66 <sup>ab</sup>	2.49 <sup>a</sup>	3.19 <sup>b</sup>	0.16	0.017
CE	1.92 <sup>a</sup>	1.81 <sup>a</sup>	2.42 <sup>b</sup>	0.13	0.0073
NEFA	0.303	0.286	0.334	0.029	0.50
PL	2.85	2.87	3.44	0.23	0.14
TAG	0.108	0.134	0.151	0.013	0.080
Follicular Fluid	2.21 <sup>a</sup>	1.99 <sup>a</sup>	2.71 <sup>b</sup>	0.13	0.004
Milk Fat	0.116	0.105	0.121	0.006	0.14
<b>22:6n-3</b>					
Blood Plasma	0.223 <sup>a</sup>	0.333 <sup>a</sup>	0.585 <sup>b</sup>	0.041	<0.001
CE	0.055 <sup>a</sup>	0.089 <sup>ab</sup>	0.111 <sup>b</sup>	0.041	0.023
NEFA	0.084 <sup>a</sup>	0.117 <sup>a</sup>	0.243 <sup>b</sup>	0.013	0.0021
PL	0.353 <sup>a</sup>	0.536 <sup>a</sup>	0.806 <sup>b</sup>	0.029	0.0017
TAG	0.131 <sup>a</sup>	0.137 <sup>a</sup>	0.218 <sup>b</sup>	0.074	0.018
Follicular Fluid	0.150 <sup>a</sup>	0.209 <sup>a</sup>	0.617 <sup>b</sup>	0.028	<0.001
Milk Fat	0.017 <sup>a</sup>	0.0232 <sup>a</sup>	0.0426 <sup>b</sup>	0.0036	<0.001

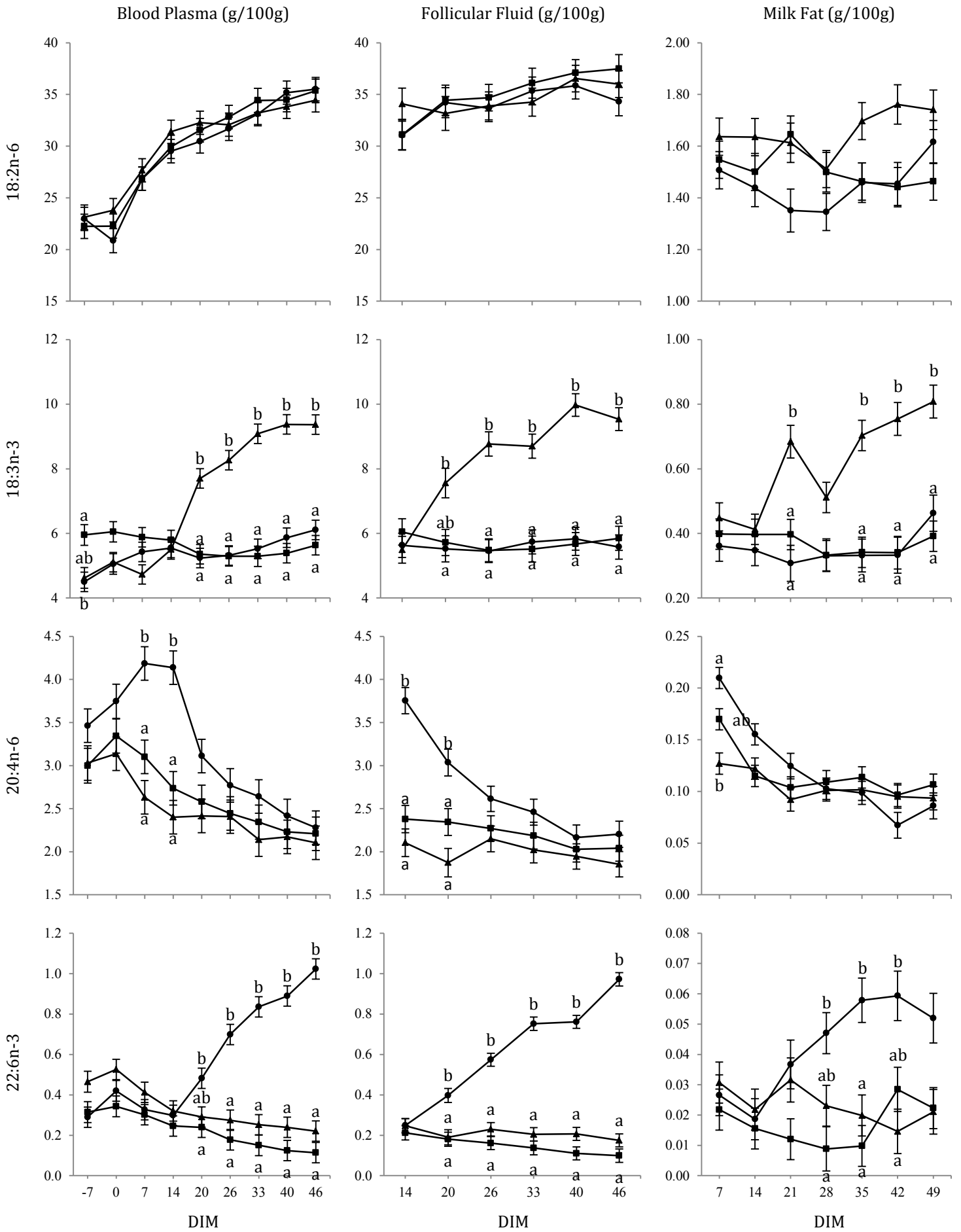
<sup>1</sup>CON = palm prills, SHORT = sequential 18:2n-6 and 18:3n-3, LONG = sequential 20:4n-6 and 22:6n-3

<sup>2</sup>Standard Error of the Mean

<sup>3</sup>Cholesterol esters; <sup>4</sup>Non esterified fatty acids; <sup>5</sup>Phospholipids; <sup>6</sup>Triacylglycerols

<sup>7</sup>Values differing in superscript significantly differ within the row

FEEDING OMEGA-6 AND OMEGA-3 FATTY ACIDS IN EARLY LACTATION



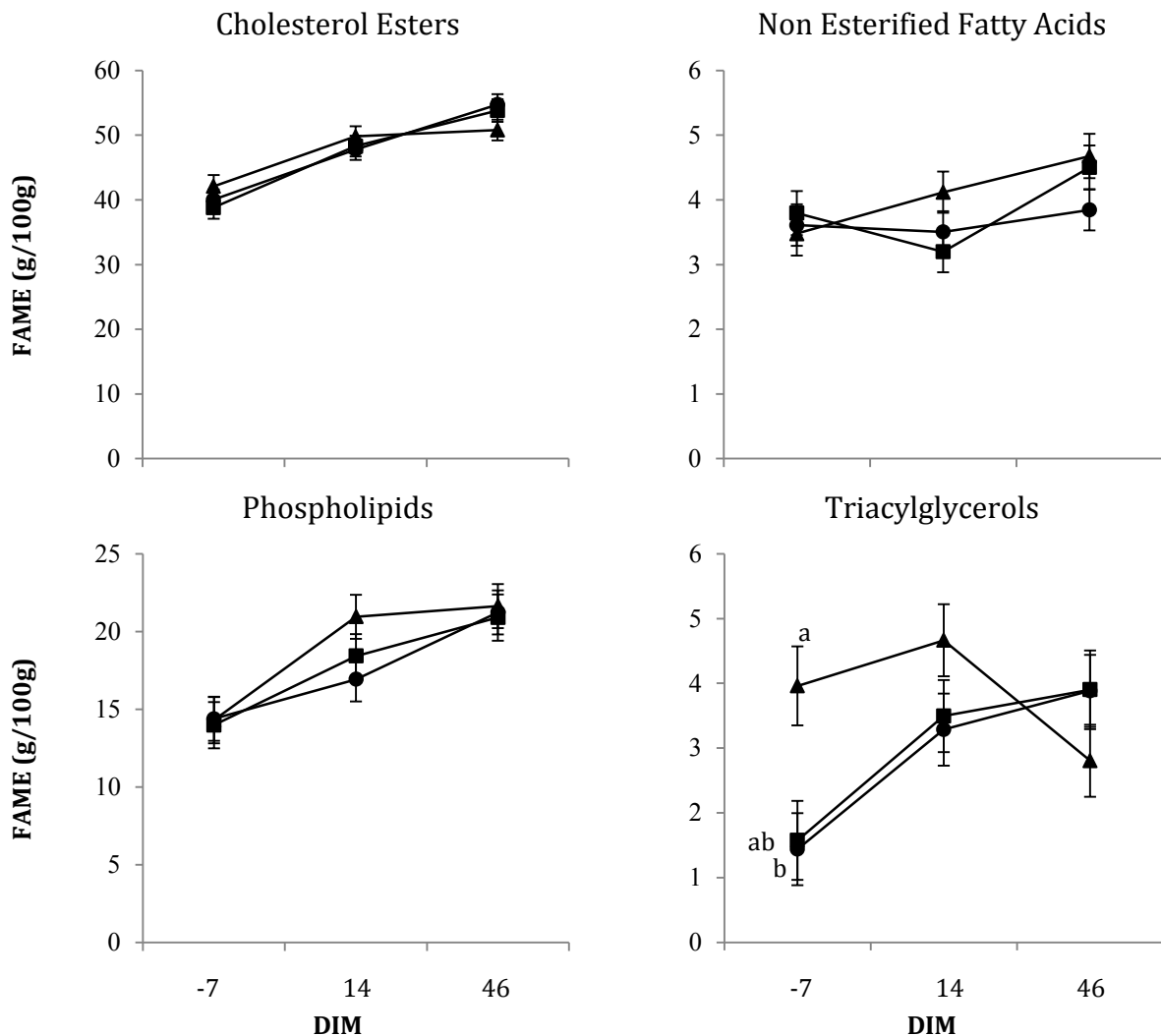
**Figure 2 (previous page).** The 18:2n-6, 18:3n-3, 20:4n-6 and 22:6n-3 content in g/100g fatty acid methyl esters (FAME) of blood plasma, follicular fluid and milk fat of cows fed palm prills (CON:■), sequential short chain n-6 and n-3 fatty acids (SHORT:▲) or sequential long chain n-6 and n-3 fatty acids (LONG:●) during the transition (n-6) and early breeding (n-3) period. Differences between diets within the same day are indicated by different labels.

## DISCUSSION

### ANIMAL PERFORMANCE

In the current study, we aimed to sequentially feed SHORT and LONG n-6 and n-3 FA to dairy cows during the transition and early breeding period. The present study shows that supplementing n-3 FA to a corn silage based diet is subsequently reflected in BP, FF and MF for both SHORT and LONG whereas n-6 FA redistribution occurred less profoundly, especially in SHORT. Sequential feeding of n-6 and n-3 FA during the transition and breeding period has been proposed as a strategy to optimize dairy cow fertility by Thatcher et al. (2010). Silvestre et al. (2011b) sequentially fed calcium salts of safflower oil during the transition period (18:2n-6) and FO during the breeding period (20:5n-3 and 22:6n-3) at 15 g/kg DM compared to a control diet with PO. Safflower oil fed during the transition period increased milk production compared to PO, without any effect of the sequential breeding diet (PO or FO). Furthermore, no effects on metabolic parameters such as NEFA, BHBA, glucose or urea were reported (Silvestre et al., 2011b). In the present experiment, DMI, milk production and content, and metabolic parameters except urea were unaffected by sequential feeding of SHORT or LONG during the experiment. More experiments on sequential feeding of SHORT are lacking, whereas 18:2n-6 fed to transition (AlZahal et al., 2008) and 18:3n-3 to early lactating dairy cows (Bilby et al., 2006c; Santschi et al., 2009) have been shown to minimally affect animal performance (milk production, metabolic parameters) except for a decreased DMI as comprehensively reviewed by Allen et al. (2000) especially in the case of 18:2n-6 (Douglas et al., 2007; Osborne et al., 2009). Beam and Butler (1998) and Chouinard et al. (1997a) suggested the need for an adaptation period when supplementing fat to transition cows which might explain the higher variability in DMI prepartum versus postpartum (SE = 0.98 vs. 0.81). Limited experiments have been conducted supplementing LONG n-3 FA, but all studies especially targeted to alter animal performance i.e. induce a milk fat depression (Franklin et al., 1999; Boeckeaert et al., 2008; Hostens et al., 2011). Furthermore, this study demonstrates the use of 20:4n-6

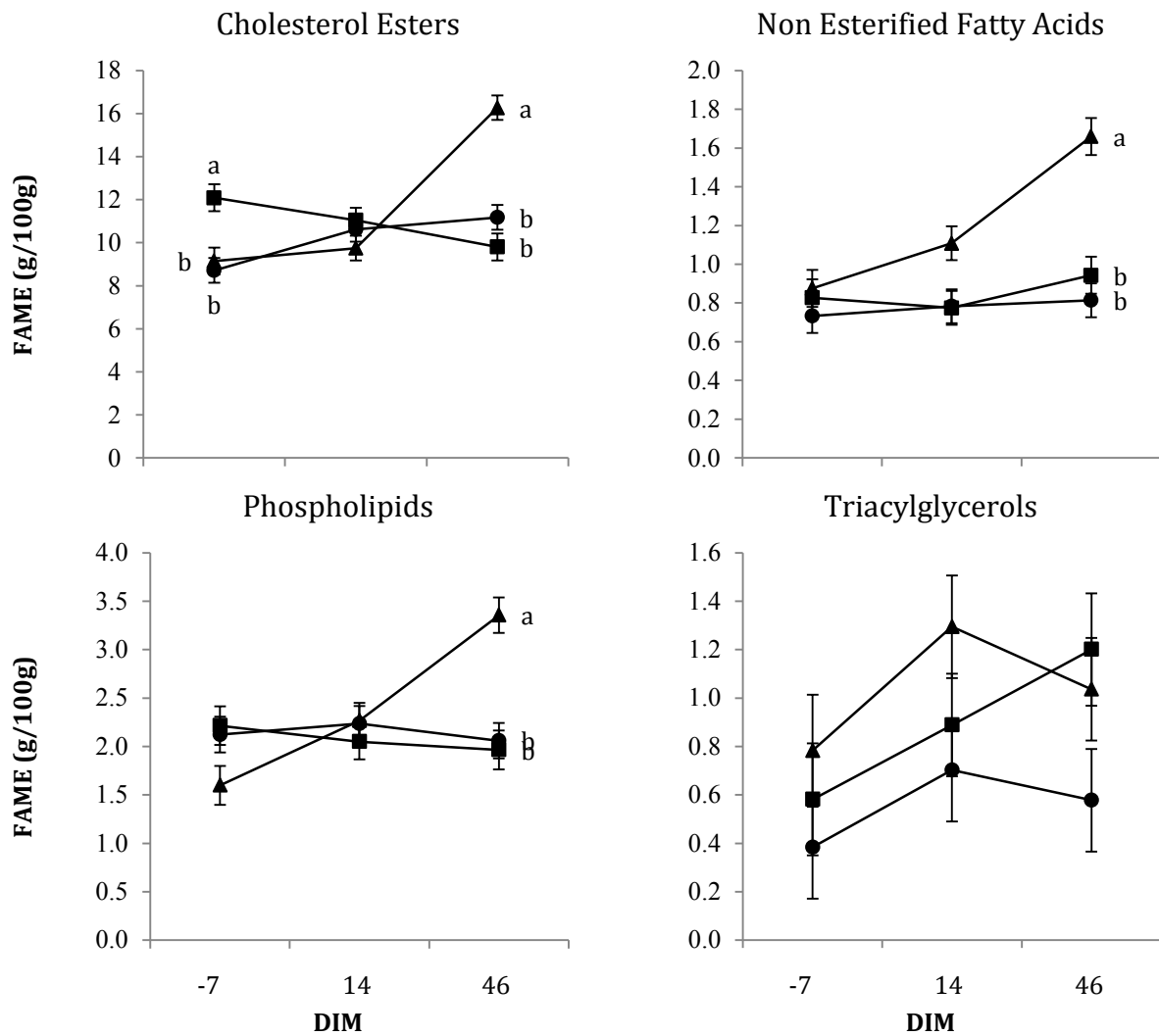
in dairy cows to increase n-6 FA supply in transition cows without affecting animal performance.



**Figure 3.** The 18:2n-6 content in g/100g fatty acid methyl esters (FAME) of separated blood plasma lipid classes of cows fed palm prills (CON:■), sequential short chain n-6 and n-3 fatty acids (SHORT:▲) or sequential long chain n-6 and n-3 fatty acids (LONG:●) during the transition (n-6) and early breeding (n-3) period. Differences between diets within the same day are indicated by different labels.

### FATTY ACID COMPOSITION OF BP, FF AND MF

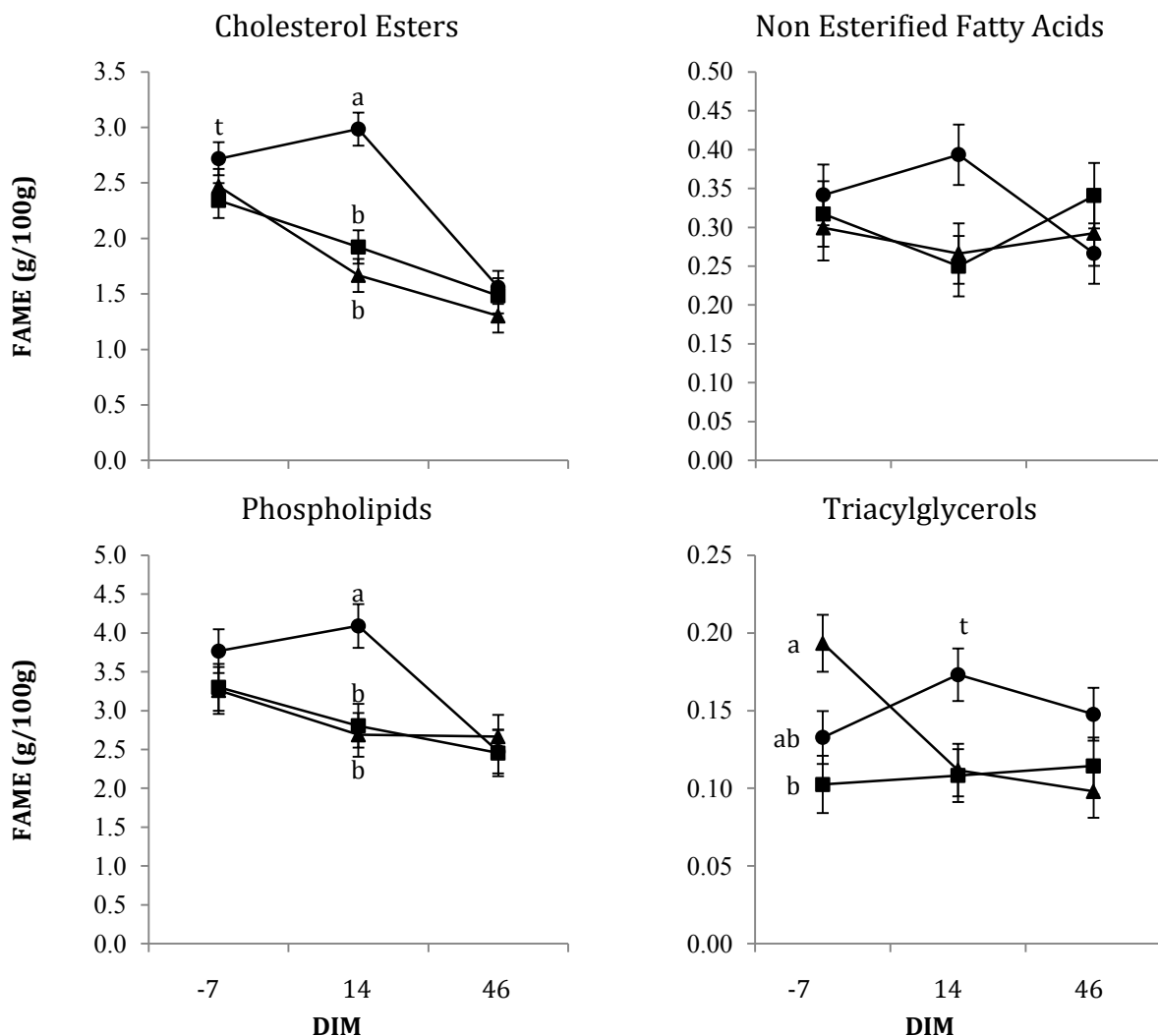
Despite the supplemental feeding of SHORT, in none of the body fluids effects on the proportion of 18:2n-6 were observed except for some increase in blood TAG. The effect of feeding soybean oil (SBO) on blood FA composition has not been investigated to a great extent, mainly at lower feed rates during the transition period. By-pass FA supplementation through abomasal infusion of 40 g/kg DM SBO to prepartum dairy cows increased BP 18:2n-6 more than two-fold when compared with infusion of saturated fat (Douglas et al., 2007) whereas doubling the dietary content of 18:2n-6



**Figure 4.** The 18:3n-3 content in g/100g fatty acid methyl esters (FAME) of separated blood plasma lipid classes of cows fed palm prills (CON:■), sequential short chain n-6 and n-3 fatty acids (SHORT:▲) or sequential long chain n-6 and n-3 fatty acids (LONG:●) during the transition (n-6) and early breeding (n-3) period. Differences between diets within the same day are indicated by different labels.

through supplementation of micronized soybeans early post partum did not alter the BP concentration of 18:2n-6 compared with calcium salts of PO (Petit and Benchaar, 2007). In established lactation, feeding 40 g/kg DM encapsulated sunflower oil rich in 18:2n-6 (Zachut et al., 2010b) or 30 g/kg DM SBO compared with canola oil (mainly 18:1 n-9, Loor and Herbein (2003) did not change total BP 18:2n-6 content. Similar to our findings, Loor and Herbein (2003) did find an increase in the 18:2n-6 TAG fraction of blood plasma. Generally, 18:2n-6 is incorporated via lecithin-cholesterol acyl transferase (LCAT) in the PL and CE fractions (Noble et al., 1977) but will be incorporated in the TAG fraction at higher dietary 18:2n-6 levels e.g. when feeding corn silage based diets

(Christie, 1979). The latter might explain why no difference in the PL fraction could be detected in our study in contrast to Looor and Herbein (2003) feeding an alfalfa based diet low in 18:2n-6. The overflow to TAG also seems to hold for 20:4n-6 when feeding SHORT, suggesting elongation from 18:2n-6. Feeding supplemental n-6 FA decreased the 18:3n-3 content in blood, mainly in the CE fraction in SHORT before parturition. Most likely, this originates from the increased concentration of 18:2n-6 compared to 18:3n-3 rather than from a difference in specificity for the acyltransferase system (Noble et al., 1972).



