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MILK FATTY ACIDS AS EARLY BIOMARKERS OF REPRODUCTION PROBLEMS IN DAIRY CATTLE

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PREFACE

"It's a lot easier to do good work when you have good words to say and work with good people."
Mark Harmon

Het einde is in zicht, maar daarbij jammer genoeg ook het einde van mijn studententijd. Daarom neem ik, tussen het verzetten van de spreekwoordelijke laatste loodjes door, graag even de tijd om enkele mensen te bedanken.

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Sasitorn, the girl who is always smiling. I was so joyous the day you arrived in Germany. Further, you were always willing to help me with my questions. So thank you very much, I hope you'll have a great time left here in Belgium and can accomplish all your dreams in Thailand. ขอบคุณ (that should be thank you according to Google translate).

Natürlich muss ich auch Martin Moos und sein Team in Deutschland danken. Ohne sie könnte ich dieser Arbeit nicht anfangen. Es wäre fünf intensiven Wochen in Deutschland, aber ich habe auch sehr viel gelernt. Danke!

Muti und Vati. Mijn weg was er een met veel hobbels en putten, maar we zijn er geraakt! Dit jaar was misschien niet het gemakkelijkste jaar voor het gezin, maar we kunnen nu allemaal vooruitkijken naar wat de toekomst ons nog te brengen heeft. Mamaatje, merci voor jouw eeuwige steun, je staat er altijd voor iedereen en krijgt daar misschien niet vaak genoeg een dankjewel voor, maar bij deze: dank je voor alles!

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Fien Lannoo
Juni 2014

ABSTRACT

Most dairy cows in the periparturient period endure a state of negative energy balance (NEB), which can result in metabolic disorders and a decreased reproductive performance. The aim of this study was to assess on the one hand the potential of milk fatty acids to diagnose detrimental blood plasma non-esterified fatty acids (NEFA) and subclinical ketosis (SCK) and on the other hand the potential of these milk fatty acids to diagnose cows with impaired reproductive performance, based here on the number of artificial inseminations ('times bred' TBRD).

The dataset consisted of 141 early lactating cows from a single farm which were calving between September 4th and September 25th and of which veterinary and specific dietary treatments and other relevant data were recorded. Milk and blood were sampled at day 3 and day 10 after parturition. Milk was analysed for milk fatty acids, blood plasma for β-hydroxybutyrate (BHBA) and NEFA. Data were classified into three groups, i.e. "at risk of SCK" (BHBA ≥ 1.2 mmol/L), "at risk of detrimental blood plasma NEFA" (NEFA ≥ 0.6 mmol/L) and "at risk of high insemination number" (TBRD > 3). Due to the low prevalence rate of subacute ketotic cases, the potential for its diagnosis based on milk biomarkers was not considered further in this master dissertation.

First, the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g milk fatty acid methyl esters (MFAME), as obtained from a former experiment at the department (Jorjong et al. in preparation) was validated as benchmark to classify observations of detrimental blood plasma NEFA (≥ 0.6 mmol/L) of the current dataset at both day 3 and day 10 after parturition. The classification performance indicators sensitivity, specificity and overall accuracy of the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME to classify detrimental blood plasma NEFA were 71%, 77% and 76%, respectively, at day 3 after parturition and 46%, 88% and 81%, respectively, at day 10 after parturition.

Secondly, the potential of this threshold to classify the observations according to the times bred (TBRD ≤ 3 and TBRD > 3) was examined. However, the performance indicators sensitivity, specificity and overall accuracy from the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME to classify the observations according the times bred (TBRD > 3) were poor. Therefore, an assessment was made whether other milk fatty acids could be identified to optimise classification. Concentrations of 45 milk fatty acids as well as milk fat C18:1 *cis*-9/C15:0 and anteisoC15:0/anteisoC17:0 ratio were subjected to a discriminant analysis. Milk fat anteisoC15:0 and C18:1 *cis*-9/C15:0 ratio revealed to be the most discriminating variables to identify high insemination number (TBRD > 3) at day 3 and day 10 after parturition, respectively, but led up to a sensitivity, specificity and overall accuracy of at most 57%, 78% and 68%, respectively.

A visual evaluation further allowed to easily assess the potential of the milk fatty acid characteristics to distinguish observations based on blood NEFA concentrations or TBRD with a false positive rate of 10% at maximum. The thresholds of milk C18:1 *cis*-9, anteisoC15:0 and C18:1 *cis*-9/C15:0 ratio with such low false positive rate, corresponded to false negative rates ranging from 22 to 35% at day 3 after parturition and 39 to 50% at day 10 after parturition for blood NEFA. But false negative rates for diagnosis of TBRD above 3 ranged from 57% to 75%, which is unacceptably high.

Hence, milk fat C18:1 *cis*-9 is an interesting biomarker for detrimental blood plasma NEFA (≥ 0.6 mmol/L) but this or other single milk fatty acids measured at an early stage after parturition seem insufficient to warn for an unacceptably high number of inseminations (TBRD > 3).

SAMENVATTING

De meeste melkkoeien krijgen de periode na het afkalven te maken met een negatieve energiebalans (NEB), wat kan leiden tot metabole stoornissen en verminderde reproductieve prestaties. Het doel van deze studie was het nagaan van enerzijds de mogelijkheid van melkvetzuren om nadelige bloedplasma concentraties van vrije vetzuren (VVZ) en subklinische ketose (SKK) te diagnosticeren en anderzijds het potentieel van deze melkvetzuren om koeien met een verminderde reproductieve prestatie te diagnosticeren, hier op basis van het aantal kunstmatige inseminaties ('Times Bred' TBRD).

De dataset bestond uit 141 vroeg-lactaire melkkoeien afkomstig van één bedrijf, die allen afkalfden tussen 4 en 25 september en waarvan de veterinaire behandelingen, specifieke voedersupplementaties en andere relevante gegevens werden geregistreerd. Melk en bloed werden bemonsterd op dag 3 en dag 10 na afkalven. Melk werd geanalyseerd voor de melkvetzuren, bloedplasma voor β-hydroxyboterzuur (BHB) en VVZ. Gegevens werden ingedeeld in drie groepen, namelijk "risico op SKK" (BHB ≥ 1,2 mmol/L), "risico op nadelige bloedplasma VVZ" (VVZ ≥ 0,6 mmol/L) en "het risico op groot aantal inseminaties" (TBRD > 3). Door de lage prevalentie van subacute ketotische gevallen werd het potentieel voor de diagnose van SKK op basis van melkbiomerkers verder buiten beschouwing gelaten in deze masterproef.

Ten eerste werd het melkvet Δ C18:1 *cis*-9 criterium van 4,11 g/100g melkvetzuur methyl esters (MVZME), zoals verkregen uit een vroeger experiment van de vakgroep, gevalideerd als drempelwaarde om waarnemingen met schadelijke bloedplasma VVZ (≥ 0,6 mmol/L) van de huidige dataset te classificeren op zowel dag 3 als dag 10 na afkalven. De classificatie prestatie-indicatoren sensitiviteit, specificiteit en algemene nauwkeurigheid van het melkvet Δ C18:1 *cis*-9 criterium van 4,11 g/100g MVZME om observaties met nadelige bloedplasma VVZ in te delen, waren 71%, 77% en 76%, respectievelijk, op dag 3 na afkalven en 46%, 88% en 81%, respectievelijk, op dag 10 na afkalven.

Ten tweede werd het potentieel van deze drempelwaarde onderzocht om observaties van aantal kunstmatige inseminaties te classificeren (TBRD ≤ 3 en TBRD > 3). De prestatie-indicatoren sensitiviteit, specificiteit en algemene nauwkeurigheid van het melkvet Δ C18:1 *cis*-9 criterium waren echter teleurstellend. Daarom werd onderzocht of er niet andere melkvetzuren geïdentificeerd konden worden die beter waren in het classificeren van groot aantal inseminaties. De concentraties van 45 melkvetzuren, evenals de melkvet verhoudingen C18:1 *cis*-9/C15:0 en anteisoC15:0/anteisoC17:0 werden onderworpen aan een discriminantanalyse. Melkvet anteisoC15:0 en C18:1 *cis*-9/C15:0 bleken de meest discriminerende variabelen om observaties met groot aantal inseminaties te classificeren op dag 3 en dag 10 na afkalven, respectievelijk, maar leidden tot een sensitiviteit, specificiteit en algehele nauwkeurigheid van hoogstens 57%, 78% en 68%, respectievelijk.

Een visuele beoordeling liet verder toe om het potentieel in te schatten van de melkvetzuurparameters om observaties in te delen op basis van bloed VVZ concentraties of TBRD met een maximaal percentage fout-positieven van 10 %. De drempelwaardes van melk C18:1 *cis*-9, anteisoC15:0 en C18:1 *cis*-9/C15:0 met zo'n laag percentage fout-positieven, kwamen overeen met een percentage fout-negatieven variërend tussen 22-35 % op dag 3 na afkalven en 39-50 % op dag 10 na afkalven voor bloed VVZ. Het percentage fout-negatieven voor de diagnose van meer dan 3 inseminaties varieerde echter van 57% tot 75%, wat onaanvaardbaar hoog is.

Als conclusie is melkvet C18:1 *cis*-9 een interessante biomerker voor schadelijke bloedplasma concentraties van VVZ (≥ 0,6 mmol/L), maar C18:1 *cis*-9 of andere melkvetzuren gemeten in een vroeg stadium na afkalven, lijken onvoldoende om te waarschuwen voor een onaanvaardbaar hoog aantal inseminaties (TBRD > 3).

OBJECTIVES

The role of animal welfare in livestock production is becoming more extensive and hereby are optimal health and fertility an important factor. In this respect, biomarkers are increasingly discussed for monitoring health. Indeed, current research indicates that specific fatty acids in milk fat can indicate for increased blood concentrations of non-esterified fatty acids (NEFA), which are indicative of a detrimental energy status of the early lactating dairy cow, which can have an adverse effect on oocyte and early embryonic development and hence on fertility. However, a direct link between the specific milk fatty acids and fertility has not yet been reported.