**Figure 5.** The 20:4n-6 content in g/100g fatty acid methyl esters (FAME) of separated blood plasma lipid classes of cows fed palm prills (CON:■), sequential short chain n-6 and n-3 fatty acids (SHORT:▲) or sequential long chain n-6 and n-3 fatty acids (LONG:●) during the transition (n-6) and early breeding (n-3) period. Differences between diets within the same day are indicated by different labels.

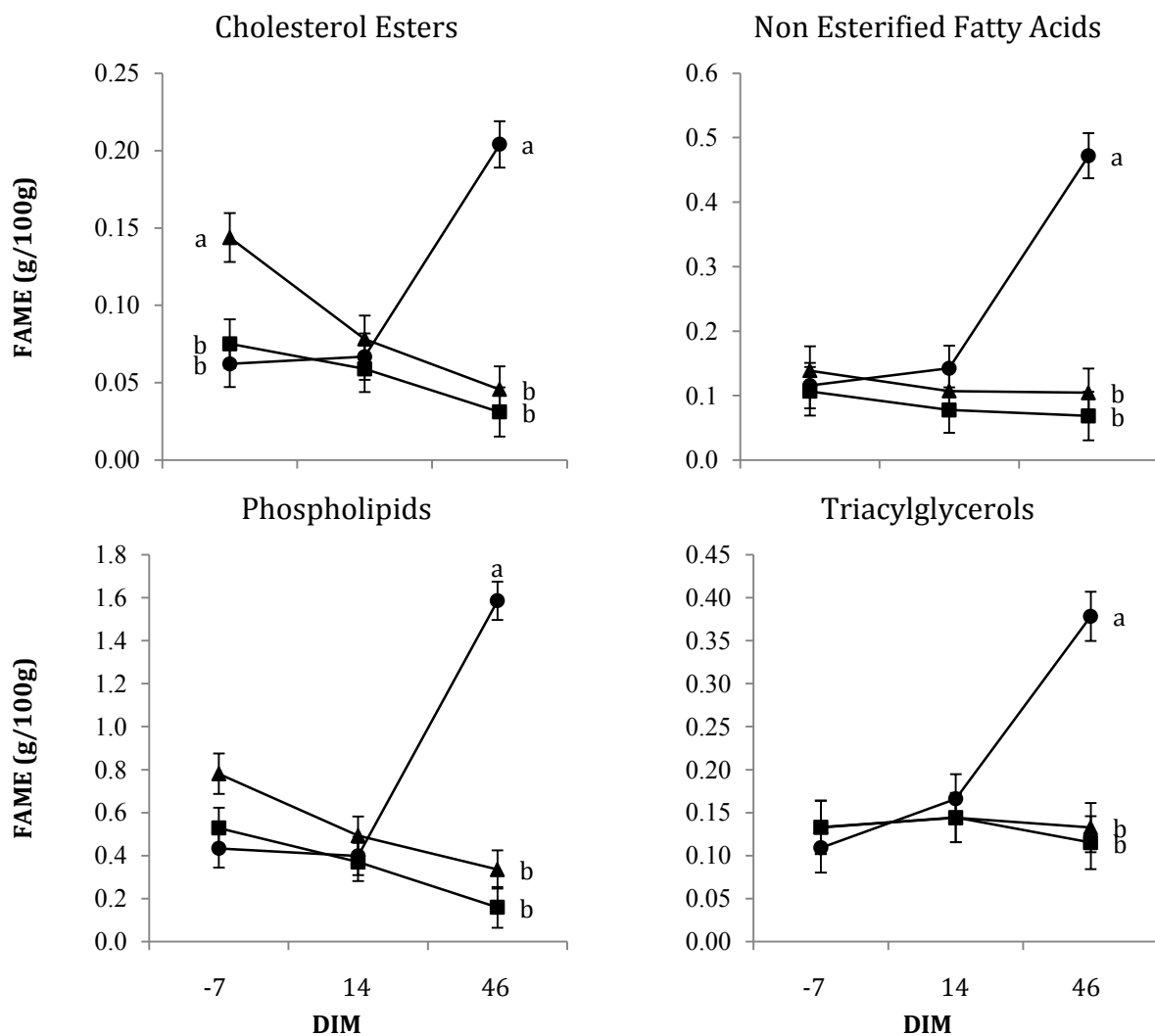
Moreover, 22:6n-3 increased in CE on d-7 when feeding SHORT which could be due to the excessive flow of n-6 and n-3 FA to the prostaglandin metabolism during the



prepartum period. At the onset of lactation, most likely due to formation of eicosanoid products of the prostaglandin series (Guilbault et al., 1984), the animals in the SHORT and CON group similarly decrease in 20:4n-6 in all blood fractions which was subsequently mirrored in FF and MF during the first 2 weeks of lactation. The response lag up to 2 weeks of 20:4n-6 supplementation to appear in BP for LONG has been reported in humans for 20:5n-3 as well (Cartwright et al., 1985; Sanders and Hinds, 1992). Overall BP 20:4n-6 remained higher compared with SHORT and CON, but despite continuous dietary supplementation, gradually decreased from week 1 to 2 in lactation.

In contrast, switching to n-3 at 14 DIM, immediately increased 18:3n-3 concentration two-fold in BP, FF and MF for SHORT at the end of the supplementation period and over a three-fold for the 22:6n-3 in BP, FF and MF for LONG. Peripartur supplementation up to 92 g/kg DM of extruded flaxseed increased 5.3-fold in BP, 5.1-fold in MF (Zachut et al., 2010a) and 8.1-fold in FF collected at 63 and 96 DIM (Zachut et al., 2011). Flaxseed, after various treatments (Mustafa et al., 2003; Petit et al., 2004; Gonthier et al., 2005) provoked similar changes in BP as observed in our study. Also, in more established lactation (114 DIM), similar results were obtained for BP and FF when feeding extruded flaxseed at dietary inclusion rate of 38 g/kg DM, but increases in MF 18:3n-3 were smaller (6.6-fold increase; Zachut et al., 2010b). From our results it can be concluded that feeding smaller amounts of 18:3n-3 is primarily reflected in the 18:3n-3 content in TAG. As the latter fraction is a major source of milk FA (Bauman and Griinari, 2003), this might explain the faster response in 18:3n-3 MF before changes are observed in BP and FF when feeding smaller amounts of flaxseed (Zachut et al., 2010b). In contrast, a clear TAG enrichment seems to occur for 22:6n-3 when fed at lower rates, possibly explaining the 3-fold increase in MF compared with a 2-fold for 18:3n-3. Furthermore, Offer et al. (2001) proved this enrichment to primarily occur in CE, NEFA and PL fraction in high-density lipoproteins (HDL), and TAG and PL in low-density lipoproteins (LDL) but not the very low-density lipoproteins (VLDL). Mammary lipoprotein lipase primarily affects the TAG fraction of chylomicrons and VLDL to obtain FA for MF synthesis (Palmquist and Mattos, 1978; Moore and Christie, 1979; Barber et al., 1997) explaining the low concentration of 22:6n-3 in MF compared to BP and FF. Compared to earlier work, we fed less than 20 g of 22:6n-3 as opposed to studies supplementing over 40 g per day of 22:6n-3 with concomitant 10 times higher 22:6n-3 concentration in MF (Boeckeaert et al., 2008; Hostens et al., 2011). Limited research has

focused on the effect of dietary 22:6n-3 on BP or FF composition but comparability to FO studies, at least for MF, has been suggested (AbuGhazaleh et al., 2009). In a study observing the FA composition in FF collected in the abattoir from crossbred heifers, higher 20:5n-3 and 22:6n-3 was found when supplementing FO at 2.08 and 41.5 g/kg DM of the diet (Childs et al., 2008). Zeron et al. (2002) found an enrichment of 18:2n-6 and 22:6n-3 in the PL fraction of FF when feeding FO to ewes. Whereas CE and PL in HDL seem to be a very poor substrate for the mammary lipoprotein lipase, the HDL fraction is the largest lipoprotein class in BP (Bauchart, 1993) and FF (Brantmeier et al., 1987; Wehrman et al., 1991), therefore allowing substantial enrichment in 22:6n-3 as proven the current and aforementioned studies.



**Figure 6.** The 22:6n-3 content in g/100g fatty acid methyl esters (FAME) of separated blood plasma lipid classes of cows fed palm prills (CON:■), sequential short chain n-6 and n-3 fatty acids (SHORT:▲) or sequential long chain n-6 and n-3 fatty acids (LONG:●) during the transition (n-6) and early breeding (n-3) period. Differences between diets within the same day are indicated by different labels.

**IMPLICATIONS FOR FERTILITY**

Silvestre et al. (2011a; 2011b) sequentially fed safflower oil during the transition period and FO during the breeding period compared to an entire pre- and postpartum period of PO supplementation. They found a reduced pregnancy loss at first service and increased the pregnancy rate at the second AI but not at first AI. Next to indirect effects by influencing the prostaglandin metabolism (Petit et al., 2002; Robinson et al., 2002), follicular growth (Bilby et al., 2006a; Mendoza et al., 2011), FA may directly affect oocyte quality through alterations of the oocyte lipids (Sturmey et al., 2009). In the present trial, feeding 20:4n-6 but not 18:2n-6 during the transition period was reflected in the FF, probably due to a high intake in 18:2n-6 from the corn silage based diet. Supplementation of n-6 might be unfavourable (Zachut et al., 2010b) as high concentrations of n-6 in FF in which the oocyte matures can inhibit resumption of meiosis at the germinal vesicle stage (Marei et al., 2010), subsequently deteriorating oocyte maturation. Interestingly, positive effects on oocyte maturation have been shown for 20:4n-6 (Marques et al., 2007). Furthermore, our results demonstrate that supplementation of 18:3n-3 and 22:6n-3 in early lactation is reflected in the FF. *In vitro* supplementation of 18:3n-3 FA improved oocyte maturation (Marei et al., 2009), which has been related to a higher n-3 concentration in the PL fraction of cumulus cells, plasma and red blood cells *in vivo* (Zeron et al., 2002). In contrast, Fouladi-Nashta et al. (2009) concluded that the ovary is able to buffer against fluctuations in plasma n-6 and n-3 FA, resulting in only modest effects on their developmental potential (Ponter et al., 2012). To the best of our knowledge, no experiments on the effect of 22:6n-3 on *in vitro* maturation and developmental capacity are available at the moment. Recently, the FF 22:6n-3 concentration has been suggested as one of the major explanations for a higher fertility in heifers as compared with cows (Bender et al., 2010) placing 22:6n-3 supplementation in order to enrich the follicular microenvironment into new perspectives.

## CONCLUSION

The present study shows that supplementing n-6 FA to a corn silage based diet during the transition period is reflected in BP, FF and MF for 20:4n-6 but not 18:2n-6 (only MF). Subsequent switching to 18:3n-3 and 22:6n-3 is reflected in BP, FF and MF for both FA. Furthermore, the follicular environment and mammary gland seem to respond in a different way to supplemental FA, most probably due to discrimination against specific FA in the different lipid classes for which body fluids show varying affinity. Further research should clarify the effects of 20:4n-6 and 22:6n-3 supplementation to dairy cows on oocyte maturation and embryo development.

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# CHAPTER 6





# MILK FAT SATURATION AND REPRODUCTIVE PERFORMANCE IN DAIRY CATTLE

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## SUMMARY

Unsaturated fatty acids (UFA) cannot be synthesized by mammalian cells due to a lack of desaturase enzymes to incorporate a double bond beyond the ninth carbon in the acyl chain. Combined with their limited supply to the small intestines, UFA have been proposed as nutraceuticals to ameliorate dairy cow fertility. However, field studies based on a large number of animals are lacking on this subject.

Therefore the aim of the present study was to analyse a large dataset containing individual cow fertility records from dairy herds and link fertility key-performance-indicators like conception rate to first insemination (CRFI), days in milk to first insemination (DIMFI) and days in milk to conception (DIMCONC), to the level of UFA in bulk tank samples, the latter being a proxy for the dietary fatty acid profile on these herds.

Within the two year study period, information from 15,055 lactations and 35,433 bulk tank milk samples was collected on 90 herds. The multilevel logistic regression model used, revealed a decreased CRFI on herds with a higher bulk tank UFA level. The decrease in CRFI was larger for higher producing herds. Increased bulk tank UFA was furthermore associated with higher DIMFI which, together with the lower CRFI, subsequently increased DIMCONC. Interestingly, higher variability in UFA, expressed by an increased coefficient of variation, was associated with an increased CRFI and decreased DIMFI and DIMCONC.

In conclusion, the present study demonstrates that increasing the UFA content of milk should not be a goal as such when supplementing UFA to dairy cows as higher bulk tank UFA are associated with worsened fertility results.



## INTRODUCTION

Continuous research in dairy cattle has focused on the link between nutrition and the on-going decline in high producing dairy cow fertility (Thatcher et al., 2011). Specific long chain fatty acids (LCFA), and not fat per se, have gained general recognition as being essential for important mammalian reproductive functions including effects on membrane fluidity, intracellular cell-signalling cascades and susceptibility for oxidative injury (Staples and Thatcher, 2005). Typically, dairy cattle diets contain approximately 2.0% of LCFA, predominantly polyunsaturated (Staples et al., 1998). The main FA in most seed lipids is 18:2n-6 (linoleic acid) whereas 18:3n-3 (linolenic acid) predominates in most forage lipids (Palmquist and Jenkins, 1980). These FA cannot be synthesised by the mammalian cells due to the lack of desaturase enzymes to incorporate a double bond beyond the ninth carbon in the acyl chain (Gurr et al., 2002). Combined with the limited supply of unsaturated FA (UFA) to the small intestine associated with the extensive biohydrogenation in the rumen (Jenkins et al., 2008), UFA have been proposed by researchers as nutraceuticals in the bovine to ameliorate dairy cow fertility (Santos et al., 2008; Silvestre et al., 2011; Thatcher et al., 2011).

Experiments focusing on the direct effect of specific FA on oocyte maturation and embryonic development have opened new perspectives to the knowledge of FA feeding. More specifically, Leroy et al. (2005) and van Hoeck et al., (2011) showed detrimental effects of saturated FA during oocyte maturation on subsequent oocyte and embryonic development as compared to their unsaturated counterparts in an *in vitro* maturation model. Fatty acid supplementation during *in vitro* bovine oocyte maturation showed contrasting results as 18:2n-6 and 18:3n-3 respectively hampered (Marei et al., 2010) and enhanced (Marei et al., 2009) the nuclear maturation rate and subsequent developmental potential of oocytes. Furthermore, post-thawing *in vitro* embryo survival was improved by supplementation of 20:4n-6 or 20:5n-3 during oocyte maturation (Marques et al., 2007). Al Darwich et al. (2010) observed a tendency for a lower blastocyst yield with increasing 18:3n-3 addition during *in vitro* embryo culture and a detrimental effect of embryo culture with the highest dose of 22:6n-3 and 18:2 *trans*-10, *cis*-12 on *in vitro* survival after vitrification and warming. The *in vivo* observation of a more UFA profile of follicular fluid, oocytes and granulosa cells in winter which might explain seasonal differences in dairy cow fertility (Zeron et al., 2001), has opened the

quest for the optimal breeding FA composition of dairy rations. This has resulted in experiments illustrating improved (Cerri et al., 2009; Fouladi-Nashta et al., 2007; Zachut et al., 2010), but also unaltered (Bilby et al., 2006; Fouladi-Nashta et al., 2009) or even hampered (Sklan et al., 1994) fertility when feeding UFA to dairy cows.

Furthermore, feeding 22:6n-3 increased the blood lipid peroxidation in transition cows, elevating the animals to a higher level of susceptibility to oxidative stress (OS) (Wullepit et al., 2012) which has been linked to embryonic death in the bovine (Fujitani et al., 1997; Olson and Seidel, 2000; Rooke et al., 2012). The latter might explain *in vitro* observations of detrimental effects at high doses of UFA and may confound *in vivo* experiments supplementing UFA to increase oocyte and embryo quality in dairy cows. A multi-study analysis of de Veth et al. (2009) documented a positive effect of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) over 5 studies on days in milk to conception (DIMCONC) in dairy cows but this kind of analysis has not been conducted for other UFA. Therefore, the hypothesis of the current study was that high milk UFA are associated with better fertility parameters in dairy cows. The objective of this study was to analyse a large dataset containing individual fertility records from dairy herds and link fertility key-performance-indicators (KPI) to the level of UFA in bulk tank samples as a proxy for the dietary fatty acid profile on that herd (Lock and Bauman, 2004; Woods and Fearon, 2009).

## MATERIALS AND METHOD

### RECORD COLLECTION AND DATA HANDLING

Herd level data was collected from 90 dairy herds in Belgium via an automated herd record collection system (Dairydatawarehouse, Assen, The Netherlands) in 2008 and 2009. Herds without official milk recording were excluded from the analysis. Herd level data such as the number of calvings within each year (HERDSIZE) and the average milk production within 305 d (M305) per year were collected. From these 90 herds, bulk milk tank information was collected via the official Milk Control Centre of Flanders (Melk Controle Centrum Vlaanderen, Lier, Belgium). For this part of Belgium, typical bulk tank milk samples are taken every 2 to 3 days depending on the size of the herd. Samples were analysed for milk protein (PROT), fat (FAT) and the percentage of UFA. For the study period, cow level information from 15,055 lactations from the

participating herds was collected and stored via the same automated herd record collection system. Additionally and for each lactation, specific information was collected: whether the cow was born on the herd, the calving date, days in milk to first insemination (DIMFI), DIMCONC, breed (BREED: 100% Holstein Friesian (HF), 50% HF, Other), days in milk to culling (see further), parity (PAR: 1, 2, >2), lactation M305 production. Lactations from cows were excluded when one of the following conditions were present: cows that were not born on the herd (incorrect lactation numbers), cows that did not have an official M305, cows without information about the breed (improper breed categorization), DIMFI before 30 DIM, culled cows (missing subsequent calving date). For the remaining 9,362 lactations, a conception was defined as an insemination followed by a realized gestation period between 267 to 295 days (no missing subsequent calving date). The final dataset was constructed by joining herd and cow level information. The information about UFA (%) was summarized by calculating the average per year (average of  $205 \pm 80$  measurements per herd over entire study period). Furthermore, the yearly coefficient of variation (CV) was calculated as a parameter for herd level variance in UFA (CVUFA). The relative milk production for each individual cow (R305) was expressed as the proportional M305 production compared to the average of the herd blocked for parity (e.g. M305 of a first parity cow is expressed relatively to the average M305 production of all first parity cows within that herd).

## **CHEMICAL ANALYSIS**

The milk analysis for PROT, FAT and UFA (mono- and polyunsaturated FA) were carried out by means of a Fourier transform infrared spectrometry (Milkoscan FT6000, Foss Electric, Hillerød, Denmark; Soyeurt et al., 2011) at the Milk Control Centre of Flanders (MCC Vlaanderen, Lier, Belgium).