The aim of this study was to assess on the one had the potential of milk fatty acids to diagnose detrimental blood plasma non-esterified fatty acids (NEFA) and subclinical ketosis (SCK) and on the other hand the potential of these milk fatty acids, monitored at an early stage in lactation to warn for impaired reproductive performance. Since fertility in high milk producing dairy cows is impacted by many events, there is a need of a large number of data to have solid and reliable results concerning the key performance indicators for reproduction, preferentially with minimal confounding effects such as difference in management and ration. Therefore, sampling in the context of this master dissertation took place at the dairy farm Gut Hohen Luckow (Satow, Germany) that consists of a large number of dairy cows (approximately 2000), which allowed to follow 141 calvings within a limited period of 3 weeks and to collect milk and blood samples during early lactation.

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LIST OF ABBREVIATIONS

AA	Amino Acids
AcAc	Acetoacetic Acid
AI	Artificial Insemination
APO	Apo lipoprotein
BCS	Body Condition Score
BHBA	Beta-Hydroxybutyrate
CoA	Coenzyme A
DMI	Dry Matter Intake
E_2	Estradiol
FSH	Follicle Stimulating Hormone
GC	Gas Chromatographic analysis
GH	Growth Hormone
GHR	Growth Hormone Receptors
GI	Gastrointestinal
GLUT	Glucose Transporter
IGF	Insulin-like Growth Factor
LH	Luteinizing Hormone
MFAME	Milk Fatty Acid Methyl Esters
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
PA	Propionic Acid
PEB	Positive Energy Balance
PG	Propylene Glycol
SCK	Subclinical Ketosis
TBRD	Times Bred
TAG	Triacyl Glycerol (triglycerides)
TG	Triglycerides
TMR	Total Mixed Ration
VFA	Volatile Fatty Acid
VLDL	Very Low Density Lipoproteins

Chapter 1

LITERATURE REVIEW

1. THE HIGH-YIELDING DAIRY COW: EVOLUTION OF PRODUCTIVE AND REPRODUCTIVE PERFORMANCE OVER THE LAST DECADES

The combination of genetic progress and improved nutritional management resulted in a dairy herd that is capable of producing vast amounts of milk (Leroy & De Kruif 2006). The annual reports of the Flemish Cattle Breeding Association indicate the increase in daily production and lifetime yield (Table 1.1). Nevertheless, there is a notable trend towards a longer calving interval, although it stagnated over the latest four reported years.

Table 1.1. Key performance indicators of Flemish dairy farms (CRV 2012)**.**

Year	days herd life	Kg milk per lifetime	Kg milk per cow per day	Number of calvings	Average calving interval
2012	2.008	28.017	27.1	3.1	418
2011	2.044	28.325	26.8	3.2	420
2010	2.079	28.534	26.5	3.3	421
2009	2.093	28.303	26.1	3.2	420
2008	2.087	27.532	25.7	3.2	413
2007	2.062	26.378	25.2	3.2	410
2006	2.039	25.348	24.8	3.2	409

In other countries, similar trends have been observed. In the Netherlands, the milk production soared from 7558 kg fat corrected milk in 305 days to 8744 kg in ten years (1988-1998). Meanwhile, the first artificial insemination (AI) success rate decreased from 55.5% to 45.5% and the number of cows showing first heat before 70 days postpartum declined (Jorritsma & Jorritsma 2000). In the UK a same fall in reproductive performance was observed. Over the last decades, the pregnancy rate to all services declined by 1% to 1.5% per year (Royal et al. 2000; Hudson et al. 2010). Between 2006/07 and 2010/11 the overall pregnancy rate to all services dropped 4.6% in total or 1.15% per year in the South-West of England (Kerby 2013).

In the US a rise in services per conception from 1.62 in 1972 to 2.91 in 1996 had been reported. Furthermore, there was an increase in days open and days to first insemination whilst the milk production had risen with approximately 20% from 1990 till 2000 (Lucy 2001). The calving interval from the US Holstein herd rose during the late nineties from 410 (1996) to 428 (2001) and slowly declined to 422 in 2007. However, the number of breedings per lactation increased by 0.3 to 0.4 from 1996 to 2007 which was somewhat confounded with a decline in days to first breeding after calving from 92 days to 85 days in the same period (Norman et al. 2009). The yearly production per cow had in the meantime risen steadily at a rate of 1.3% from 2000-2010 (Santos et al. 2010). In Iran, the calving interval rose from 394 days in 1994 to 413 days in 2008. Per 1000-kg increase in milk yield, there was an increase of 6.55 days calving interval (Atashi et al. 2012).

2. THE TRANSITION PERIOD: A CRITICAL TIME

Hence, dairy production systems that use dairy breeds selected for milk production, seem to have a decline in fertility over the recent decades (Walsh et al. 2011). At the onset of lactation, the high milk producing dairy cow encounters a steep increase in energy and protein requirements, concomitant with a reduced feed intake, which is commonly inadequate to meet the maintenance and production demands. Therefore, the high yielding dairy cow enters a state of negative energy balance (NEB), characterized by metabolic changes (see Chapter 1, 3. The metabolic changes during the transition period) (Butler 2000).