## **STATISTICAL ANALYSIS**

All statistical analyses were performed using SAS software (Release 9.2, SAS Institute Inc., Cary, NC). Descriptive statistics mean, CV, SD, median and quartiles were done using the MEANS and UNIVARIATE procedure on herd and cow level variables and represented in Table 1. The Pearson correlation was calculated between continuous variables with the CORR procedure. The distribution of all dependent and independent variables was checked to approximate the Gaussian distribution. DIMFI and DIMCONC



could not be approximated by the Gaussian distribution and were therefore transformed before analysis using the LOG10 function. Days to first insemination was used as a predictor in the conception rate to first insemination (CRFI) model and was categorized into  $\leq 61$ , 61 to 81, 82 to 102, 103 to 123, and  $\geq 124$  DIM. The predictor HERDSIZE was categorised using quartiles as cut-off points in all models. At first, multi-collinearity ( $r > 0.6$ ) was checked by regressing each predictor on all other predictors. Separate models were built using the GLIMMIX procedure (method=Laplace) of SAS for CRFI, DIMFI and DIMCONC. In the case of the CRFI a binomial distribution was assumed with the logit link. The models were built by initially including a random intercept for cow nested within the herd, possible confounders based on earlier research (BREED, PAR, YEAR), the cow level predictors R305 and herd level predictors HERDSIZE, UFA, CVUFA and M305. In the CRFI model, the categorised DIMFI was considered as confounder as well. Effects were retained at a type-3 significance level of 0.05, or when variables confounded other effects by significantly altering the remaining parameter estimates. Relevant first-order interactions were included in the model and retained at type-3 significance level lower than 0.02. This alpha level was chosen to prevent falsely significant associations due to the large number of possible interactions. The final CRFI model was found acceptable according to the Pearson chi-square statistic divided by its degrees of freedom being close to one (0.98), not indicating any lack of fit. The residual plots of the final DIMFI and DIMCONC models were graphically found to be normally distributed and did not show any abnormalities. Results from the DIMFI and DIMCONC model were back-transformed and reported with 95% confidence limits.

**Table 1.** Descriptive statistics (number of observations, mean, SD, median and inter quartile range) of the herd and cow level variables

Herd level	n	Mean $\pm$ SD	Median	IQR <sup>1</sup>
Herd size, n	90	96.6 $\pm$ 49.6	83.5	65 - 118
M305 <sup>2</sup> , kg	90	8706 $\pm$ 821	8657	8281 - 9350
Fat, g/kg	90	42.2 $\pm$ 1.84	42.2	41.1 - 43.2
Protein, g/kg	90	35.0 $\pm$ 0.80	35.0	34.5 - 35.6
Bulk tank UFA <sup>3</sup> , %	90	30.8 $\pm$ 1.47	30.6	30.0 - 31.5
Bulk tank CVUFA <sup>4</sup> , %	90	6.89 $\pm$ 2.11	6.65	5.34 - 8.20
<b>Cow level</b>				
DIM to first AI, d	8247	89.6 $\pm$ 43.9	79.0	61.0 - 105.0
DIM to conception, d	5895	135.7 $\pm$ 78.9	114.0	79.0 - 171.0
M305, kg	8247	9096 $\pm$ 1795	9060	7843 - 10277

<sup>1</sup> Inter quartile range

<sup>3</sup> Unsaturated FA

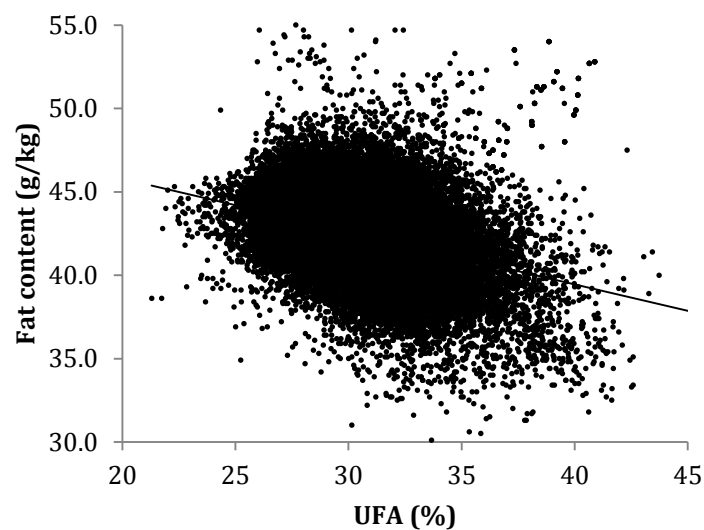
<sup>2</sup> Milkproduction within 305d

<sup>4</sup> Coefficient of variation of UFA

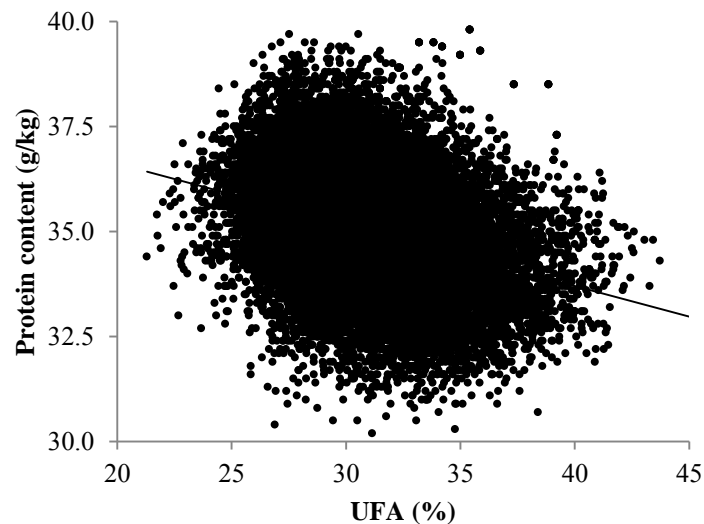
## RESULTS

### DESCRIPTIVE STATISTICS AND BULK TANK MILK SAMPLES

During the study period, 35,433 bulk tank milk samples were collected and analyzed from the participating herds for FAT, PROT and UFA. The overall fat and protein content was  $42.3 \pm 2.58$  g/kg and  $35.0 \pm 1.25$  g/kg, respectively. The average UFA percentage was  $30.8 \pm 1.47$  %. This percentage was negatively correlated with both FAT ( $r = -0.32$ ,  $P < 0.0001$ , Figure 1) and PROT ( $r = -0.31$ ,  $P < 0.0001$ , Figure 2).



**Figure 1.** Scatterplot presenting the correlation between the percentage of unsaturated fatty acids (UFA) and the fat content (g/kg) in bulk tank milk samples ( $n=35,433$ ) of Belgian herds ( $n=90$ ) in 2008 and 2009.



**Figure 2.** Scatterplot presenting the correlation between the percentage of unsaturated fatty acids (UFA) and the protein content (g/kg) in bulk tank milk samples ( $n=35,433$ ) of Belgian herds ( $n=90$ ) in 2008 and 2009.

**EFFECT OF UFA AND CVUFA ON CRFI**

Parameter estimates and odds ratios (OR) of the final multivariable logistic regression model on CRFI are provided in Table 2. The variance partition coefficient (VPC), representing the proportion of the total variation in the conception rate which resides at the herd level, was estimated at 12.3%, calculated using the latent-variable method (Goldstein et al., 2002; Snijders and Bosker, 1999).

**Table 2.** Final multivariable logistic regression model for conception rate to first insemination, based on 8,247 first inseminations on 90 Belgian herds in 2008 and 2009 using herd as random effect in the model ( $\lambda = 0.46$ ,  $SE = 0.1$ ,  $P < 0.0001$ )

Covariate	Class	OR <sup>1</sup>	95% CI of OR	P-value
Cow level				
Parity	1	1.00 <sup>2</sup>		<0.0001
	2	0.85	0.75-0.98	
	>2	0.73	0.65-0.83	
DIM	≤60 d	1.00		0.0003
	61-81 d	1.21	1.05-1.39	
	82 – 102 d	1.27	1.09-1.49	
	103 – 123 d	1.11	0.92-1.34	
	≥124 d	1.45	1.22-1.71	
Relative M305 <sup>3</sup> , %	Cont. <sup>4</sup>	0.98	0.98-0.99	<0.0001
Herd level				
M305, x 1,000 kg	Cont.	0.87	0.75-1.01	0.0021
Bulk tank UFA <sup>5</sup> , %	Cont.	0.85	0.79-0.92	0.0042
Bulk tank CVUFA <sup>6</sup> , %	Cont.	1.07	1.02-1.12	0.0036
Cow level interactions				
Parity x DIM <sup>7</sup>				0.0045
Herd level interactions				
M305 x Bulk tank UFA				0.0014

<sup>1</sup> Odds ratio

<sup>2</sup> Reference class of categorized variable

<sup>3</sup> Milkproduction within 305d

<sup>4</sup> Continuous variable without classes

<sup>5</sup> Unsaturated Fatty Acids

<sup>6</sup> Coefficient of variation of UFA

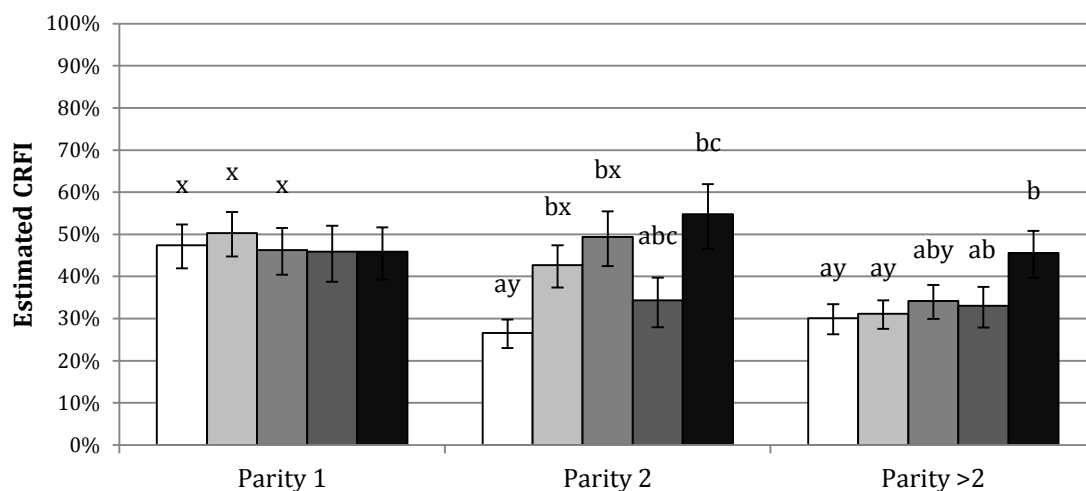
<sup>7</sup> Categories not shown

The CRFI was estimated at 47.1%, 40.2% and 34.4% for parity 1, parity 2 and older parity cows, respectively ( $P < 0.0001$ ). The main variables of interest, UFA and CVUFA, decreased (OR: 0.85,  $P = 0.0042$ ) and increased (OR: 1.07,  $P = 0.0036$ ), respectively, the odds to conceive to first AI (Table 2). A one percent increase in UFA consequently decreased CRFI to 36.9% for first parity, to 34.2% for second parity and

29.2% for older parity cows. A one percent increase in CVUFA increased CRFI to 50.4% for first parity, to 43.0% for second parity and 36.8% for older parity cows. Furthermore, the interaction between M305 and UFA showed a decreased odds to conceive with increasing UFA especially in the higher producing herds ( $P = 0.0014$ ). The IQR range of M305 was used to demonstrate this interaction in low (25<sup>th</sup> quartile M305 = 8,281 kg milk, Table 1) and high (75<sup>th</sup> quartile M305 = 9,350 kg milk) yielding herds. For example, in heifers, a one percent increase in UFA resulted in 3.8% vs. 9.8% lower CRFI in low and high yielding herds, respectively. For older parity cows, a one percent increase in UFA resulted in a 2.8% vs. 7.2% lower CRFI on low and high yielding herds, respectively. The DIMFI significantly affected the CRFI ( $P = 0.0003$ ), and the interaction with parity is represented in Figure 3 ( $P = 0.0045$ ).

### EFFECT OF UFA AND CVUFA ON DIMFI

The intercept variance was estimated at  $0.0041 \pm 0.00072$  ( $P < 0.0001$ ) which resulted in an intraclass correlation (ICC) of 12.6%. The largest proportion of the total variance remains at the cow level (83.4%). Both main variables of interest, UFA ( $P = 0.009$ ) and CVUFA ( $P = 0.0074$ ) increased DIMFI, but the negative coefficient for the interaction between UFA and CVUFA complicated the interpretation of their effect. Therefore, the interaction was illustrated in Figure 4 showing the largest DIMFI for



**Figure 3.** The effect of the interaction between parity (1,2 or >2) and DIM to first AI ( $\leq 60$  d (white bars), 61 to 81 d (light gray bars), 82 to 102 d (gray bars), 103 to 123 d (dark gray bars), and  $\geq 124$  d (black bars)) in the final multivariable logistic regression model for conception rate to first insemination (CRFI). Significant differences at  $P < 0.05$  within parity between DIM to first AI are indicated by abc, whereas differences within DIM to first AI between parities are indicated by xyz.

**Table 3.** Final multivariable linear regression model for the log 10 transformed days to first insemination, based on 8,247 first inseminations on 90 Belgian herds in 2008 and 2009 using herd as random effect in the model ( $\lambda = 0.0041$ , SE = 0.00072,  $P < 0.0001$ )

Covariate	Class	$\beta^1$	SE	P-value
<b>Cow level</b>				
Parity	1	-0.024	0.0045	<0.0001
	2	-0.017	0.0048	
	>2	Ref. <sup>2</sup>		
Relative M305 <sup>3</sup> , %	Cont. <sup>4</sup>	0.00087	0.000183	<0.0001
<b>Herd level</b>				
Herd size <sup>5</sup> , n/year	≤65	0.0072	0.01449	0.0479
	66-83	-0.0190	0.01328	
	84-118	0.0023	0.00982	
	≥119	Ref.		
Bulk tank UFA <sup>6</sup> , %	Cont.	0.027	0.0082	0.0009
Bulk tank CVUFA <sup>7</sup> , %	Cont.	0.10	0.0377	0.0074
<b>Cow level interactions</b>				
Parity x Relative M305	1	0.000118	0.000272	0.016
	2	0.000804	0.000292	
	>2	Ref.		
<b>Herd level interactions</b>				
Bulk tank UFA x CV UFA	Cont.	-0.0033	0.00121	0.0056

<sup>1</sup> Estimated coefficient ( $\beta$ ), SE for the coefficient, and significance level

<sup>2</sup> Reference class of categorized variable

<sup>3</sup> Milkproduction within 305d

<sup>4</sup> Continuous variable without classes

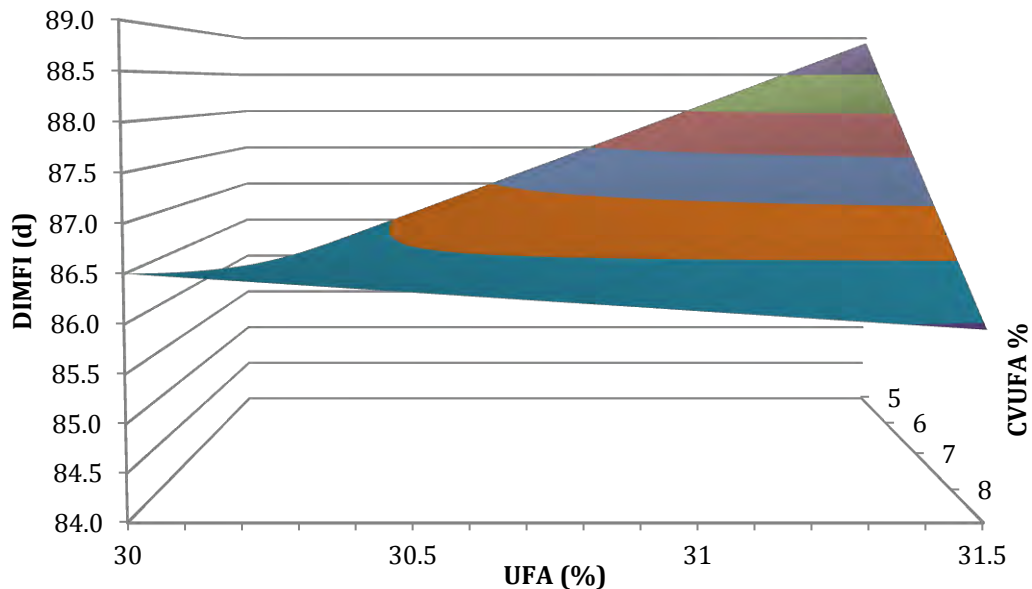
<sup>5</sup> Herd size expressed as number of calvings per year

<sup>6</sup> Unsaturated Fatty Acids

<sup>7</sup> Coefficient of variation of UFA

herds with a high UFA and low CVUFA ( $P = 0.0056$ ). Overall, the effect remained smaller than 5d for the inter-quartile range (IQR) of both variables and their interaction. The herd level predictor HERDSIZE remained in the final multivariable linear regression model (Table 3,  $P = 0.0479$ ). Back-transformed confidence limits for DIMFI were 82-90 d for ≤65 cows, 77-85 d for 66-83 cows, 81-89 d for 84-118 cows and 80-89 d for ≥119 cow dairies and differed between the 66-83 cows and smaller (≤65 cows) and larger HERDSIZE (84-118 cows) classes. At the cow level, DIMFI differed between all parities with 79-85 d, 81-87 d and 84-89 d for parity 1, 2 and >2 respectively ( $P < 0.0001$ ). High producing cows had a longer DIMFI compared to their lower producing herdmates ( $P <$

0.0001). The latter effect was additionally enlarged for the lower parity cows as a result of the significant interaction with parity ( $P = 0.016$ ).



**Figure 4** The effect of the percentage of unsaturated fatty acids in the bulk tank (UFA), its coefficient of variation (CVUFA), and the interaction between both on days in milk to first insemination (DIMFI) based on 8247 first inseminations on 90 Belgian herds in 2008 and 2009.

### EFFECT OF UFA AND CVUFA ON DIMCONC

Compared to the DIMFI, a smaller proportion of the total model variance of the model predicting DIMCONC, remained at the herd level (ICC = 4.9%), leaving the largest proportion of variance at the cow level (95.1%,  $P < 0.0001$ ). The main variables of interest, UFA ( $P = 0.0057$ ) and CVUFA ( $P = 0.0102$ ) increased DIMCONC. As in the DIMFI model, a negative coefficient for the interaction between UFA and CVUFA ( $P = 0.0083$ ) was illustrated (Figure 5), showing the largest DIMCONC for herds with a high UFA and low CVUFA. Even though a minor part of the variance resided at the herd level (see higher), the herd level predictor HERDSIZE remained in the final multivariable linear regression (Table 4,  $P = 0.001$ ). Back-transformed confidence limits for DIMCONC were 119-134 d for  $\leq 65$  cows, 117-130 d for 66-83 cows, 113-124 d for 84-118 cows and 105-116 d for  $\geq 119$  cow dairies. The largest HERDSIZE class had lower DIMCONC compared to all other size classes. At the cow level, DIMCONC was lower for the heifers (110-119 d)

compared with second (115-125 d) and older parity cows (119-128 d,  $P < 0.0001$ ). As in the DIMFI model, the highest producing cows in the herd had a longer DIMCONC compared to their lower producing herdmates ( $P < 0.0001$ ). Pure Holstein cows had a 5 d larger DIMCONC (119-126d) compared to crossbred Holsteins (112-121 d,  $P = 0.0042$ ). Other breeds showed an intermediate DIMCONC (111-129 d).

**Table 4.** Final multivariable linear regression model of log10 transformed days to conception, based on 5,839 cows that conceived on 90 Belgian herds in 2008 and 2009 using herd as random effect in the model ( $\lambda = 0.0024$ ,  $SE = 0.00057$ ,  $P < 0.0001$ )

Covariate	Class	$\beta^1$	SE	P-value
Cow level				
Parity	1	-0.032	0.0067	<0.0001
	2	-0.011	0.0074	
	>2	Ref. <sup>2</sup>		
Breed	100% HF <sup>3</sup>	0.0094	0.01763	0.0166
	50% HF	-0.0114	0.01796	
	Other	Ref.		
Relative M305 <sup>4</sup> , %	Cont. <sup>5</sup>	0.002967	0.00018	<0.0001
Herd level				
Herd size <sup>6</sup> , n/year	≤65	0.059	0.0157	0.0010
	66-83	0.049	0.0147	
	84-118	0.032	0.012	
	≥119	Ref.		
Bulk tank UFA <sup>7</sup> , %	Cont.	0.035	0.0125	0.0057
Bulk tank CVUFA <sup>8</sup> , %	Cont.	0.14	0.056	0.0102
Herd level interactions				
Bulk tank UFA x CV UFA	Cont.	-0.0048	0.00181	0.0083

<sup>1</sup> Estimated coefficient ( $\beta$ ), SE for the coefficient, and significance level

<sup>2</sup> Reference class of categorized variable

<sup>3</sup> Holstein Friesian

<sup>4</sup> Milkproduction within 305d

<sup>5</sup> Continuous variable without classes

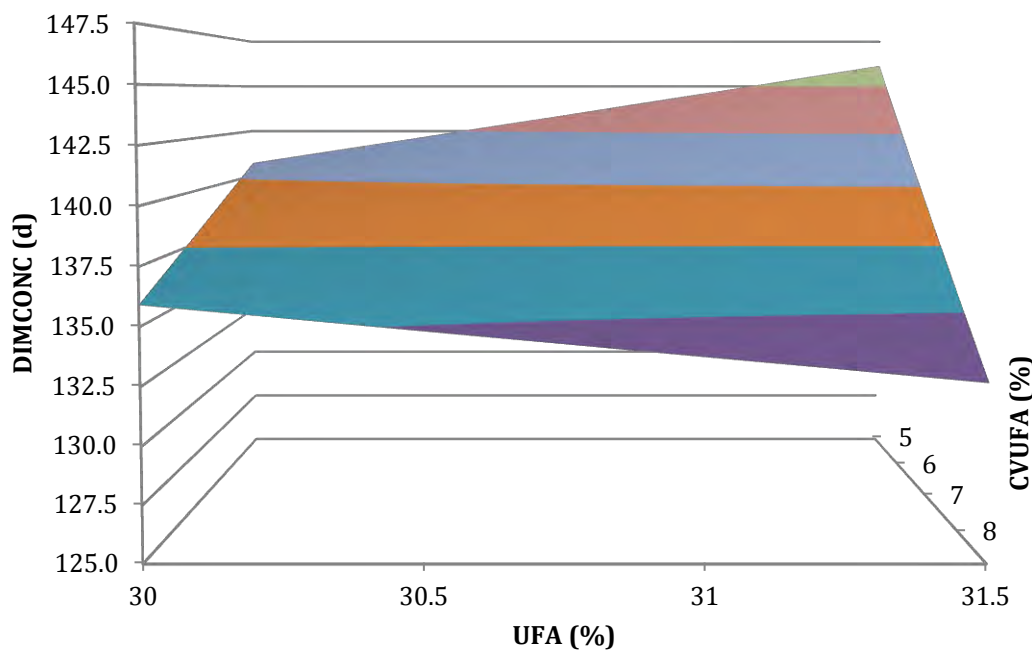
<sup>6</sup> Herd size expressed as number of calvings per year

<sup>7</sup> Unsaturated FA

<sup>8</sup> Coefficient of variation of UFA

## DISCUSSION

The aim of this study was to detect associations between fertility parameters in dairy cows and the level of UFA in bulk tank samples as a proxy for the dietary fatty acid profile on that herd. Milk fat typically contains about 30% UFA, of which the main proportion are 18:1 FA (20-30%), followed by 16:1 and 18:2, both representing about 1-3% of FA (Jensen, 2002). There is considerable interest in increasing the UFA as it contains more saturated FA compared to plant oils which could harm long-term health of consumers (Dewhurst et al., 2006). The level of UFA is affected by animal factors such as breed (Soyeurt et al., 2006; Schennink et al., 2008), stage of lactation and parity (Kelsey et al., 2003), subclinical mastitis (Auld et al., 1996) but the largest variation is accounted for by ruminant nutrition as reviewed by many (e.g. Glasser et al., 2008; Schmidely et al., 2008).



**Figure 5.** The effect of the percentage of unsaturated fatty acids in the bulk tank (UFA), its coefficient of variation (CVUFA), and the interaction between both on days in milk to conception (DIMCONC) based on 8247 first inseminations on 90 Belgian herds in 2008 and 2009.

In Belgium over the last decade, producers have been encouraged by dairy industries to produce milk with a more unsaturated FA profile, mainly by feeding extruded linseed. As herds participating in this specific project were paid depending on the UFA content of the bulk tank milk samples, UFA content was determined continuously on all Flemish herds providing the possibility to link UFA to herd fertility



records. The UFA content of selected herds was 30.8% and aligns with literature on milk UFA (Jensen, 2002; Lock and Bauman, 2004). From our data it cannot be concluded whether a higher UFA content originates from an increase in mono unsaturated FA (MUFA), poly unsaturated FA (PUFA) or both, which mainly depends of the FA profile of dietary lipids (Chilliard and Ferlay, 2004). However, next to PUFA supplementation, increased UFA in bulk tank milk samples can be explained through other pathways. The highest proportion of UFA in MF is accounted for by 18:1 *cis*-9 (Jensen, 2002). An increased concentration in 18:1 *cis*-9 in MF can originate from both an increased supply of 18:1 *cis*-9 or its precursor 18:0 to the mammary gland (Glasser et al., 2008). An increase in 18:1 *cis*-9 has been associated with increased body fat mobilisation during ketosis (Van Haelst et al., 2008). However, a contribution of FA from lipomobilisation in cows in positive EBAL to milk FA cannot be excluded, especially for 18:0 and 18:1 *cis*-9 (Chilliard et al., 1991). Furthermore, the induction of a MFD has been associated with an increase in *trans*-FA and proportional decrease in saturated FA increasing the milk UFA (Griinari et al., 1998; Bauman and Griinari, 2003). An increased rumen unsaturated FA load (RUFAL) has been determined as one of the risk factors for a lower FAT content when accompanied by an altered rumen fermentation (Relling and Reynolds, 2007; Harvatine et al., 2009). The prerequisite of both risk factors is mirrored in the significant but small proportion of the variation in FAT explained by UFA in our study (10.2%).

Researchers have focussed on many aspects of dairy cow fertility but the complete biological window in which UFA attenuate fertility is yet to be revealed. Though small differences were found (IQR max 3 d) which might not be of real biological relevance, we did demonstrate a positive association between DIMFI and milk UFA. However, earlier resumption of postpartum cyclicity has not been observed when feeding FA to dairy cows (Juchem et al., 2010; Silvestre et al., 2011). Interestingly, high UFA was associated with the highest DIMFI especially when accompanied by a low level of variation, which has to our most recent knowledge not been described. Feeding n-6 UFA to dairy cows has been shown to stimulate PGF $2\alpha$  metabolism improving uterine health (Petit et al., 2004; Robinson et al., 2002). In contrast, feeding n-3 UFA reduced endometrial secretion of PGF $2\alpha$ , thereby inducing antiluteolytic effects on the corpus luteum (Staples et al., 1998). These subtle differences between n-6 and n-3 FA could explain differences in DIMFI but cannot be attributed to our study from the parameter milk UFA which does not distinct between FA.

We demonstrated a negative association between the milk UFA content and the CRFI which, together with the aforementioned increased DIMFI, results in higher DIMCONC. *In vivo* observations on conception rates (CR) and milk UFA when supplementing FA to dairy cows are scarce and contradicting. On top, our results show an association with the average herd bulk tank UFA per year, whereas experiments supplementing UFA have often targeted specific supplementation during the pre-breeding or breeding period which makes comparison to our data intriguing. Nevertheless, the observation of a more unsaturated FA profile of follicular fluid, oocytes and granulosa cells in winter as an explanation for seasonal differences in dairy cow fertility (Zeron et al., 2001), has triggered *in vivo* research to increase the UFA content of reproductive tissues. Many diverse experiments with supplemental UFA have tried to increase CR in dairy cows. Compared to a source of SFA, feeding flaxseed and sunflower seeds increased the UFA content in granulosa cells and milk fat respectively (Zachut et al., 2010). Subsequently, the number of follicles aspirated during ovum pickup and the subsequent cleavage rate of collected oocytes in the flaxseed fed cows was increased whereas cows fed sunflower had a lower proportion of grade 1 oocytes. Fouladi-Nashta et al. (2009) increased milk UFA by feeding both full fat toasted soybean or extruded linseed. Although a higher PUFA content was found in granulosa cells for the soybean group, they were unable to show any differences in blastocyst yield or embryo quality compared with SFA. The authors concluded that bovine ovaries are able to buffer oocytes against FA fluctuations, only modestly affecting their developmental potential. Furthermore, increasing the level of UFA in milk by feeding rolled sunflower seeds compared with rolled flaxseed has been associated with a higher pregnancy loss and tendency for a lower CRFI (Ambrose et al., 2006). The latter studies illustrate the diversity in reproductive studies supplementing FA from different sources (saturated FA vs. soybean vs. linseed) and different treatments (toasted vs. extruded vs. rolled) as comprehensively reviewed by Gulliver et al. (2012). When Petit et al. (2001) compared a mixture of SFA/UFA (calcium salts of FA (Megalac) plus flaxseed meal) to an UFA source (formaldehyde treated whole flaxseed) in an experiment with 30 cows, they found an increased CRFI (87.5 vs. 50%) in cows fed whole flaxseed. When Fuentes et al. (2008) tried to repeat the results of the previous study, they were not able to find any differences in reproductive performance on a total of 356 cows fed either a mixture of SFA/UFA (calcium salts of 16:0 and extruded soybeans) or extruded flaxseed. This nicely

demonstrate the need for a large number of cows per group when trying to detect significant and biologically relevant differences in CR in dairy cattle (Wathes et al., 2007).

Direct effects of specific FA on *in vitro* oocyte maturation and embryonic development might not always mimic *in vivo* responses (Santos et al., 2008) but can help understanding contrasting *in vivo* observations. A possible explanation for the negative association between high milk UFA and CRFI and subsequent longer DIMCONC might be proven by Al Darwich et al. (2010). They showed a tendency for a lower blastocyst yield with increasing 18:3n-3 addition during *in vitro* embryo culture and a detrimental effect of embryo culture with the highest dose of 22:6n-3 and 18:2 *trans*-10, *cis*-12 on *in vitro* embryo survival after vitrification and warming. Feeding transition cows 22:6n-3 increased the blood lipid peroxidation increasing their susceptibility to oxidative stress (Wullepit et al., 2012). The latter has been linked to embryonic death in humans (Guerin et al., 2001; Agarwal et al., 2005) and the bovine (Fujitani et al., 1997; Olson and Seidel, 2000; Rooke et al., 2012), possibly explaining *in vivo* and *in vitro* observations when supplementing UFA to increase oocyte and embryo quality in dairy cows. The level of free radicals in blood of dairy cows has been linked to their milk production (Castillo et al., 2005; Lohrke et al., 2005) which could explain the most negative impact of high UFA on CRFI in the higher yielding herds. The positive effect of the higher CVUFA remains to be clarified but suggests that especially the herds with high UFA are relieved from the high UFA by a higher CVUFA and hence experience a less hampered fertility.

## CONCLUSION

In summary, researchers have not been able to prove a consistent increase in dairy cow fertility by feeding UFA making a general conclusion on the state-of-the-art on UFA feeding difficult. Although some experiments have shown positive effects of UFA supplementation to dairy cows, we demonstrated that increasing the UFA content of bulk tank milk should not be a goal as such as this was associated with worsened fertility parameters. Further research should focus on the effect of specific individual FA on reproduction in dairy cows rather than increasing the overall level of unsaturation in the animal.

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# GENERAL DISCUSSION



The scope of the present thesis was to get a better insight (Chapter 4) in the transition period which cows have to endure every time they have calved and (Chapter 5) in the challenges when implementing dietary fatty acids (FA) to improve metabolic health and subsequent fertility in dairy cows. More specifically, in the first part of Chapter 4 (Chapter 4.1) we focused on the effect of metabolic diseases (MD) on milk production and the shape of the lactation curve. In the second part (Chapter 4.2), we estimated the preferential site from which fat is mobilized during negative energy balance (NEBAL), in cows suffering from a left displacement of the abomasum (LDA). Furthermore, in the first part of Chapter 5, we examined the effect of a milk fat depression (MFD) induced by feeding long chain FA on the degree of NEBAL (Chapter 5.1). In Chapter 5.2, the effect of supplementation with short and long chain FA on blood (BL), follicular fluid (FF) and milk FA composition was investigated. In finalizing chapter 6, the association between fertility parameters and the level of unsaturated FA (UFA) in bulk tank samples was examined in a large dataset. Here, we aimed to interpret the latter observations using knowledge acquired in the former chapters in order to get an answer to the question: *“Unsaturated fatty acids in dairy cows: Do’s and Don’ts?”*

## WHERE IT ALL STARTS : ‘TRANSITIONOLOGY’

During the last decades, dairy cows have been intensively selected to produce more milk, and as a result are nowadays generally called high yielding (Shook, 2006). The increase in milk yield per cow has been reported worldwide (Belgium: Hostens and Opsomer, 2012; Canada: Leblanc, 2010; the United States of America: Lucy, 2001 and England: Royal et al., 2000).

*“The current milk production world record is held by “Hartje Meyer Beacon 9792” who produced 34,512 kg of milk in 365 days (32g/kg fat and 29g/kg protein).”*

Although high yielding dairy cows convert feed (nutrients) more efficiently into milk, and thereby provoking a smaller environmental impact when compared to lower yielding cows (Capper et al., 2009; Garnsworthy, 2011), there are some important downsides associated with the increased level of milk production and its accompanying NEBAL which are described in Chapter 4.



## **METABOLIC DISEASES DURING THE TRANSITION PERIOD**

During the transition period, which is defined as the time span from 3 weeks before until 3 weeks after calving (Drackley, 1999), high yielding dairy cows are facing marked changes in their endocrine, metabolic and physiological status (Drackley et al., 2005; Leroy et al., 2008). At the onset of lactation, most glucose is insulin independently partitioned towards the udder to sustain lactogenesis (Zhao et al., 1996; Bell and Bauman, 1997). Simultaneously homeorethnic controls ensure the cow switches from an anabolic to a catabolic state, which coincides with the fact that the cows are at that moment not able to take up the amount of energy and glucose they lose by producing milk. This is generally referred to as the negative energy balance (NEBAL; Bauman and Currie, 1980). Briefly, low insulin concentrations uncouple the growth hormone (GH)–insulin like growth factor 1 (IGF-I) axis in the liver due to a downregulation of GH 1 A receptors (Lucy, 2008). The uncoupled somatotropic axis causes GH to increase, despite the negative feedback of low IGF-I, which enhances liver gluconeogenesis, thus milk production and induces lipolysis in the different fat depots (Butler et al., 2003). The lipolysis results in a steep increase in blood non esterified FA (NEFA) concentrations around parturition. Although some have argued that at least a part of this mobilization is genetically driven, and is hence not detrimental *in se* (Friggens et al., 2007), side products of this body fat mobilization have been negatively associated with production (Ospina et al., 2010) and reproduction (Leroy et al., 2011; Walsh et al., 2011).

Both the decreased dry matter intake (DMI) and the increased NEFA levels have been associated with a suppression of the immune function typically occurring in the early lactation stage of modern dairy cows (Rukkwamsuk et al., 1999). High levels of NEFA (Scalia et al., 2006) and BHBA (Suriyasathaporn et al., 2000) have been associated with a compromised inflammatory response in dairy cows (Sordillo and Aitken, 2009; Contreras and Sordillo, 2011). As a result, fresh dairy cows are more vulnerable to MD such as retained placenta (Kimura et al., 2002), metritis (Goff, 2006; Hammon et al., 2008) and mastitis (Chagunda et al., 2006; Moyes et al., 2009). This vulnerability is further increased by the lower DMI which imbalances the availability versus the requirement of minerals and antioxidants (Mulligan and Doherty, 2008). Besides an important negative impact on reproduction (Walsh et al., 2011), these MD do cause profound economic losses to the industry, mainly through a decreased milk production

(Hogeveen, 2012). Although, the effect of MD during the transition period on the level of milk production has already often been described (Fourichon et al., 1999), studies yielded contrasting results and estimations of short- and long-term effects are currently missing for most MD. Moreover, statistical pitfalls hamper adequate interpretation of the effect of MD on milk production:

- ✓ In early lactation, dairy cows show a high variability in daily milk production as cows proceed towards peak milk yield (Kessel et al., 2008; Quist et al., 2008; Lovendahl et al., 2010). Hence, short term evaluation by comparing daily milk production before and after clinical diagnosis (Detilleux et al., 1994; Detilleux et al., 1997; Bareille et al., 2003) is hugely affected by the lactation stage. This makes comparability among the different studies nearly impossible.
- ✓ Monthly test day milk productions lag time before they are measured. Especially lower producing diseased animals may have been culled before their first test day (Bartlett et al., 1997) provoking bias and an underestimation of the effect of MD on milk production (Fourichon et al., 1999).
- ✓ Similarly, cows culled on 59, 99 or 304 DIM, are excluded from any analysis when using milk production data within the first 60 (M60; Sheldon et al., 2004); 100 (van Werven et al., 1992) or 305 DIM (M305; Dubuc et al., 2011) respectively.
- ✓ Using first test day milk or M60 has the benefit of including more animals but may not provide accurate predictions of the entire lactation (Leblanc, 2010).
- ✓ Finally, differences in definition and severity of diseases (van Werven et al., 1992), and interrelationships between the different transition diseases (Ingvartsen et al., 2003) require controlling for bias (Rajala and Grohn, 1998; Fourichon et al., 1999).

Lactation curve models have been used to model residuals between predicted and observed milk yield in healthy and diseased animals (Lucey et al., 1986; Rowlands and Lucey, 1986). Therefore, in Chapter 4.1, we demonstrated the use of the new Milkbot model (Ehrlich, 2011) on a large dataset of 1,555 lactations from one herd to estimate the effect of MD on the shape of the lactation curve. The proposed model can be fitted to milk production data to summarize an individual lactation as a set of 4 fitted parameter values, each corresponding to a specific aspect of the lactation curve with

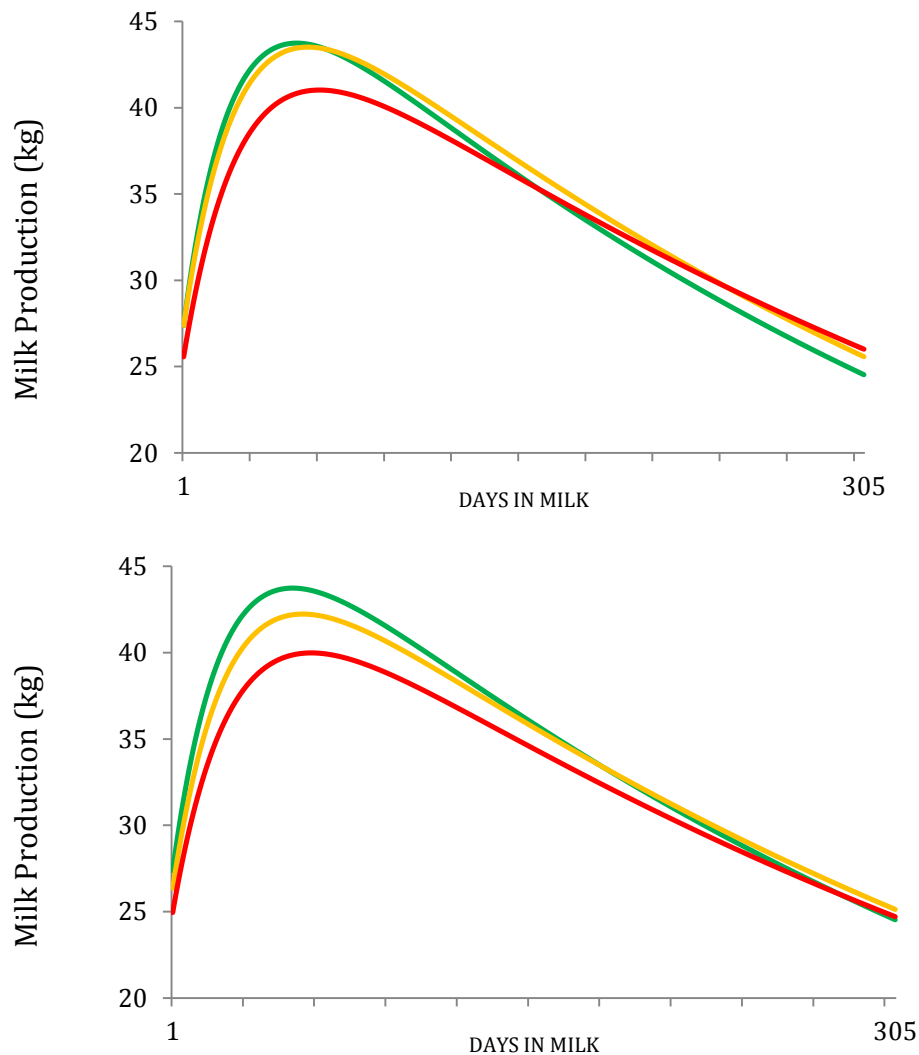
regards to its shape or magnitude (Ehrlich, 2011; Cole et al., 2012). We especially focused on differences between cows which remained healthy (H) during the entire transition period as opposed to cows which suffered from one metabolic disease (uncomplicated; MD) and cows which suffered from more than one metabolic disease (complicated; MD+).

Our results indicate that the fitted parameter values were more sensitive to the effects of MD than totals or averages calculated from raw production data as they allow to detect specific changes in the distribution of production that are not apparent when only totals or averages are analyzed. For example, although we were able to detect differences in the shape of the lactation curve between healthy and diseased animals, a slower rise to a lower peak seemed to be compensated for by better persistency, so the change in the total production (M305) was only lowered in cows suffering from complicated MD+ (-377 kg milk). So far, we are only aware of one study reporting an overall estimation of the daily milk loss of 7.2 kg due to MD in the first 20d post partum (Wallace et al., 1996).