The transition period, defined as 3 weeks before to 3 weeks after parturition, is the critical time for the dairy cow, since the cow experiences the stress of parturition, starts lactation and recommences a reproductive cycle. Simultaneously, most diseases and metabolic disorders occur during this period (Drackley 1999). Assumably, almost 75% of diseases in dairy cattle take place the first month after parturition (LeBlanc 2010). Indeed, the transition cow is potentially faced with e.g. milk fever, ketosis, fatty liver syndrome, retained foetal membranes, metritis and displaced abomasum (Ospina et al. 2010a). Further, half of the clinical mastitis cases occur during the first 30 days postpartum (Schukken et al. 2013). All of former diseases are related to or enhanced by the negative energy status of the early lactating cow (Walsh et al. 2011). Detrimental NEB, metabolic disorders and periparturient diseases complicate the subsequent reproductive performance and can result in milk income losses (Block 2010; Patton et al. 2007). Indeed, cows diagnosed with a clinical or subclinical disease had reduced estrous cyclicity on day 49 after parturition and pregnancy at first AI, and increased risk of pregnancy loss (Ribeiro et al. 2013).

Although it remains debatable to what extent the higher production has a direct negative effect on the reproductive performance (LeBlanc 2013; Patton et al. 2007), it is clear that a loss in reproductive efficiency is a worldwide concern in the dairy industry. Indeed, the reproductive efficiency is an important economic parameter as it influences average daily milk production, percent days in milk, average days in milk, calves born per year and generation interval (Johnson & Gentry 2000).

3. THE METABOLIC CHANGES DURING THE TRANSITION PERIOD

3.1. DRY MATTER INTAKE

The energy balance is defined as the difference between energy consumed and the energy needed for maintenance, growth, gestation and lactation (Grummer 2008). It is often argued that the energy status of pre-fresh transition cows is endangered due to decreasing dry matter intake (DMI) and increasing gestation requirements. The latter is normally of minor importance since the pregnancy energy requirements remain roughly the same during the final 60 days of gestation (NRC 2001). In the meantime the DMI before parturition declines 30-35% during the final 3 weeks from which 89% occurs in the last week of gestation (Hayirli et al. 2002). This decrease in DMI is partially due to the growing foetus, but mainly caused by hormonal changes of e.g. enhanced oestrogen, glucocorticoids (NRC 2001).

The nadir of DMI is just prior to calving (Figure 1.1) and slowly recovers postpartum peaking at 10 to 22 weeks in lactation. Meanwhile the milk production culminates already 4 to 8 weeks after parturition which causes the cow to fall into a negative energy status (NRC 2001; Grummer 2008; Butler 2000) as illustrated in Figure 1.2. The most negative value for the energy balance generally appears between 2.5 and 12 days postpartum and NEB lasts approximately until day 72 in milk (Jorritsma et al. 2003), but can differ strongly between cows (Bossaert 2010). From experiments by Bossaert et al. (2008) similar conclusions could be drawn: the nadir concentrations of glucose and insulin occurred on day 8 and 12 postpartum respectively, while the non-esterified fatty acids (NEFA) peaked at day 4 postpartum, which confirmed the nadir of NEB to occur between day 4 and 12 postpartum (Bossaert et al. 2008).

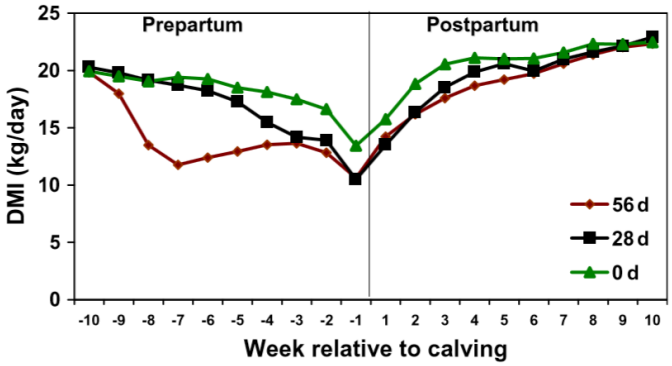
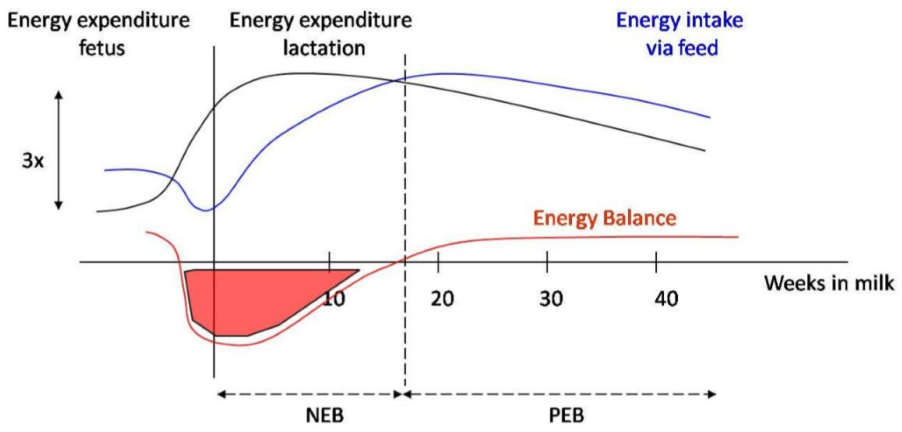


Figure 1.1. DMI intake of cows with different dry period lengths. Treatments were no planned dry period (0 d), 28-day dray period (28 d) or 56-day dry period (56 d) (Grummer 2008).



NEB = negative energy balance; PEB = positive energy balance.

Figure 1.2. Hypothetical presentation of energy requirements (black line), energy intake (blue line) and net energy balance (red line) in high-yielding dairy cows before and after calving (represented by the solid vertical line) (Bossaert 2010)**.**

The severity of NEB depends to a larger extent on the reduced and lagging dry matter intake than on the increased milk production. Remaining influencing factors of the dry matter intake are e.g. body score index at calving (negatively correlated with body condition score > 3, optimum = 3 at 1-5 scale), ration composition and nutrient content (feeds low in digestibility can inhibit high DMI due to slow clearance of the rumen and passage through digestive tract), periparturient diseases (sick animals eat less) and social conflicts (cows in lower social rank spend less time at the feed bunk) (NRC 2001; Jorritsma et al. 2003).

3.2. NEGATIVE GLUCOSE BALANCE

Efforts to alleviate NEB of high-yielding dairy cows through dietary supplementation of lipogenic nutrients have consistently failed, using glucogenic nutrients improved the energetic status (Van Knegsel et al. 2005; Grummer 2008). Indeed, glucose is the primary precursor for lactose synthesis which acts as an osmotic agent and regulates milk volume by attracting water to the mammary gland (Zhao & Keating 2007). To produce 1 kg of milk, 72g of glucose is needed and thus up to 3 kg of glucose is required for a cow producing 40 kg per day (Kronfeld 1982). The glucose uptake by the mammary gland can be as much as 60 to 85% of the total glucose that enters the blood (Zhao & Keating 2007). Therefore, glucose is the 'bottleneck' molecule of milk production and makes NEB basically a negative glucose balance (Bossaert 2010).

The major milk yield potential in the modern dairy cows, notwithstanding depressed DMI, is only possible via favouring glucose uptake by the mammary gland. This is established by 1) limiting glucose consumption in extra-mammary tissues, 2) maximizing the hepatic glucose production, and 3) mobilizing energy reserves to supply alternative fuel for the extra-mammary tissues (Bossaert 2010).

3.3. INSULIN REGULATION

The genetic selection for maximizing milk yield has exploited the cows fundamental mammal strategy to prioritize nutrients for lactation whereby survival of the offspring is insured (Leroy et al. 2008). This resulting 'glucose highway' from ration and body reserves to the udder is mainly regulated by the insulin concentration and responsiveness and sensitivity from peripheral tissues (Bossaert 2010).

Glucose cannot permeate the plasma membrane, so intracellular uptake is mediated by membrane-bound glucose transporter (GLUT) molecules of which up till now 13 isoforms have been described (De Koster & Opsomer 2013). The tissue-specific expression pattern of those different GLUT grants insulin to control the glucose partitioning all over the organism. GLUT 1 is considered to be the primary transporter responsible for basal glucose uptake and is independent of insulin (Zhao & Keating 2007).

The mammary gland of lactating cows expressed a large quantity of GLUT 1, whilst the mammary gland of non-lactating cows did not. On the contrary, adipose tissue of late and non-lactating cows expressed a large quantity of GLUT 1, while the adipose tissue of peak-lactating cows did not (Komatsu et al. 2005). Meanwhile, the expression of GLUT 4, the insulin-regulated glucose transporter, was abundant in adipose tissue and muscle and did not show significant differences in expression throughout lactation, whereas it was not noticed in the mammary gland (Komatsu et al. 2005) (Figure 1.3 and Figure 1.4).