In the individual disease models, specific changes in the shape of the lactation curve were found except for twinning. Milk fever, retained placenta, ketosis, and mastitis mainly affected the lactation curve when they were accompanied by another MD (MD+), but overall M305 was not affected due to a higher persistency. As illustrated in Figure 1 for ketosis, this MD can be named “a compensated metabolic disease” which is in contrast with e.g. ‘complicated metritis’ as the amount of milk produced by cows suffering from this disease, was not compensated for though lactation curves were even more persistent. These cows have a 489.6 kg lower M305 compared to healthy herdmates.

The higher persistency in diseased animals that compensates for the early loss in milk has been reported for cows suffering from metritis (Markusfeld, 2003) and ketosis (Gustafsson and Emanuelson, 1996; Markusfeld, 2003). However, when unraveling this result more carefully, it is clear that most MD such as milk fever (Hayes et al., 2012), retained placenta (van Werven et al., 1992; Hayes et al., 2012), metritis (Opsomer et al., 2000; Melendez et al., 2004), ketosis (Leroy et al., 2005; Walsh et al., 2007; Ospina et al., 2010) and LDA (Jorritsma et al., 2008) have been associated with reproductive failure. As MD cows are less likely to conceive at an early stage in lactation (Walsh et al., 2007; Walsh et al., 2011), the reduced negative effect of pregnancy on milk production

(Svennersten-Sjaunja and Olsson, 2005) might explain the observed higher persistency in cows confronted with MD. Further research should focus on the effect of MD on culling and life time production and further elaborate the interaction between pregnancy and the shape of the lactation curve i.e. persistency.



**Figure 1.** The effect of metabolic diseases on lactation curve shape in cows that encountered no metabolic problem during the transition period (green line), cows that encountered 1 metabolic problem during the transition period (orange line) and cows that encountered more than 1 metabolic problem during the transition period (red line). The upper and lower figure demonstrate a compensated (i.e. ketosis) and uncompensated metabolic disease (i.e. metritis) respectively.

## REPRODUCTIVE PERFORMANCE IN DAIRY COWS

Friggens et al. (2010) stated: “In order to be successful in evolutionary terms, the animal needs to reproduce as intensively as possible (number of viable offspring per time unit) whilst surviving as long as possible (number of units of time)”.

*“Today, the highest recorded lifetime yield of milk in the world is 216,891 kg in 10 lactations, and is held by Smurf, a 15 year old Holstein cow.”*

Although exceptional, this observation supports that at least some cows are able to successfully combine high milk production with sound fertility. High yielding dairy cows are genetically programmed to mobilize body reserves, particularly FA, to sustain milk production in early lactation (Friggens et al., 2007). It has been argued by some researchers that the direct side effects of this mobilization (NEFA, BHBA) should be considered as a ‘pollution’ deteriorating reproductive function in high yielding dairy cows (O’Callaghan and Boland, 1999; Leroy et al., 2008). In contrast, others have found it, from an evolutionary perspective difficult to believe that animals would compromise their reproductive capacity through adaptive mechanisms that guarantee the survival of their offspring (Friggens et al., 2010).

Seen from the same perspective, it might also be difficult to believe that dairy cows which possess evolved mechanisms to conserve essential UFA within their body, would simultaneously hamper their reproductive capacity as suggested by the results in Chapter 6. In that study we aimed to find associations between the level of UFA in milk fat at the bulk tank level, and fertility parameters in a large dataset of Belgian herds. Wathes et al. (2007) stressed the need for large numbers of animals per group (>300) when trying to detect statistically significant and biologically relevant differences in pregnancy rates. We were able to collect data from 15,055 lactations from 90 herds by which we tackled the challenge of sufficient power to detect statistical differences.

Surprisingly however, in herds with bulk tank milk samples high in UFA, cows experienced a lower CR to first insemination, whereas a high variation in UFA in bulk milk samples (measured as the coefficient of variation in UFA) was associated with higher CR. As these results are contradictory to the initial hypothesis of improved fertility parameters with increasing milk UFA, some clarification is needed.

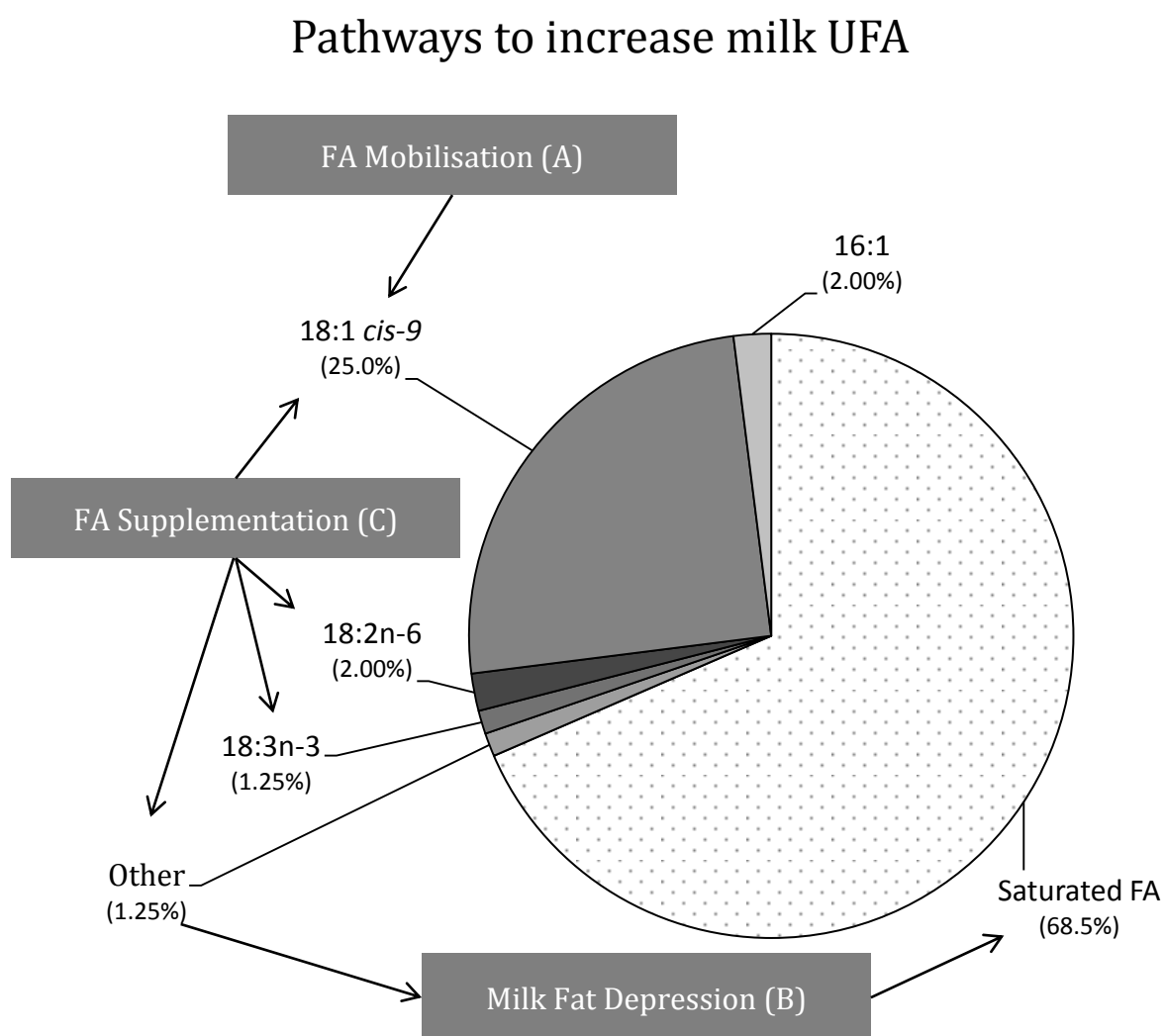
First, researchers have focused on many aspects of dairy cow fertility but the complete biological window in which UFA may attenuate fertility, has yet to be revealed. Certainly, dietary FA are able to improve fertility beyond their ability to increase the energy density of fresh cow diets (Staples et al., 1998; Staples and Thatcher, 2005), and most probably through the stereochemistry of particular FA (Gulliver et al., 2012). Besides the work of de Veth and co-workers (2009) in which an overall positive effect on days open was shown following dietary supplementation of *trans*-10, *cis*-12 CLA, other meta-analyses examining the effect of PUFA supplementation on fertility are to the best of our knowledge lacking.

Many studies have focused on specific aspects of reproduction such as an increase in the number (Lucy et al., 1991) or size of the preovulatory follicles (Lucy et al., 1993; Moallem et al., 1999; Bilby et al., 2006b) when feeding calcium soaps of FA. Furthermore, feeding n-6 UFA to dairy cows has been shown to stimulate endometrial secretion of PGF $2\alpha$  improving uterine health in the early postpartum period (Robinson et al., 2002; Petit et al., 2004). On the other hand, feeding n-3 PUFA reduced PGF $2\alpha$  secretion, thereby diminishing luteolytic effects on the corpus luteum which is beneficial at the time of recognition of pregnancy (Staples et al., 1998). *In vitro* assessed oocyte and embryo quality was improved (Cerri et al., 2009; Fouladi-Nashta et al., 2007; Zachut et al., 2010), unaltered (Bilby et al., 2006a; Fouladi-Nashta et al., 2009; Ponter et al., 2012) or even hampered (Petit et al., 2008) after PUFA supplementation of dairy cows. These varying results might explain the observed limited changes in CR when feeding PUFA (Ambrose et al., 2006; Petit and Twagiramungu, 2006; Fuentes et al., 2008). As stated above, another reason for the inconsistent results might be the low number of animals used in many experiments (Wathes et al., 2007). One study by Silvestre et al. (2011) involving a large number of animals (n=1,582) showed an increased CR to the second but not to the first AI when sequentially supplementing n-6 and n-3 FA.

However, next to PUFA supplementation, increased UFA in bulk tank milk samples can be explained through other pathways. The highest proportion of UFA in MF is accounted for by 18:1 *cis*-9 (Jensen, 2002). An increased concentration in 18:1 *cis*-9 in MF can originate from both an increased supply of 18:1 *cis*-9 or its precursor 18:0 to the mammary gland (Glasser et al., 2008). An increase in 18:1 *cis*-9 has been associated with

increased body fat mobilisation during ketosis (Van Haelst et al., 2008). However, a contribution of FA from lipomobilisation in cows in positive EBAL to milk FA cannot be excluded, especially for 18:0 and 18:1 *cis*-9 (Chilliard et al., 1991). Furthermore, the induction of a MFD has been associated with an increase in *trans*-FA and proportional decrease in saturated FA increasing the milk UFA (Griinari et al., 1998; Bauman and Griinari, 2003).

All aforementioned pathways are schematically represented in Figure 2 and will be discussed in the following sections with a particular focus on the metabolism in transition dairy cows.



**Figure 2:** Schematic representation of the average fatty acid composition of milk fat (Jensen, 2002) and possible pathways to increase the proportion of unsaturated fatty acids (UFA; grey sections).

## FATTY ACID MOBILIZATION IN DAIRY COWS

In order to get a better insight in the pathogenesis of the NEBAL, we hypothesized (Chapter 4.2) site specific differences in the saturation profile of subcutaneously (SUBC) versus abdominally (ABD) stored fat depots. Differences would, in the case of lipolysis render both depots to a different level of pathogenicity as saturated FA have been linked with impaired leukocyte (Contreras et al., 2010; Contreras and Sordillo, 2011) and granulosa cell (Vanholder et al., 2005) function and impaired oocyte maturation (Van Hoeck et al., 2011). We furthermore aimed to identify the main site of lipolysis during severe NEBAL (i.e. LDA).

Left abomasal displacement has been studied intensively over the last decades, as its lactation incidence has increased concurrently (Doll et al., 2009). Economic losses mainly occur through the higher risk of culling (Jorritsma et al., 2008) and treatment costs, rather than production losses which can be compensated for by a higher persistency in established lactation (Chapter 4.1). Though not fully understood, risk factors continuously have been linked with the transition period (Cameron et al., 1998; Stengarde et al., 2008; Stengarde et al., 2011; Stengarde et al., 2012), making animals with LDA an appropriate subject to study the effects of severe NEBAL on the composition of NEFA.

### BLOOD FA PROFILE IN HEALTHY AND DISEASED ANIMALS

In contrast to what has been shown for healthy cows in early lactation (Moallem et al., 1999; Contreras et al., 2010), a remarkably lower 16:0 (22.0 vs. 27.8 and 46.4 g/100g FA) and 18:0 (20.1 vs. 39.5 and 30.1g/100g FA) while higher 18:1 *cis*-9 (26.2 vs. 6.57 and 11.8g/100g FA) content have been found in NEFA from LDA cows in our experiment (Chapter 4.2). A higher level of saturated FA in NEFA *per se* has been linked to deteriorated leukocyte function early post partum enhancing susceptibility to transition diseases (Contreras and Sordillo, 2011) and to detrimental effects on oocyte maturation later post partum (Leroy et al., 2005; Van Hoeck et al., 2011). Therefore, when ignoring the absolute higher NEFA level and focusing on the relative FA composition of NEFA, cows with LDA would be experiencing a less severe pro-inflammatory environment. Are cows suffering from excessive NEBAL shifting to a preferential mobilization of SUBC which is due to its lower saturation level, less harmful



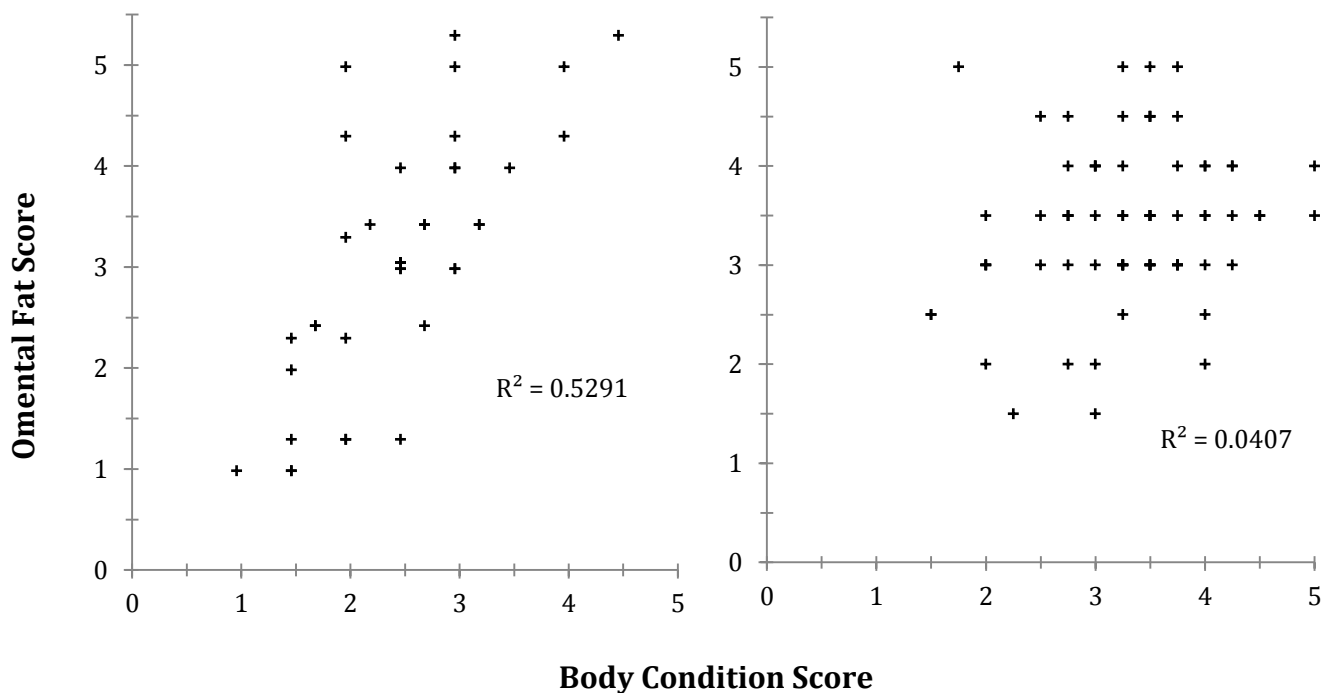
for the animal? Future research to further distinguish the role of physiological (stage of lactation, diet, breed) and pathophysiological effects on the mobilization of NEFA in early lactating dairy cows, is therefore needed.

The FA profiles in Chapter 4.2 support the hypothesis of a preferential mobilization of ABD in comparison to SUBC during severe NEBAL. Recently, retroperitoneal fat was shown to be more sensitive to periparturient challenges (Locher et al., 2011) and is preferentially mobilized in early lactation (Akter et al., 2011). This is in contrast with the higher lipolytic (Etherton et al., 1977) and lipogenic rate (Baldwin et al., 2007) of SUBC versus perirenal adipocytes. However, when adjusted for the smaller adipocyte size in SUBC adipocytes, a higher net lipogenic activity was found in ABD fat in nonlacting heifers (Eguinoa et al., 2003). The hypothesis of an increased ABD FA metabolism has also been supported by Nikkhah et al. (2008) who demonstrated an increased deposition of the omental fat mass when cows were moderately overfed during an 8-week dry period without concomitant increase in BCS.

### **BODY CONDITION SCORE VERSUS ABDOMINAL FAT SCORE**

The different FA metabolism in ABD and SUBC is interesting as it questions the use of BCS as an accurate estimator of lipolysis and hence of the NEBAL in early lactating dairy cows. For example, when compared to dairy cows in the eighties, 21<sup>th</sup> century Holstein cows seem to have lowered their optimal BCS at calving regarding subsequent reproductive performance (Garnsworthy, 2006). These results may have been confounded as modern high yielding dairy cows may have shifted towards a preferential deposition and subsequent mobilization of abdominal fat which is not measured when using BCS. Therefore, in Chapter 4.2 we also estimated the amount of intra-abdominally stored fat by using the omental fat score (OFS) as developed by Van Eetvelde et al. (2011). Of particular interest are the animals observed in our study with a moderately low BCS but with a concomitantly high OFS (Figure 3: left scatterplot). In contrast with the aforementionedly higher metabolic activity in ABD, this observation suggests a higher FA mobilization in SUBC. We furthermore compared our data to the individual data from the 74 healthy dairy cows at slaughter used by Van Eetvelde et al. (2011). In comparison to our study on cows suffering from LDA, Van Eetvelde and co-workers found a lower correlation between BCS and OFS (Figure 3: right scatterplot). Although Van Eetvelde et al. (2011) did not have background information about the

slaughtered cows, another interesting observation can be made compared to our data. Whereas cows with a low BCS and a concomitantly high OFS could be found in both healthy and LDA cows, cows with an oppositely high BCS and low OFS were not observed in our LDA study. Further research is needed to clarify the contradiction between the preferential mobilization of ABD fat in Chapter 4.2 and observations on OFS. Finding non-invasive techniques to estimate OFS will be one of the most challenging parts of future research as applicability of three-dimensional body scanning, computed tomography and magnetic resonance imaging used to assess body composition in humans (Mattsson and Thomas, 2006) is economically not feasible in farm animals at this moment.



**Figure 3.** Body Condition Score and Omental Fat Score of dairy cows at the moment of correction for a left abomasal displacement (left: n=46; data from Hostens et al., 2012) and healthy cows at slaughter (right: n=74; data from Van Eetvelde et al., 2011).

## FATTY ACID SUPPLEMENTATION IN DAIRY COWS

### MILK FAT DEPRESSION AND NEGATIVE ENERGY BALANCE

The induction of MFD to reduce the NEBAL early post partum has been proposed by many researchers to target a reduced energy loss via a decreased loss of the energetically most expensive compound (50 % of total milk energy; Tyrrell and Reid, 1965), while at the same time, the most easily manageable milk component through dietary modifications (Odens et al., 2007; Medeiros et al., 2010; Moallem et al., 2010). In Chapter 5.1, we initially tried to induce a MFD using 2 different approaches. The first method involved the supplementation of *trans*-10, *cis*-12 CLA which has been shown to depress milk fat synthesis in a significant and predictable manner (de Veth et al., 2004). At the end of the experiment described in Chapter 5.1, data analysis revealed the absence of a MFD for the *trans*-10, *cis*-12 CLA group (data not shown). We hypothesized that the processing of the *trans*-10, *cis*-12 CLA increased its susceptibility to rumen biohydrogenation which was confirmed by *in vitro* incubations of the unprocessed and processed supplement. Biohydrogenation of the *trans*-10, *cis*-12 CLA was 45 and 60% for two batches of unprocessed *trans*-10, *cis*-12 CLA whereas processing caused biohydrogenation to rise as high as 96% for both batches (Dehkordi et al., 2008). Further analysis of this treatment group was cancelled. The second approach was dietary supplementation of 22:6n-3 (DHA).