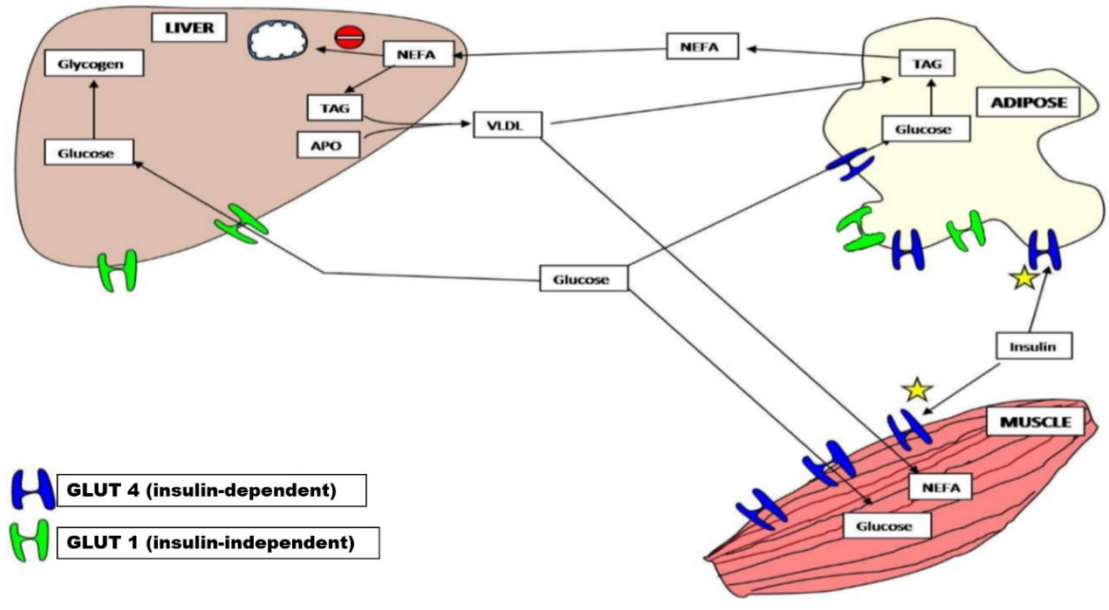


Figure 1.3. Glucose uptake by peripheral tissues and consequences for lipolysis and circulation of its metabolites during the dry period. APO: apolipoprotein, TAG: triacyl glycerol (triglycerides) (redrafted after Bossaert 2010)**.**

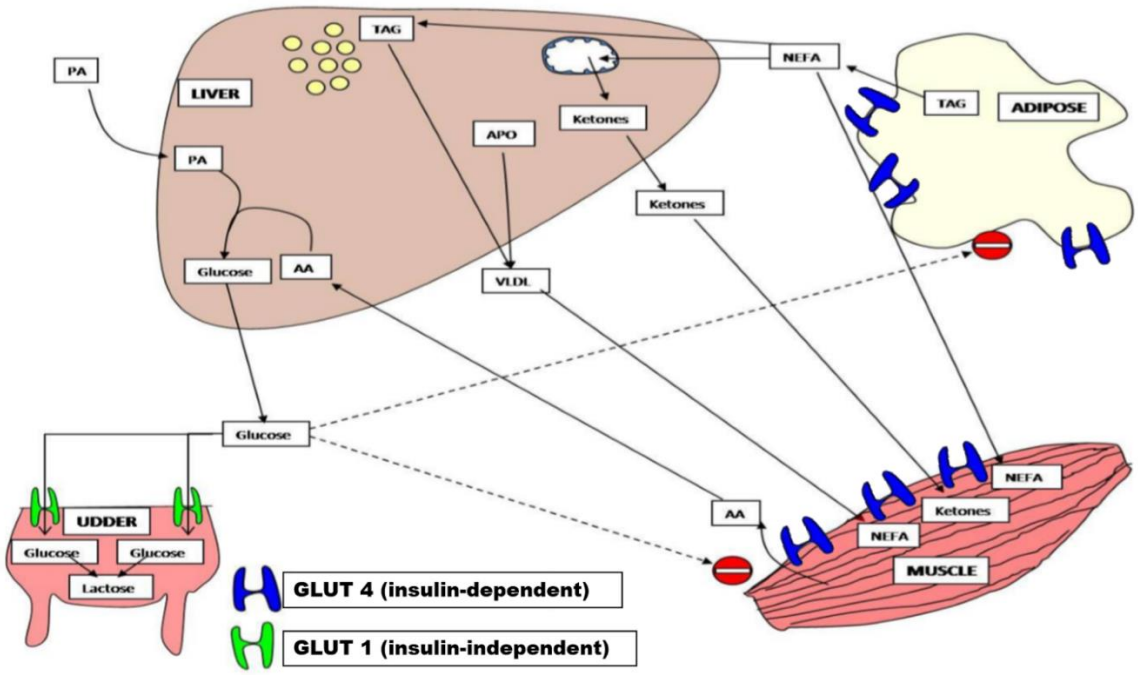


Figure 1.4. Glucose uptake by peripheral tissues and consequences for lipolysis and circulation of its metabolites during the dry period. PA: propionic acid, AA: amino acid (redrafted after Bossaert 2010)**.**

In summary, during the periparturient period a normal concentration of insulin induces a decreased biological response in the insulin-sensitive tissues, a state defined as insulin resistance (De Koster & Opsomer 2013). Meanwhile, there is a lower plasma insulin concentration during lactation compared with the concentration in non-lactating cows. The combination of the latter with the insulin resistant state facilitates a preferential insulin-independent glucose supply for the mammary gland and thus plays a specific role in favouring milk synthesis (Zhao & Keating 2007).

3.4. LIPOLYSIS

The mismatch between increased energy expenditure (end of gestation and early lactation) and the lagging DMI peak (energy intake) causes the cow to enter a negative energy status. Therefore, energy is mobilized out of the body reserves, which is externally visible as the loss of body condition score (BCS), commonly expressed on a 1 to 5 scale for dairy cattle (NRC 2001). The BCS loss from calving until the end of NEB varies from 0.5 to 1 point which can account for a 7 to 8% decrease in body weight (Bossaert 2010).

The release of non-esterified fatty acids (NEFA) from the adipose tissue is the result of the joined effect of lipolysis of triglycerides and reesterification of NEFA in the adipocytes or lipogenesis. At late gestation and the onset of lactation, the blood NEFA levels incline by increased lipolysis and decreased lipogenesis, partially regulated by reduced insulin and high catecholamines or glucocorticoids concentrations during this period (Figure 1.5) (De Koster & Opsomer 2013; Leroy et al. 2008).