Whereas *trans*-10, *cis*-12 CLA has been broadly accepted as a potent inhibitor of milk fat synthesis at the level of the mammary gland (Chouinard et al., 1999; Baumgard et al., 2000), the physiological pathway by which 22:6n-3 induces MFD remains unclear leaving unidentified biohydrogenation intermediates (Bauman and Griinari, 2003) as well as a decreased supply of 18:0 for triacylglycerol (TAG) synthesis in the udder as possible explanations (Shingfield et al., 2010). Recently, the higher 22:6n-3 concentration in FF has been suggested as one of the explanations for a higher fertility in heifers compared with cows (Bender et al., 2010) placing 22:6n-3 supplementation into prosperous perspectives by simultaneously enriching the follicular microenvironment.

The main objective of the study in Chapter 5.1 was to induce a MFD in early lactation with concomitant registration of NEBAL indicators. We were able to induce a MFD of 21% which is in accordance with other studies (Franklin et al., 1999; Boeckaert et al., 2008). As discussed in Chapter 5.1, studies simultaneously reporting metabolic parameters are scarce, especially those using marine products in early lactation. Moreover, different types of marine products such as fish meal (Heravi Moussavi et al., 2007), fish oil (FO: Mattos et al., 2004; Ballou et al., 2009) or FO on a carrier (Kupczynski et al., 2011), and the differences in daily supplementation of 20:5n-3 + 22:6n-3 (e.g. Mattos et al., 2004: 128g/d vs Ballou et al., 2009: 46.7g/d) make it difficult to compare production responses and blood metabolites.

Literature on the effect of *trans*-10, *cis*-12 CLA on the energy status in early lactating dairy cows is more abundant (Table 1). Even though higher doses of milk fat depressing isomers of *trans*-10, *cis*-12 CLA are needed in early lactation (Moore et al., 2004; Kay et al., 2006), the response in milk fat content and yield is consistently depressed (de Veth et al., 2004) which is in contrast with the variable milk yield response during MFD (Table 1). Generally, the milk yield was increased in pasture based production systems during MFD (Kay et al., 2006; Medeiros et al., 2010; Hutchinson et al., 2012). In TMR based systems, both increased (de Veth et al., 2006; Odens et al., 2007; Moallem et al., 2010) and equal (Moore et al., 2004; Castaneda-Gutierrez et al., 2005; von Soosten et al., 2011) milk yields have been observed during MFD. The differences in milk yield response might explain why merely 3 studies (Table 2) have demonstrated an improved calculated EBAL during MFD (Kay et al., 2006; Kay et al., 2007; Odens et al., 2007).

We demonstrated in Chapter 5.1 that a metabolic approach to MFD based on nutrient requirement per mole of milk fat, though induced by supplementing 22:6n-3 in our study, might be more appropriate than a caloric approach when trying to estimate the increase in milk yield following a MFD. We showed that the increased milk production is most likely caused by a propionate saving effect, while the increased concentration of one metabolite (BHBA in FF) might not always be attributed to a worsened energy status of the animals, but rather can be seen as a leftover metabolite when *de novo* milk fat synthesis is depressed.

An increased BHBA level has been shown in cows >63 DIM during MFD (Bernal-Santos et al., 2003). As shown in Table 2, until now only 1 study has been able to successfully ameliorate the metabolic energy status in early lactating dairy cows (Odens et al., 2007). More recent studies have however reported an unexplained lower body weight increase (Moallem et al., 2010) and lower back-fat thickness (von Soosten et al., 2011) during MFD. Castaneda-Gutiérrez et al. (2007) were the first to demonstrate an increased IGF-I concentration in plasma during MFD, the latter being brought forward to explain the overall improved reproductive performance in the multi-study analysis from de Veth et al. (2009) on *trans*-10, *cis*-12 CLA supplementation. The approach of altered signaling metabolites such as IGF-I was suggested to explain enhanced reproduction as new studies failed to repeat the results of Odens et al. (2007) indicating an improved energetic status.

**Table 1.** Overview of published literature on production response to *trans*-10, *cis*-12 CLA supplementation in dairy cows.

Reference	DIM <sup>1</sup>	Milk <sup>2</sup>			Fat <sup>3</sup>		Protein	
		Yield	FCM	Energy	Cont	Yield	Cont	Yield
<i>TMR and PMR<sup>4</sup> – based designs</i>								
Bernal-Santos et al., 2003	-14 - 140	<sup>-5</sup>		-	-12.5	-	-	-
Moore et al., 2004	-10 - 21	-			-32.2	-30.0	-	-
Perfield et al., 2004	78	-			-27.6	22.0	(+1.18)	8.49
Castaneda-Gutierrez et al., 2005	-21 - 63	-		-11.3	-19.4	-21.2	-	-
de Veth et al., 2006	112	+3.27			-22.0	-19.6	-	+2.56
Castaneda-Gutierrez et al., 2007	20 - 56	-		-	-	-	-	-
Odens et al., 2007	-9 - 40	+7.51	-		-26.0	-22.6	-	-
Moallem et al., 2010	21 - 100	+4.66	-3.85	-3.48	-12.5	-8.93	-3.25	-
von Soosten et al., 2011	-21 - 41	-	-	-	-14.1	-	-	-
von Soosten et al., 2011	42 - 105	-	-	-	-25.4	-17.1	-10.4	-
Pappritz et al., 2012	1 - 49	+1.64	-		-4.29	-	-	-
<i>Pasture – based designs</i>								
Mackle et al., 2003	80	+11.2			-42.6	-36.1	-	-
Kay et al., 2006	-27-36	-			-29.6	-29.4	-	-
Kay et al., 2007	204	-			-43.2	-43.5	+7.25	+5.77
Medeiros et al., 2010	28 - 84	+7.24		-	-26.2	-20.4	+11.5	+19.4
Hutchinson et al., 2012	0 - 60	+10.1	-	-	-16.8	-7.69	-4.88	-

<sup>1</sup> Days In Milk at the start and the end of supplementation. Single DIM indicate start of factorial design.

<sup>2</sup> FCM: Fat Corrected Milk; Energy: Milk Energy Output.

<sup>3</sup> Content (g/kg) and yield (g/d) of milk components

<sup>4</sup> Total Mixed Ration and Partially Mixed Ration

<sup>5</sup> Parameter was increased (↑), decreased (↓) or equal(-) compared with control; tendency indicated between brackets.

When parameter was not measured, cell was left blank.

Even though some of these findings might be of particular interest, when interpreting metabolites in early lactation dairy cows, some caution should be taken:

- ✓ As described for the statistical analyses done in Chapter 5.1 and 5.2, the distribution of metabolic parameters such as NEFA and BHBA are often right skewed (Cozzi et al., 2011). This necessitates data normalization before analysis with parametric tests (Duffield et al., 2003), which unfortunately is rarely described. Even the residual errors from non-normally distributed outcomes are unlikely to be 'normalized' by regression on the predictor variables in linear regression models which are often used to analyze the effect of e.g. feeding strategies on the metabolism in dairy cattle (Dohoo et al., 2003).
- ✓ Especially in dairy cows which have a highly variable metabolism in early lactation (Grummer, 2008), special care should be taken to prevent mis- or over-interpretation of experiments with low number of animals.
- ✓ Furthermore, as stipulated by Friggens et al. (2010), cows have a genetically driven fat mobilization, which occurs in an environment that is in no way constraining. By excluding diseased animals in research trials, which does not represent the actual situation in most dairy herds, it might be difficult to find statistically significant differences in metabolic parameters as mobilization in these cows is mainly driven by their genetics rather than by the environment. Recently, von Soosten et al. (2012) showed a decreased body mass mobilization in early lactating dairy cows suggesting a protective effect of *trans*-10, *cis*-12 CLA supplementation against excessive mobilization of body reserves. The induction of a MFD might in other words be more useful in cows suffering from excessive (environmentally driven) body reserve mobilization as compared to the more intensively studied animals which remain healthy and solely suffer genetically driven mobilization. Can in other words a MFD be used as a cure for diseased animals rather than prevention of excessive NEBAL?

Further research is needed to document the variability in production response to a MFD especially in combination with the energy status of the animals. In this, meta-analysis and meta-regression might provide greater statistical power in quantifying overall production or reproduction responses than individual experimental studies (Lean et al., 2009). The metabolic approach to a MFD has provided a better

understanding in the response to increase the milk yield. In conclusion, from our study and other *trans*-10, *cis*-12 CLA studies, it can be concluded that, although the animals spare calories, the energy is often repartitioned to increased milk production, finally leaving the net EBAL of the animals unchanged.

Interestingly, when milk production was unchanged, *trans*-10, *cis*-12 CLA was shown to upregulate genes involved in lipid synthesis in hepatic and adipose tissue during short-term MFD (Harvatine et al., 2009). The increased expression of these genes (especially peroxisome proliferator-activated receptor- $\gamma$ ; PPAR- $\gamma$ ) is associated with increased EBAL and improved insulin sensitivity (Walczak and Tontonoz, 2002). Of particular interest during MFD through n-3 FA (e.g. 22:6n-3), is their ability to simultaneously modulate the action of insulin as comprehensively discussed by Pires and Grummer (2008). Indeed, insulin-sensitizing effects have been observed in dairy cows fed 18:3n-3 (Pires et al., 2008; Salin et al., 2012). At this moment, it might be questionable to implement the induction of a MFD in early lactation as a general practice in high yielding dairy cows to reduce the NEBAL. Regardless, the aforementioned findings leave opportunities for further research on the use of n-3 FA to induce MFD.

**Table 2.** Overview of published literature on the effect of *trans*-10, *cis*-12 CLA supplementation on energy status and metabolic parameters in dairy cows.

Reference	Energy Status <sup>1</sup>			Metabolic Parameters <sup>2</sup>				Comments <sup>3</sup>
	EBAL	BW	BCS/ BFT	NEFA	BHBA	Glucose	Insulin	
<i>TMR and PMR<sup>4</sup>- based designs</i>								
Bernal-Santos et al., 2003	- <sup>5</sup>	(↑)	-	-	-	-	-	BHBA ↑ > 63DIM
Moore et al., 2004	-	-	-	-	-	-	-	EBAL nadir ↓
Perfield et al., 2004								Ruminal infusion (3 x 3)
Castaneda-Gutierrez et al., 2005	-	-	-	-	-	-	-	
de Veth et al., 2006				-		-	-	CLA induced energy repartition (2 x 2)
Castaneda-Gutierrez et al., 2007	-	-	-	-				IGF-I ↑
Odens et al., 2007	↑	↑	↑	↓		↑		ΔBW/ΔBCS ↓
Moallem et al., 2010	-	↓	-					No effect on fertility parameters
von Soosten et al., 2011	-	-	-/-	-	-	-	-	
von Soosten et al., 2011	(↓)	-	-/↓	-	-	-	-	
Pappritz et al., 2012	↓		-/-	-	-	-	-	
<i>Pasture – based designs</i>								
Mackle et al., 2003				↓	-	-	-	Abomasal infusion (4 x 4)
Kay et al., 2006	-	-	-	-		-	-	
Kay et al., 2007	↑			-	-	-	-	Abomasal infusion (2 x 2)
Medeiros et al., 2010		-	-	(↓)				Zebu x Holstein crossbreds
Hutchinson et al., 2012								No effect on fertility parameters No effect on fertility parameters

<sup>1</sup> EBAL: Calculated Energy Balance; BW: Body Weight; BCS: Body Condition Score; BFT: Back Fat Thickness

<sup>2</sup> NEFA: Non Esterified Fatty Acids; BHBA: Beta Hydroxy Butyric Acid

<sup>3</sup> Factorial design between brackets; IGF-I: Insulin-like Growth Factor-1; Δ: change; PMR: Partial Mixed Ration

<sup>4</sup> Total Mixed Ration and Partially Mixed Ration

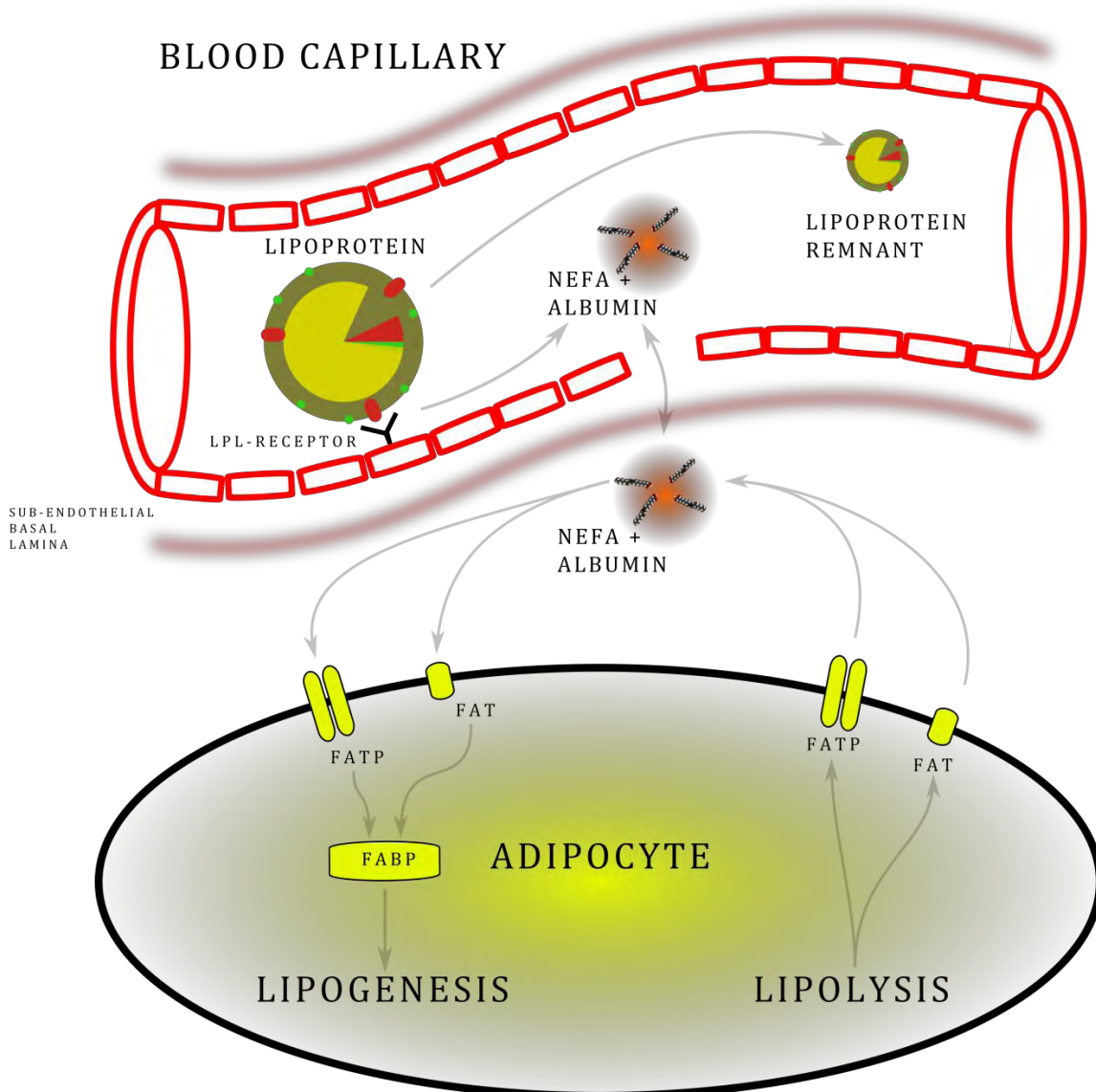
<sup>5</sup> Parameter was increased (↑), decreased (↓) or equal(-) compared with control; tendency indicated between brackets. When parameter was not measured, cell was left blank



## **DISTRIBUTION OR REDISTRIBUTION OF FATTY ACIDS TO THE REPRODUCTIVE TRACT**

One of the most trivial processes when evaluating the link between the metabolism and the ovarian compartment, especially when evaluating the effect of FA, is the physical barrier between the blood and the follicular cavity which serves as an avascular compartment in which the oocyte is subdued to a highly orchestrated process of growth, prematuration and maturation (Picton et al., 2008; Ferreira et al., 2009). The pathway via which FA intervene in these processes is partly mediated in the way they are being released into the bloodstream, transported to and subsequently are taken up by e.g. the ovary, but experiments in this research area are rare.

During lipolysis, TAG catabolism is initiated by adipose tissue lipases which transform the insoluble TAG into NEFA which are released via FA transfer proteins (FATP) and FA translocase (FAT) into the blood (Bionaz and Loo, 2008; Contreras and Sordillo, 2011; Figure 4). Typically, NEFA are bound to albumin, although a very small portion can be transported as unbound monomers in aqueous solution (Richieri and Kleinfeld, 1995). The follicular wall has been documented to be permeable via paracellular transport for molecules such as albumin and other proteins (Edwards, 1974; Gosden et al., 1988). In Chapters 5.1 and 5.2, albumin concentration did not differ between plasma and FF, which implies differences in NEFA concentrations cannot be attributed to differences in the concentration of their carrier molecule. The higher NEFA in plasma compared to the FF, as in our (Chapters 5.1 and 5.2) and other studies (Leroy et al., 2005; Shehab-El-Deen et al., 2010), has been explained by Chung et al. (1995) by a substantial partitioning of NEFA into low density lipoproteins (LDL) in case of excessive plasma NEFA concentrations. Interestingly in the same study, the partitioning of FA was shown to be 'saturation dependent' with saturated FA mainly being partitioned to the LDL whereas unsaturated FA remained albumin bound (Chung et al., 1995). The latter might explain the higher PUFA content which has been observed in NEFA FF when compared to plasma (Moallem et al., 1999; Leroy et al., 2005; Renaville et al., 2010).



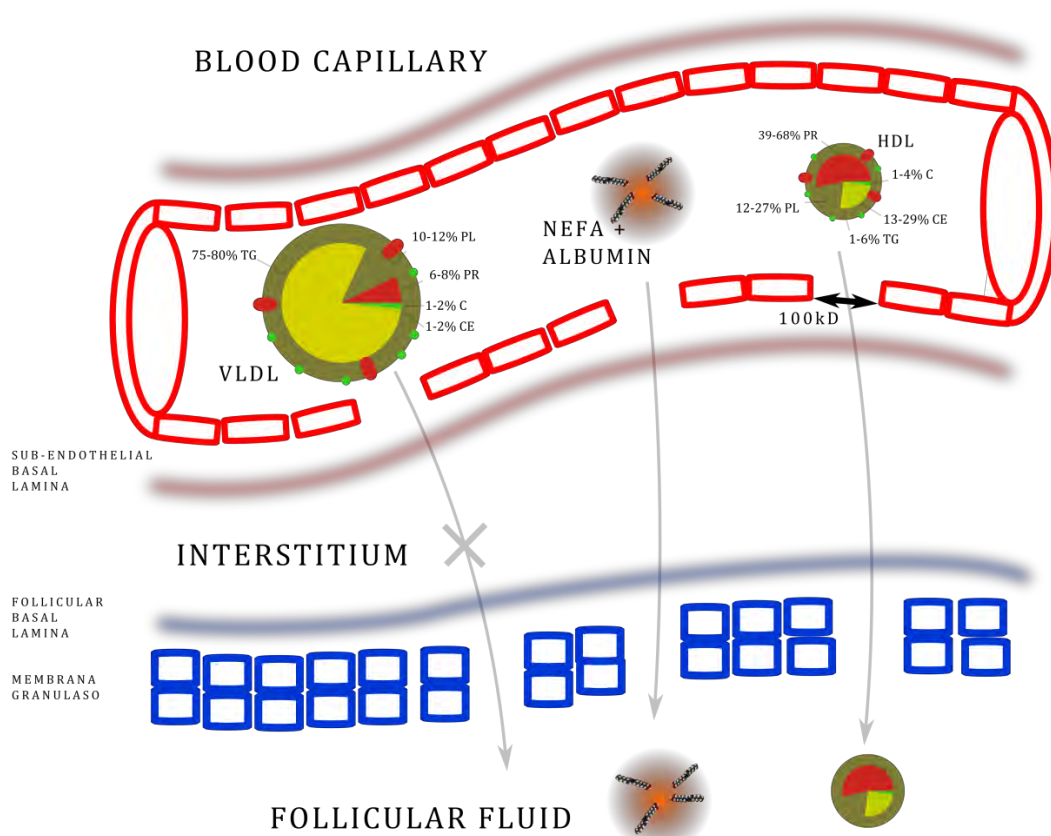
**Figure 4.** Fatty acid release from lipoproteins when bound to the lipoprotein lipase receptor (LPL-R), giving rise to non-esterified fatty acids (NEFA) and the formation of remnant lipoprotein particles. Fatty acids move via the interstitium to the fatty acid transfer proteins (FATP) and fatty acid translocase (FAT) to be internalized. Fatty acids can act as signaling molecules after binding to fatty acid binding proteins (FABP) or can be incorporated into triglycerides via lipogenesis. The opposed pathway, lipolysis will release fatty acids to the blood NEFA pool (Adapted from Chilliard et al., 2000; Bionaz and Loor, 2008; Contreras and Sordillo, 2011).