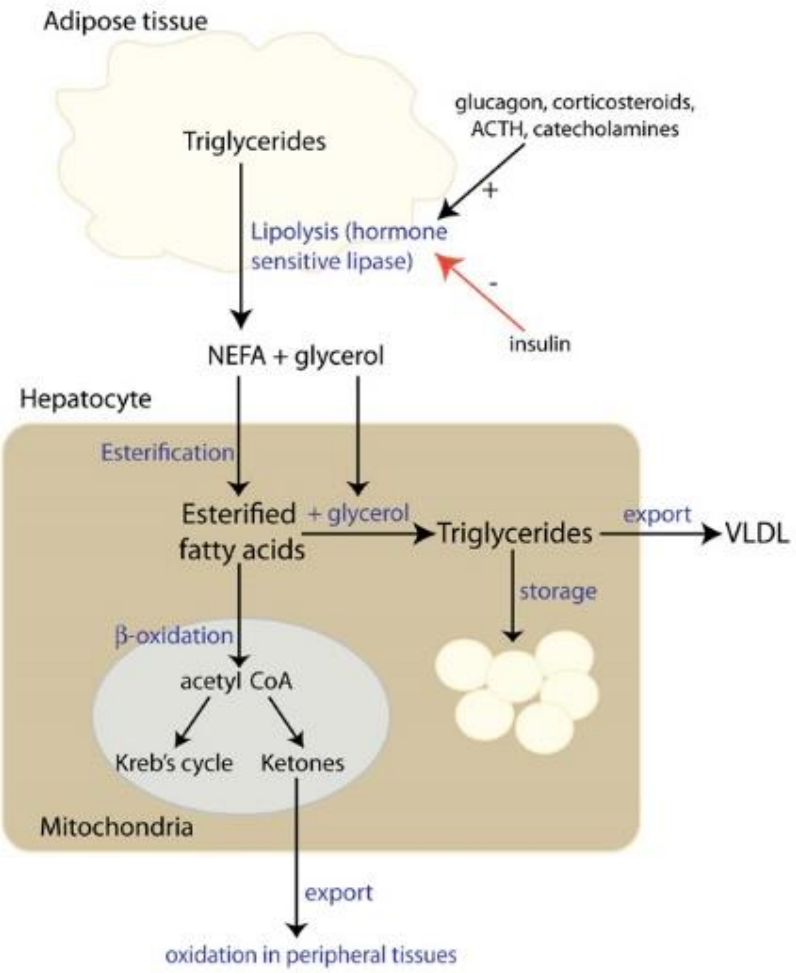


Figure 1.5. The lipolysis of adipose tissue leads to the uptake of blood NEFA by the liver and the circulation of its metabolites (Cornell University 2013)**.**

Plasma NEFA increased two-fold or more between 2 to 3 weeks prepartum and 2 to 3 days prepartum, followed by a substantial rise until parturition, partly due to the stress of calving (NRC 2001; Leroy et al. 2011). The NEFA concentration peaked at day 4 postpartum and it declined throughout lactation (Bossaert et al. 2008; NRC 2001). The mobilized NEFA serve as an alternative energy source for non-mammary tissues to preserve glucose and can be used to synthesize milk fat (Leroy et al. 2008). In the liver some NEFA provide energy, or are oxidized to Acetyl-CoA or reesterified into triglycerides (TG) that are either exported as very low density lipoproteins (VLDL) or stored in the liver (Ospina et al. 2010b; Van Knegsel et al. 2005) (Figure 1.5).

3.5. KETOSIS AND FATTY LIVER

The NEFA are taken up by the liver proportionally to their concentration in blood and blood flow (Emery et al. 1992). The metabolite acetyl coenzyme A (CoA) formed by the complete oxidation of NEFA can be used in the Krebs cycle to generate energy. However, when redundant NEFA enter the liver, the Krebs cycle gets saturated and the excess of acetyl CoA is redirected to produce the ketone bodies acetoacetic acid, acetone and beta-hydroxybutyrate (BHBA) (Esposito et al. 2013; Walsh et al. 2007) (Figure 1.6).