In contrast with mobilized FA that are bound to albumin, once absorbed, digested FA are bound to plasma lipoproteins which are soluble complexes of lipid-fractions (NEFA, TAG, phospholipids (PL) and cholesterol esters (CE)) with specialized proteins (apolipoproteins; Vance and Vance, 2008). These lipoproteins circulate in the blood plasma, which allows interaction through the vascular wall with parenchymal cells. Although only low lipoprotein lipase (LPL) activity has been found in the bovine ovary (Hocquette et al., 1998), the enzyme can release FA from the lipoproteins and locally increase the concentration of NEFA (Olivecrona and Olivecrona, 1999). These NEFA, as mobilized NEFA, can transverse the blood-follicle barrier. In contrast, the basement membrane between the blood circulation and FF is impermeable to macromolecular particles > 400,000 Dalton (Shalgi et al., 1973). Due to their large molecular weight, very low density lipoproteins (VLDL) and LDL cannot transverse the intact blood-follicle barrier, in contrast with high density lipoproteins (HDL), which subsequently is the predominant lipoprotein in bovine FF (Figure 5; Brantmeier et al., 1987; Grummer and Carroll, 1988). As the follicle grows, the concentration of LDL and VLDL has been shown to increase (Argov et al., 2004). The HDL contain relatively more FA and less cholesterol, hence the enrichment of the LDL fraction in the developing follicle may increase the sensitivity to insulin and increase the estradiol production and secretion (Hodgen, 1982; Wiltbank et al., 2011). Whether the lower PUFA content of larger follicles can be attributed to relative differences in LDL and HDL (Argov et al., 2004) or may indicate a selective use of polyunsaturated FA during oocyte maturation (Zeron et al., 2002; Adamiak et al., 2006), needs further investigation.

The HDL, typically low in TAG, mainly contain PL and CE rich in PUFA (Bauchart, 1993). Most essential PUFA have a carbon length of more than 18 carbon atoms (Wathes et al., 2007; Palmquist, 2010). Due to the higher molecular weight of these FA and the relatively large size of bovine HDL (Chapman, 1980), it is not unreasonable that HDL loaded with these PUFA are less able to transverse the blood-follicle barrier. However, the 22:6n-3 concentration in FF has been suggested as one of the explanations for a higher fertility in heifers compared to cows (Bender et al., 2010). Therefore, in Chapter 5.2 we aimed to enrich the FF with 18:3n-3 or 22:6n-3 during the early breeding period. Moreover, we particularly aimed to feed these FA without altering the animal performance in early lactation as we learned from the experiment in Chapter 5.1 that a MFD might affect conclusions of experimental results. The results showed that 18:3n-3

and 22:6n-3 were successfully transferred into the FF without any change in animal performance early post partum (equal milk yield and yield of milk components).

During the transition period, n-6 sources were supplemented (see further for biological background). Interestingly, this n-6 feeding increased the 20:4n-6 but not 18:2n-6 in FF. Preferentially, 18:2n-6 is incorporated via lecithin-cholesterol acyl transferase (LCAT) into the PL and CE (Noble et al., 1977), but can be incorporated in the TAG fraction at higher dietary 18:2n-6 levels when e.g. feeding corn silage based diets (Christie, 1979). Conclusively, when feeding a corn silage based diet to dairy cows, supplemental 18:2n-6 floods to the TAG fraction of the blood lipids which is subsequently mirrored in the milk fat but not in the FF (Chapter 5.2). In contrast, most probably because of smaller amounts of 18:3n-3, 20:4n-6 and 22:6n-3 in the basal diet of dairy cows (Woods and Fearon, 2009) and the lower basal concentration of these FA in the blood plasma (Chapter 5.2), 18:3n-3, 20:4n-6 and 22:6n-3 are more successfully transferred into all blood lipid classes, and hence are mirrored in both FF and milk fat.



**Figure 5.** Fatty acid transport in very-low density lipoproteins (VLDL), high density lipoproteins (HDL) and non-esterified fatty acids (NEFA) bound to albumin from the blood capillary to the follicular fluid. The proportion of lipid fractions triglycerides (TG), phospholipids (PL), cholesterol-esters (CE), and cholesterol (C) and apoproteins (PR) within each lipoprotein is depicted (Adapted from Edwards, 1974; Grummer and Carroll, 1988; Argov et al., 2004).

**IMPLICATIONS FOR *IN VITRO* MATURATION MODELS IN THE BOVINE**

*In vitro* experiments focusing on the direct effect of specific FA on oocyte maturation and embryonic development have opened new perspectives to the knowledge of FA feeding. More specifically, Leroy et al. (2005) indicated the detrimental effect of saturated FA as compared to their unsaturated counterparts in an *in vitro* maturation model. Aardema et al. (2011) showed that 18:1 *cis*-9 is able to alleviate the detrimental effects of saturated fatty acids *in vitro*.

It might be important to mention that both studies used ethanol and bovine serum albumin (BSA) respectively, to add the specific FA to the *in vitro* maturation media which mimics the *in vivo* presence of NEFA in the FF. More recently, FA supplementation during *in vitro* bovine maturation showed contrasting results as supplementation of 18:2n-6 hampered (Marei et al., 2010) whereas 18:3n-3 (Marei et al., 2009) and 20:4n-6 or 20:5n-3 (Marques et al., 2007) enhanced the developmental capacity of oocytes. In all aforementioned studies, FA were added to the medium as if they were NEFA; therefore we would argue whether these *in vitro* experiments mimic the *in vivo* situation when feeding these specific FA to dairy cows. From the results of Chapter 5.2, it can be clearly seen that only a small proportion of these FA are present in NEFA as opposed to CE and PL. *In vitro* results on the direct effect of specific FA on *in vitro* oocyte maturation and embryonic development might not always mimic *in vivo* responses (Santos et al., 2008) but can help understanding contrasting *in vivo* observations. The latter was shown by Fouladi-Nashta et al. (2009) feeding different FA sources to examine the developmental potential of oocytes in high yielding dairy cows. From their results it was concluded that the ovary is capable of buffering oocytes against the effects of fluctuations in plasma n-6 and n-3 FA, resulting in only modest effects on their developmental potential.

Further research is needed to understand the different effects of mobilized (NEFA) and absorbed (mainly in PL and CE in HDL) FA on the developmental capacity of oocytes.

## IMPLICATIONS FOR *IN VIVO* MODELS IN THE BOVINE

To combine the successful transfer of 18:3n-3, 20:4n-6 and 22:6n-3 to FF (Chapter 5.2) with the negative association between high milk UFA and lower CR (Chapter 6) is more intriguing. One of the hypothesis might be an increased level of oxidative stress (OS) in the animal, resulting in an impaired fertility (Roth, 2008). Even though we did not measure OS in Chapter 6, blood samples from the animals in Chapter 5.1 were analysed in a complementary study on OS by Wullepit et al. (2012). Albeit we fed high amounts of 22:6n-3 to induce a MFD, a higher level of blood lipid peroxidation was demonstrated in dairy cows fed 22:6n-3 during the transition period. As elevated oxidative stress has been linked to both human (Guerin et al., 2001; Agarwal and Gupta, 2005) and bovine (Olson and Seidel, 2000) reproductive failure, the results from our study described in Chapter 6 might suggest that when FA are fed at levels that are able to increase the level of UFA in milk, the supplementation is associated with decreased CR and longer days open. Results from a recent study by Al Darwich et al. (2010) showed a tendency for a lower blastocyst yield with increasing 18:3n-3 addition during *in vitro* embryo culture. Furthermore, cultures to which the highest dose of 22:6n-3 and 18:2 *trans*-10, *cis*-12 had been added, gave rise to embryos with significantly lower survival rates after vitrification and thawing, which again provides evidence for deleterious effects of over supplementation with UFA. On top, we were able to show that the negative association between CR and the bulk milk UFA level was especially evident in higher producing herds. It could be hypothesized that a possibly increased level of OS concomitant with the higher level of UFA in milk, is additive to the increased level of free radicals in high yielding dairy cows (Castillo et al., 2005; Lohrke et al., 2005), subsequently further hampering fertility.

In conclusion, our results are indicative that certain aspects of UFA feeding to improve dairy cow fertility are yet to be revealed as illustrated by the negative association between the increased milk UFA levels and fertility parameters (lower CR and longer days open). Although some experiments have shown positive effects of UFA supplementation on dairy cow fertility (Santos et al., 2008; Thatcher et al., 2011), we demonstrated that increasing the UFA content of bulk tank milk should not be a goal as such as this was associated with worsened fertility parameters.

## CONCLUSIVE REMARKS & TARGETS FOR FURTHER RESEARCH

In the present thesis, we were able to shed light on some specific health and fertility challenges in high yielding dairy cows during the transition period with a particular focus on the supplementation of FA in order to tackle these challenges. Although we did answer some important questions, the following points need further clarification when implementing FA as an optimization strategy during the transition period in the dairy cow:

- ✓ We were able to demonstrate profound effects of MD on the shape of the lactation curve of dairy cows. As herd profitability is not only affected by the shape of the lactation curve, it is essential to also demonstrate the distinct effects of MD on the culling of dairy cows during (early) lactation (Ducrocq et al., 1988; Kristensen et al., 2008).
- ✓ Moreover, many experiments in early lactation are biased by exclusion (or culling) of the often high number of diseased animals. We speculate that this might not only bias the estimated effect of diseases on milk production (Chapter 4.1), but can also bias experiments with early lactating animals. By excluding diseased animals in research trials, it might be difficult to find statistically significant differences in metabolic parameters as mobilization in these cows is mainly driven by their genetics rather than by the environment. We hypothesize that the induction of a MFD might be more useful in cows suffering from environmentally driven body reserve mobilization as compared to the more intensively studied animals which remain healthy and solely suffer genetically driven mobilization.
- ✓ We clearly demonstrated that mobilized endogenous fats versus dietary supplemented fats are delivering FA to the animal and hence also to the animal's reproductive tissues in different biologically active forms, i.e. NEFA and lipoproteins. Whether these different active forms are of biological relevance when assessing e.g. the effect of specific FA on oocyte quality and embryonic development remains unclear and should be subjected to further research.

- ✓ We found a negative association between the level of UFA in the milk fat at bulk tank level and several fertility parameters which contrasts with the general assumption that feeding PUFA can be beneficial for dairy cow fertility (Santos et al., 2008; Thatcher et al., 2011). One of the hypotheses explaining this observation might be a direct deleterious effect of the increased level of OS on the oocyte when feeding PUFA to transition cows (Wullepit et al., 2012). Literature on OS when supplementing PUFA to dairy cows is rare. Future research in this area should be conducted to differentiate between positive effects of a more unsaturated FA profile of oocytes, granulosa cells and embryos (Zeron et al., 2001), versus the negative effect from which these reproductive key players might suffer as they are susceptible to OS.
  
- ✓ Whereas in Chapter 6 we focused on the overall UFA content of milk fat, we were unable to associate fertility parameters with levels of individual FA. Recently, the 22:6n-3 concentration in FF has been suggested as one of the main explanations for a higher fertility in heifers when compared to the fertility of cows (Bender et al., 2010). It would be of particular interest to associate the level of specific FA in milk and/or blood with the reproductive performance of dairy cows as has been proposed to use these levels to monitor rumen function (Vlaeminck et al., 2006; Colman et al., 2010) and ketosis (Van Haelst et al., 2008).



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# SUMMARY





The time span during which dairy cows transition from late gestation into lactation is one of the most challenging periods in their production cycle. In **Chapter 1**, we described how the high yielding dairy cows' metabolism adapts to the limited substrate availability to sustain foetal growth and lactogenesis during the transition period, generally called the negative energy balance (NEBAL). The dairy cow switches to a catabolic state, depleting body reserves which mainly consist of fatty acids (FA). However, some specific aspects in the pathophysiology of the NEBAL and its effect on production and reproduction remain unclear. Furthermore and next to FA mobilisation, supplementation of FA has been generally accepted as a management strategy to reduce the NEBAL and directly ameliorate dairy cow fertility which is comprehensively discussed in **Chapter 2**. However, these effects might be masked by different physiological processes that typically take place in early lactating dairy cows, with a special focus on the NEBAL. Therefore, in **Chapter 3**, we formulated the specific aims of the dissertation.

In the first part of our research (**Chapter 4**), we emphasised on some specific health challenges dairy cows are facing during the transition period. In **Chapter 4.1**, the effects of metabolic diseases (MD) associated with the NEBAL on milk production of dairy cows were described. These effects have been evaluated in many different ways, all too often leading to conflicting conclusions. We proposed the use of a fitted lactation model to analyze specific aspects of lactation curve shape and magnitude in cows that avoided culling or death in the first 120 days in milk. Production and health records of 1,946 lactations in a one year follow up study design were collected from a transition management facility in Germany in order to evaluate both short and long term effects of MD on milk production. Milk production data were fitted with the nonlinear MilkBot lactation model while health records were used to classify cows as healthy (H), affected by one MD (MD), or by multiple MD (MD+). The final dataset contained 1,071 H, 348 MD and 136 MD+ cows with distinct incidences of 3.7 % twinning, 4.8 % milk fever, 3.6 % retained placenta, 15.4 % metritis, 8.3 % ketosis, 2.0 % displaced abomasum and 3.7% mastitis in the first 30 DIM. The model containing all healthy and diseased cows showed that lactations classified as H have a milk production that rises faster (lower ramp) but also declines faster (lower persistence) in comparison with cows which encounter one or more metabolic problems. The level of production (scale) was only lowered in MD+

cows when compared to H and MD cows. Though the shape of the lactation curve is changed when cows encounter uncomplicated (single MD) or complicated MD (more than one MD), a slower rise to a lower peak seems to be compensated for by higher persistency resulting in the overall 305-d milk production only being lowered in MD+ cows. In the individual disease models, specific changes in the shape of the lactation curve were found for all MD except twinning. Milk fever, retained placenta, ketosis and mastitis mainly affected the lactation curve when accompanied with another MD whereas metritis and displaced abomasum affected the lactation curve equally with or without another MD. Overall, the 305-d milk production was decreased in complicated metritis ( $10,603 \pm 50$  kg vs.  $10,114 \pm 172$  kg). Though care should be taken in generalizing conclusions from studies carried out on a highly specialized transition management facility, Chapter 4.1 demonstrates that lactation curve analysis may contribute substantially to the evaluation of both short and long term effects of metabolic diseases on milk production by detecting changes in the lactation curve that are not apparent when only totals or averages are analyzed.

Preliminary to the next section which focuses on the supplementation of FA, we conducted a trial in **Chapter 4.2** to assess the main site of FA mobilisation during severe NEBAL in lactating dairy cows. Therefore we determined the FA profile in blood non-esterified FA (NEFA) as well as in the abdominal (ABD) and subcutaneous (SUBC) fat stores in dairy cows suffering from a left displacement of the abomasum (LDA). Blood, ABD and SUBC samples were taken from 50 Holstein cows offered for surgery to correct LDA. The FA profile of the 3 compartments was determined by gas chromatography after lipid extraction, methylation and, in the case of blood plasma, separation of lipid classes. The most abundant FA in all three compartments were 16:0, 18:0 and 18:1 *cis*-9, with a total proportion of 82.5, 68.0 and 74.1 g/100 g FA in ABD, NEFA and SUBC, respectively. A principal component analysis (PCA) was performed on the entire FA profile as well as on the  $\Delta$ 9-desaturase indices (14:1 *cis*-9/14:0, 16:1 *cis*-9/16:0, 18:1 *cis*-9/18:0). The PCA extracted 2 principal components (PC) representing 51.6 % (PC1) and 21.1 % (PC2) of the total variance in FA composition of the three compartments. The loading plot for the regression factors revealed a strong positive correlation between PC1 and the  $\Delta$ 9-desaturase indices, the proportions of 14:1 *cis*-9 and 16:1 *cis*-9, while negatively correlated with the proportion of 18:0 and saturated FA. The

correlation with PC2 was positive for the proportion of unsaturated FA, 18:2n-6 and 18:3n-3 and negative for the proportion of 14:0, 16:0 and saturated FA. The SUBC could be distinguished from the NEFA and ABD by a positive score for PC1 while differentiation among the latter 2 compartments could be made by a positive (NEFA) or negative (ABD) score for PC2. The  $\Delta 9$ -desaturase indices for C14 and C16 differed between all compartments but were numerically closer for NEFA and ABD versus NEFA and SUBC. The desaturase indices of the main FA (18:1 *cis*-9 and 18:0) did not differ between NEFA and ABD. In conclusion, these results suggest the existence of a different FA composition in ABD versus SUBC. The findings of a greater similarity between the FA profiles of ABD and NEFA compared to SUBC and NEFA and the closer desaturase indices of ABD and NEFA, support our hypothesis of a preferential mobilization of ABD fat in dairy cows during severe NEBAL.

Unsaturated FA cannot be synthesized by mammalian cells due to a lack of desaturase enzymes. Combined with their limited supply to the small intestines, unsaturated FA have been proposed as nutraceuticals to ameliorate dairy cow fertility. In **Chapter 5** we focused on the intriguing interaction between supplemented unsaturated FA and the metabolic adaptation towards lactation in early lactating dairy cows. Therefore, in the first part (**Chapter 5.1**), sixteen Holstein cows were assigned to 2 groups to evaluate the caloric and metabolic effect of feeding marine algae (ALG) from 3 weeks pre partum until 12 weeks post partum. Milk production characteristics and the profiles of hormones and metabolites in the serum were monitored from -7 to 46 days in milk and in follicular fluid (FF) from 14 to 46 days in milk. All cows received a corn- and grass silage based partially mixed ration supplemented with concentrates and a protein supplement. In the diet of the ALG group, 2 kg of the concentrates were replaced by a concentrate containing 44 g docosahexaenoic acid. Diets were isocaloric (net energy basis) and equal in intestinal digestible protein. ALG increased milk yield, whereas milk fat yield and milk fat content decreased. Protein yield was not affected but a tendency towards reduced milk protein content was observed. Marine algae supplementation increased the  $\beta$ -hydroxy butyric acid (BHBA) concentration in the FF of the ALG compared to the control. The total protein concentration in FF was decreased in the ALG. The plasma and serum metabolites did not significantly differ between treatments except for a tendency towards a lower urea concentration in the serum of the control

versus the ALG. Based on metabolizable energy calculations, a daily energy sparing effect of 3.48 MCal was obtained due to the milk fat depression (MFD). The concomitant milk yield increase suggests that at least part of this spared energy is used to stimulate milk production. Theoretically, 3.48 MCal of ME could lead to an increase in milk yield of 7.43 kg/d which is higher than the observed 3 kg/d. However, when evaluating nutrient requirements during MFD in early lactation, we calculated that increased milk production is caused by a propionate saving effect of 2.71 mol in the udder when milk fat is depressed. Concurrent increased BHBA concentration in the FF in the ALG cannot be attributed to a worsened energy status of the animals as all other indicators contradict any change in energy balance, indicating that BHBA might not be an appropriate metabolic parameter to estimate the energy balance in early lactating dairy cows during MFD.

In the previous chapter, we showed an effect of a MFD on production in early lactation. Therefore in **Chapter 5.2** we aimed to feed fat supplements, varying in saturation and length of the FA during the transition and early breeding period without altering milk production variables. Production variables and the FA composition of blood plasma (BP), blood cholesterol esters (CE), NEFA, phospholipids (PL) and triacylglycerols (TAG), FF and milk fat (MF) were determined. Eighteen Holstein Friesian dairy cows were assigned in a randomized complete block design to one of three isoenergetic, isonitrogenous and isolipidic diets containing either palm prills (CON), sequential 18:2n-6 and 18:3n-3 (SHORT), or sequential 20:4n-6 and 22:6n-3 (LONG). The fat supplements were fed from approximately 14d before parturition until 46 d in milk. Production variables (dry matter intake, milk production and content, body condition score) did not differ between treatments throughout the experimental period. Metabolic parameters in BP and FF did not differ between treatments except the BP urea content which was higher in LONG versus SHORT. Feeding SHORT increased 18:2n-6 in MF compared with LONG but not CON. Effects on BP fractions were minimal except for the increased BP TAG 18:2n-6 content compared with CON prepartum. Compared to CON, feeding SHORT increased the 18:3n-3 content in BP, FF and MF 1.25, 1.46 and 1.66 times respectively which was reflected in a higher 18:3n-3 content in all BP fractions on d 46 except TAG. Feeding LONG increased the 20:4n-6 in BP compared with SHORT, and in the FF compared with SHORT and CON, but not in MF. As opposed to 18:2n-6, this was

reflected in CE and PL but not NEFA and TAG. Furthermore, compared with CON a 2.6, 4.1 and 2.5-fold increase in 22:6n-3 was observed in BP, FF and MF respectively, when feeding LONG. This was consistently reflected in all BP fractions. The follicular environment and mammary gland seem to respond in a different way to supplemental SHORT and LONG n-6 and n-3, most probably due to discrimination against specific FA in the different lipid classes. We were able to show limited effects on the FA 18:2n-6 proportion in BP and FF, while substantially increasing 18:3n-3 in BP and FF when feeding SHORT. In contrast, sequential feeding of LONG n-6 and n-3 FA did increase both 20:4n-6 and 22:6n-3 in BP and FF without disturbing animal performance.

In Chapter 5.1 and 5.2 we clarified some unique challenges when feeding unsaturated FA to dairy cows in early lactation. However, field studies based on a large number of dairy cows that support our main hypothesis of improved fertility by feeding unsaturated FA, are lacking. Therefore the aim of **Chapter 6** was to analyse a large dataset containing individual cow fertility records from dairy herds and link fertility key-performance-indicators like conception rate to first insemination (CRFI), days in milk to first insemination (DIMFI) and days in milk to conception (DIMCONC), to the level of UFA in bulk tank samples, the latter being a proxy for the dietary fatty acid profile on these herds. Within the two year study period, information from 15,055 lactations and 35,433 bulk tank milk samples was collected on 90 herds. The multilevel logistic regression model used, revealed a decreased CRFI on herds with a higher bulk tank unsaturated FA level. The decrease in CRFI was larger for higher producing herds. Increased bulk tank unsaturated FA was furthermore associated with higher DIMFI which, together with the lower CRFI, subsequently increased DIMCONC. Interestingly, the higher variability in unsaturated FA, expressed by an increased coefficient of variation, was associated with an increased CRFI and decreased DIMFI and DIMCONC. In conclusion, the present study demonstrates that increasing the unsaturated FA content of milk should not be a goal as such when supplementing unsaturated FA to dairy cows, as higher bulk tank unsaturated FA are associated with worsened fertility results.

In the final chapter of this dissertation, we comprehensively discussed the results of our studies and placed them into the most current scientific perspectives. From this, the following conclusions can be drawn:

- ✓ Lactation curve analysis can serve as a useful tool to evaluate short and long term effects of metabolic diseases during the transition period on milk production. Subsequent culling analysis after metabolic diseases should allow for a better estimation of the possible economic losses as e.g. milk fever and retained placenta were shown to have limited effects on milk production.
- ✓ During severe NEBAL in the transition period, FA are mobilised preferentially from the abdominal fat.

When supplementing FA to dairy cows to improve reproduction, the following considerations should be taken:

- ✓ Substantial knowledge is lacking to implement the induction of a MFD in early lactation as a general practice in high yielding dairy cows to reduce the NEBAL.
- ✓ Supplemented n-6 and n-3 FA are successfully transferred into all blood lipid classes, and hence are mirrored in both FF and milk fat.
- ✓ Our data questions whether dairy cows which possess evolved mechanisms to conserve essential FA within their body, are being overfed with FA supplements hampering their reproductive capacity by mechanisms which should be subject of further research.







# SAMENVATTING



De overgang van dracht naar lactatie, is één van de meest uitdagende periodes in de lactatiecyclus van een hoogproductieve melkkoe. In **Hoofdstuk 1** van dit doctoraat werd beschreven op welke manier een melkkoe tijdens deze zogenaamde transitieperiode haar metabolisme afstemt op een beperkte aanvoer van nutriënten, eerst voor de foetale groei en later voor de toenemende melkproductie. Deze nutritionele imbalance die wordt gekenmerkt door een algemeen energietekort wordt vaak aangeduid als de negatieve energie balans (NEBAL). In eerste instantie zal de melkkoe hierop reageren door haar lichaamsreserves aan te spreken en voornamelijk lichaamsvet onder de vorm van vetzuren (VZ) te mobiliseren in het bloed. Een aantal aspecten met betrekking tot de pathogenese van de NEBAL en het effect van de hiermee geassocieerde metabole stoornissen op de melkproductie en de vruchtbaarheid zijn momenteel nog niet volledig duidelijk. VZ worden steeds vaker via de voeding gesupplementeerd aan hoogproductieve melkkoeien en dit met het doel om zowel indirect een vermindering van de NEBAL te verkrijgen als rechtstreeks de vruchtbaarheid te verbeteren (**Hoofdstuk 2**). Het is echter nog niet volledig uitgeklaard hoe de mobilisatie van lichaamsvet aan het begin van de lactatie, de directe effecten van gesupplementeerde VZ kunnen beïnvloeden of deze zelfs volledig kunnen maskeren. De algemene doelstelling van deze doctoraatsthesis was om een beter inzicht te verwerven inzake enkele specifieke aspecten van de transitieperiode bij melkkoeien, in het bijzonder de mogelijkheid tot supplementatie van VZ via het rantsoen. Verder werden in **Hoofdstuk 3** een aantal specifieke doelstellingen voor dit proefschrift gedefinieerd om de in Hoofdstuk 1 en 2 gestelde vragen te beantwoorden.

In het eerste deel van het onderzoek (**Hoofdstuk 4**) lag de nadruk op enkele metabole uitdagingen die melkkoeien doormaken tijdens de transitieperiode. Zo werd in **Hoofdstuk 4.1** het effect van metabole stoornissen geassocieerd met de NEBAL, op de melkproductie onderzocht. De gevolgen van metabole stoornissen zijn eerder reeds op veel verschillende manieren geëvalueerd, wat geleid heeft tot tegenstrijdige resultaten in de literatuur. In onze studie werd gebruik gemaakt van het recent ontwikkelde 'Milkbot' model om zowel de vorm als de hoogte van de lactatiecurve van hoogproductieve koeien (die niet opgeruimd werden in de eerste 120 dagen van hun lactatie) te analyseren. Daartoe werden de melkproductie- en gezondheidsgegevens van 1,946 lactaties verzameld tijdens een één jaar durende studie op een Duits melkveebedrijf dat beschikte over aangepaste afkalf-faciliteiten (TMF: Transition

Management Facility). Op basis van de beschikbare gezondheidsgegevens werden de koeien ingedeeld in gezonde koeien (G; n=1,071), koeien met één metabole stoornis (MD; n=348), en koeien met meerdere metabole stoornissen (MD+; n=136). Bij 3.7% van de dieren werd een tweelinggeboorte geregistreerd, bij 4.8% kalfziekte, bij 3.6% retentio secundinarum, bij 15.4% een baarmoederontsteking, bij 8.3% slepende melkziekte, bij 2.0% een lebmaagverplaatsing en bij 3.7% een mastitis. Het finaal model dat alle ziektes omvatte, toonde aan dat gezonde koeien in vergelijking met koeien die één of meerdere metabole stoornissen hadden gekregen een snellere melkproductiestijging combineren met een lagere persistentie. De melkproductiepiek was enkel lager voor koeien die leden aan meerdere metabole stoornissen (MD+). Hoewel de vorm van de lactatiecurve in het algemeen negatief beïnvloed werd door het voorkomen van metabole stoornissen, werd de trage stijging naar een lagere melkproductiepiek over het algemeen gecompenseerd door een hogere persistentie. Dit resulteerde erin dat de totale 305d productie enkel lager was voor de MD+ koeien. Voor alle individuele ziektemodellen werd een effect op de lactatiecurve aangetoond behalve in het geval van een tweeling. Kalfziekte, retentio secundinarum, slepende melkziekte en mastitis beïnvloedden de lactatiecurve voornamelijk wanneer ze vergezeld waren van een andere stoornis, daar waar een baarmoederontsteking en een lebmaagverplaatsing de lactatiecurve zowel in de ongecompliceerde (MB) als gecompliceerde groep (MB+) beïnvloedden. Het effect op de totale productie was opnieuw beperkt met enkel een lagere 305d productie in het geval van een gecompliceerde metritis ( $10,603 \pm 50$  kg vs.  $10,114 \pm 172$  kg). Dit toont de beperktheid aan van het gebruik van totale lactatieproducties bij het inschatten van het effect van metabole stoornissen op de melkproductie. Hoewel men behoedzaam moet zijn bij het extrapoleren van gegevens die werden verkregen op een bedrijf met aangepaste afkalf-faciliteiten, toont deze studie aan dat het gebruik van lactatiecurve-analyse een accurate tool kan zijn om de korte en lange termijn gevolgen van metabole stoornissen op de melkproductie exact in te schatten.

Vervolgens werd een studie uitgevoerd die als doel had de oorsprong van de gemobiliseerde vetzuren tijdens een ernstige NEBAL te achterhalen (**Hoofdstuk 4.2**). Hiertoe werd het VZ-profiel van het gemobiliseerd vet in het bloed (NEFA) vergeleken met het VZ-profiel van abdominaal (ABD) en subcutaan (SUBC) vet van melkkoeien met

een lebmaagverplaatsing ( $n = 50$ ). De VZ-profielen werden bepaald door middel van gaschromatografie na VZ-extractie, methylatie en in het geval van het bloed, scheiding van de NEFA fractie. De meest voorkomende VZ waren 16:0, 18:0 en 18:1 *cis*-9, die samen goed waren voor een respectievelijke bijdrage van 82.5, 68.0 en 74.1g/100g vetzuren in ABD vet, NEFA en SUBC vet. Daarnaast werd een principale componenten analyse (PCA) uitgevoerd op het volledige VZ-profiel en de  $\Delta$ 9-desaturase indexen (14:1 *cis*-9/14:0, 16:1 *cis*-9/16:0, 18:1 *cis*-9/18:0). Met deze analyse werden 2 principale componenten (PC) geëxtraheerd waarvan de eerste (PC1) 51.6% van de totale variatie in het VZ-profiel verklaarde. Op basis van de regressie factoren van PC1 kon een scheiding gemaakt worden tussen SUBC enerzijds en NEFA en ABD anderzijds. Principale component 2, die een kleinere fractie van de totale VZ-variatie verklaarde (21.2%), liet toe onderscheid te maken tussen NEFA en ABD. Daarnaast waren de C14- en C16- $\Delta$ 9-desaturase indexen tussen NEFA en ABD meer vergelijkbaar dan tussen NEFA en SUBC en was de  $\Delta$ 9-desaturase index van C18 niet verschillend tussen NEFA en ABD. De bevindingen van een meer vergelijkbaar VZ-profiel tussen ABD en NEFA ondersteunen onze initiële hypothese waarin we preferentiële VZ-mobilisatie vanuit ABD versus SUBC aannamen bij melkkoeien tijdens een ernstige NEBAL.

Onverzadigde VZ (OVZ) kunnen niet aangemaakt worden door de (meeste) lichaamscellen van zoogdieren vanwege de afwezigheid van bepaalde desaturase enzymes. Gecombineerd met een beperkte aanvoer via de dunne darm, heeft dit ertoe geleid dat in de laatste decennia, geopperd werd dat OVZ essentieel zijn in het rantsoen van hoogproductief melkvee. In het tweede luik van dit proefschrift (**Hoofdstuk 5**) werd daarom verdergegaan op de intrigerende interactie tussen supplementatie van VZ via het rantsoen en de metabole veranderingen in het begin van de lactatie. In een eerste deel (**Hoofdstuk 5.1**) werd het calorisch en metabool effect van een melkvetdepressie (MVD) onderzocht. Daartoe werden de melkproductie (week 0 t/m 12), enkele metabole parameters in het serum (dag -7 t/m 46 tov het kalven) en het follikelvocht (dag 14 tem 46) geanalyseerd bij 16 Holstein koeien, die een rantsoen kregen dat aangerijkt was met marine algen (ALG) versus een iso-energetisch en iso-stikstof controle rantsoen (CON). Alle koeien kregen hetzelfde op gras- en maaskuil gebaseerde basisrantsoen aangevuld met evenwichtig en eiwitrijk krachtvoeder. In het experimentele rantsoen (ALG) werd 2 kg evenwichtig krachtvoeder vervangen door een krachtvoeder aangerijkt met 44 g 22:6n-3 (DHA). In de ALG groep was de melkproductie hoger, terwijl zowel de

totale opbrengst als het gehalte aan melkvet lager waren. De eiwitopbrengst bleef ongewijzigd ondanks de trend voor een lager eiwitgehalte in de ALG. Door het voederen van ALG steeg het beta-hydroxy-boterzuur (BHB) gehalte en daalde het totaal eiwitgehalte in het follikelvocht. De metabole parameters in het serum waren niet verschillend behalve een trend tot een hoger ureum gehalte in de ALG. Theoretisch spaarde de MVD in dit experiment gemiddeld 3.48 MCal metaboliseerbare energie per koe per dag uit, wat kan omgezet worden in een melkproductiestijging van 7.43 kg/d. Een metabole benadering van een MVD in het begin van de lactatie is echter beter in staat het melkproductieverschil in te schatten. Zo berekenden wij dat door de MVD op het niveau van de uier 2.71 mol propionzuur werd gespaard wat slechts kan leiden tot een melkproductiestijging van 2.95 kg/d wat dichterbij de waargenomen 3 kg/d. Daarenboven wordt tijdens een MVD ook minder BHB gebruikt door de uier wat aanleiding geeft tot een gestegen BHB gehalte zoals waargenomen in het follikelvocht in dit experiment. Dit stelt het gebruik van BHB als geschikte indicator van de NEBAL tijdens MVD in vraag.

Aangezien in het vorige hoofdstuk een duidelijk effect aangetoond werd van een MVD, werd in **Hoofdstuk 5.2** getracht VZ, verschillend in lengte en saturatiegraad, te supplementeren tijdens de transitie en pre-inseminatie periode zonder de melkproductie te wijzigen. Achttien melkkoeien werden in een gerandomiseerd block design toegewezen aan één van de 3 rantsoenen gelijk in energie, eiwit- en vetgehalte met of enkel palmolie (CON), of opeenvolgend 18:2n-6 en 18:3n-3 (KORT) of opeenvolgend 20:4n-6 en 22:6n-3 (LANG). De VZ werden gevoederd vanaf 2 weken voor de vermoedelijke kalfdatum tot en met week 7 van de lactatie. Daarop volgend werd de VZ-samenstelling van het bloedplasma, de verschillende VZ-fracties (cholesterolesters, NEFA, fosfolipiden, en triacylglycerol), het follikelvocht en het melkvet bepaald. Tussen de 3 groepen, konden er geen verschillen in melkproductie en -gehalten, droge stof opname en body conditie score worden gevonden. Geen van de metabole parameters was verschillend noch in het bloed noch in het follikelvocht, behalve een hoger ureumgehalte in het serum bij de KORT vergeleken met de LANG. Het 18:2n-6 gehalte in het bloed was gestegen door het voeren van KORT vergeleken met LANG. In de vetfracties van het bloed werd een toegenomen 18:2n-6 gehalte in de triacylglycerolen gevonden voor het kalven. Na het omschakelen naar n-3 VZ steeg het 18:3n-3 gehalte in

het bloed, in het follikelvocht en in het melkvet respectievelijk 1.25, 1.46 en 1.66 maal. Dit werd weerspiegeld in alle vetfracties in het bloed, op de triacylglycerolen op dag 46 na. Het voederen van LANG zorgde voor een stijging van 20:4n-6 in het bloed vergeleken met KORT, in het follikelvocht vergeleken met zowel KORT als CON maar niet in het melkvet. In tegenstelling met de kleine verschillen in 18:2n-6 waargenomen in de KORT, werd het 20:4n-6 voederen duidelijk weerspiegeld in de cholesterolesters en fosfolipiden van het bloed maar niet de NEFA of triacylglycerolen. Na het omschakelen naar n-3 VZ werd het 22:6n-3 gehalte in het bloed, follikelvocht en melkvet 2.6, 4.1 en 2.5-maal hoger, wat opnieuw weerspiegeld werd in alle fracties van het bloed. Uit deze bevindingen kan besloten worden dat het follikelvocht en de uier op een verschillende manier reageren op een verhoogde aanvoer van n-6 en n-3 VZ. Dit is waarschijnlijk een gevolg van een selectieve opname van VZ uit bepaalde vetfracties in het bloed die elk verschillen in VZ-samenstelling. Samengevat toonden we aan dat het supplementeren van KORT weinig effect heeft op de 18:2n-6 samenstelling van het bloed en het follikelvocht daar waar er een duidelijke aanrijking met 18:3n-3 plaats vindt. In tegenstelling hiermee, leidt het supplementeren van LANG tot een duidelijke stijging van 20:4n-6 en 22:6n-3 in zowel het bloed als het follikelvocht.

In de twee voorgaande hoofdstukken werden enkele specifieke aspecten van het supplementeren van OVZ aan melkkoeien in het begin van de lactatie toegelicht. Echter, grote veldstudies waarmee beoogd werd het uiteindelijke effect van het supplementeren van OVZ tijdens de lactatie op de vruchtbaarheid te onderzoeken, ontbraken op dat moment. Daarom werd in **Hoofdstuk 6** een grote dataset onderzocht met als doel de vruchtbaarheidsresultaten van 90 Belgische bedrijven te koppelen aan het percentage OVZ op tankmelk niveau. Gedurende 2 jaar werd op deze bedrijven van in totaal 15,055 lactaties de kans op drachtigheid van de eerste inseminatie, het aantal dagen tot de eerste inseminatie en het aantal dagen tot de conceptie onderzocht. In diezelfde periode was het percentage OVZ van 35,433 tankmelkstalen van de betreffende bedrijven beschikbaar. Het multilevel logistische regressie model toonde aan dat de kans op drachtigheid van de eerste inseminatie op bedrijven met een hoger OVZ-gehalte op tankniveau lager was. Deze daling was meer uitgesproken op hoogproductieve bedrijven. Verder was een hoger OVZ-gehalte geassocieerd met een hoger aantal dagen tot de eerste inseminatie wat in combinatie met het voorgaande, leidde tot een hoger aantal dagen tot de conceptie. Met deze studie kon worden aangetoond dat het verhogen



van het OVZ-gehalte van de melk geen doel op zich mag zijn bij het supplementeren van OVZ aangezien dit over het algemeen geassocieerd is met minder goede vruchtbaarheidsresultaten. Opmerkelijk was echter dat in het finale model, meer variatie in het OVZ gehalte van de tankmelk zorgde voor een stijging van de kans op drachtigheid van de eerste inseminatie, net als een lager aantal dagen tot eerste inseminatie én dracht. De exacte oorzaak voor deze associatie dient verder onderzocht te worden. In het laatste deel van dit proefschrift werden de verschillende deelaspecten van het onderzoek samengevat en werden deze getoetst aan de literatuur. Aldus kunnen de volgende conclusies geformuleerd worden:

- ✓ Lactatie curve analyse is een goede methode om korte en lange termijn gevolgen van metabole stoornissen op de melkproductie in te schatten. Onze analyse toonde aan dat bepaalde metabole stoornissen nagenoeg geen effect hebben op de melkproductie (bijv. kalfziekte en retentio secundinarum in niet gecompliceerde vorm) waardoor de economische verliezen beperkt blijven. De gevolgen van metabole stoornissen reiken echter verder dan de melkproductie. Zo kan een bijkomende analyse van het vervangingspercentage helpen om de totale economische impact van metabole stoornissen beter in te schatten.
- ✓ Tijdens een ernstige NEBAL worden VZ preferentieel gemobiliseerd vanuit het abdominale lichaamsvet.

Bij het supplementeren van VZ aan melkkoeien om de reproductie te verbeteren, dient met het volgende rekening te worden gehouden:

- ✓ Op dit moment is er te weinig wetenschappelijk bewijs om standaard bij alle hoogproductieve melkkoeien in het begin van de lactatie een MVD te induceren met als doel het verminderen van de NEBAL.
- ✓ Na supplementatie is er een duidelijke aanrijking van het follikelvocht aan n-6 en n-3 VZ.
- ✓ Onze experimenten en studies doen de vraag rijzen of koeien, die van nature uit mechanismen ontwikkeld hebben om essentiële VZ binnen het lichaam te houden, niet 'over-gesupplementeerd' kunnen worden met OVZ waardoor hun vruchtbaarheid eerder verminderd dan wel verbeterd wordt. Hoe dit precies plaats vindt moet nog verder onderzocht worden.





# CURRICULUM VITAE



Miel Hostens werd geboren op 27 april 1982 te Gent. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Lievenscollege te Gent in de richting Latijn-Wetenschappen, begon hij in 2000 met de studie Diergeneeskunde aan de Universiteit Gent. Hij behaalde zijn diploma van Dierenarts in 2006 met onderscheiding, optie Herkauwers.

Na een 7 maanden durende tewerkstelling als dierenarts bij Dierenkliniek Den Ham in Nederland, werd hij in september 2007 te werk gesteld aan de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde van de Faculteit Diergeneeskunde te Gent. Hij werkte er gedurende 4 jaar als doctoraatsstudent in het kader van een IWT-landbouw project getiteld "Inductie van melkvetdepressie dmv specifieke vetzuren voor vermindering van de negatieve energiebalans in het begin van de lactatie van hoogproductief melkvee en relatie met vruchtbaarheid". In 2011 werd hij aanvaard als voltijds assistent Bedrijfsdiergeneeskunde. Zijn taak bestond uit het afwerken van zijn onderzoek, het geven van klinisch onderwijs aan de laatstejaarsstudenten diergeneeskunde en dienstverlening bij klanten van de Buitenpraktijk en Bedrijfsbegeleiding. Daarnaast participeerde hij actief in de nacht- en weekend-diensten.

Miel Hostens is auteur en mede-auteur van diverse wetenschappelijke publicaties in (inter)nationale tijdschriften en stelde zijn onderzoek voor op verschillende (inter)nationale congressen. Tevens is hij reviewer voor diverse veterinaire tijdschriften.



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