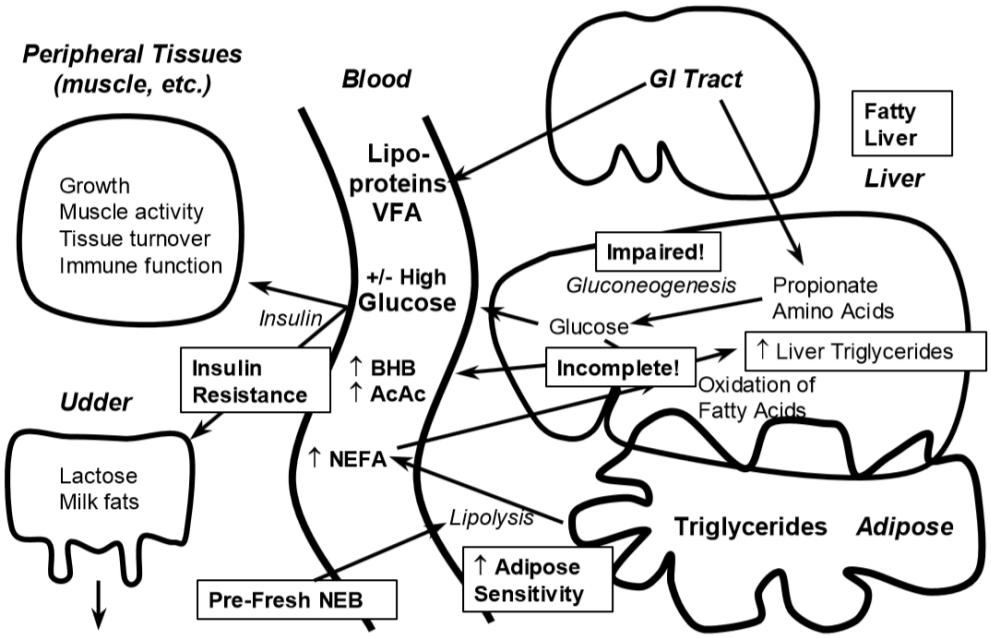


Figure 1.6. The circulation of excessive blood NEFA and BHBA leading up to ketosis and fatty liver syndrome. GI tract: gastrointestinal tract, VFA: volatile fatty acids, BHB: β-Hydroxybutyrate, AcAc: acetoacetic acid (Oetzel 2007)**.**

Subclinical and clinical ketosis arises when ketones (using blood BHBA as an indicator) are elevated above 1.0 to 1.4 mmol/L and 3,36 mmol/L respectively (Block 2010). Presumably at least 50% of all dairy cows endure briefly subclinical ketosis (SCK) in the first month of lactation as a rewarding strategy to preserve blood glucose and/or prevent fatty liver. Despite a considerable increase in glucose requirement, the blood glucose commonly only shows a temporary decline for around 1-2 weeks at parturition (Esposito et al. 2013).

Remaining excess NEFA in the liver are re-esterified into TG, but the hepatocytes are inefficient in exporting the TG as VLDL, which leads to the accumulation of TG in the liver or fatty liver during NEB (Brickner et al. 2009; Emery et al. 1992; Ospina et al. 2010b). The fatty liver syndrome lessens the hepatic gluconeogenic capacity whereby the incidence of ketosis is further enhanced (Oetzel 2007). To avoid hindered liver function it is important to prevent ketosis and fatty liver syndrome by maintaining the body condition score at 3.5 at calving (Esposito et al. 2013).

4. CONSEQUENCES FOR REPRODUCTION

High reproductive performance is achieved by high submission rates (percent of cows submitted for service for the first time in a specified period) and high first artificial insemination (AI) success rates (Fulkerson 1984). However, to accomplish a good submission and AI success rate, cows need to quickly resume ovarian cyclicity, to have a normal follicular development and a good oocyte quality and to be heat detected and inseminated at the right time (Crowe & Mullen 2013). Usually, resumption of ovarian cyclicity starts within 5 to 10 days postpartum with first ovulation on average at 30 days postpartum (Leroy et al. 2005; Butler 2003). The number of ovulatory estrous cycles prior to insemination influences conception rate and is hence an important factor in reproductive efficiency (Butler 2003) (Figure 1.7).

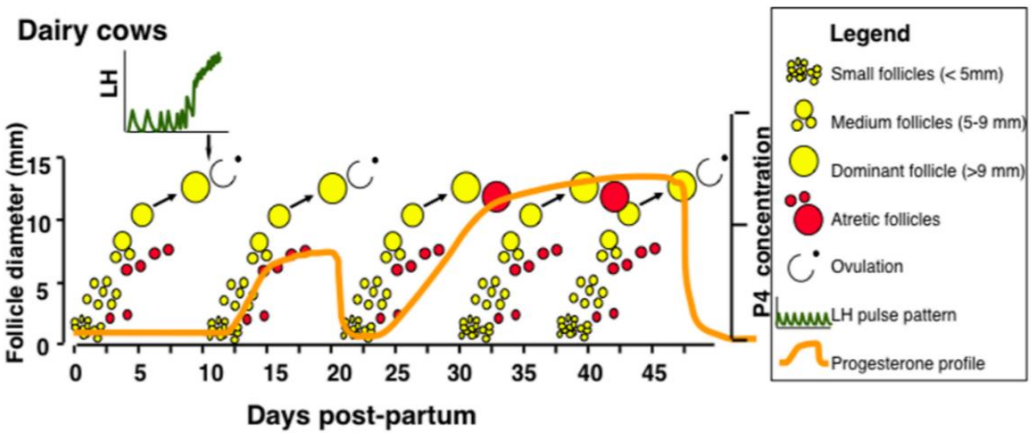


Figure 1.7. Scheme of postpartum resumption of dominant follicles and ovarian cycles (Crowe & Mullen 2013)**.**

However, the ovarian activity coincidences with the nadir of NEB (Figure 1.8), whereby the latter negatively affects direct or indirect reproductive performance via alterations in hormones e.g. insulin-like growth factor-I (IGF-I), insulin and growth hormone (GH) and metabolites e.g. NEFA and BHBA (Matoba et al. 2012; Wathes et al. 2007; Leroy et al. 2005).

4.1. HORMONES

So far NEB was mainly characterized by an increase in blood NEFA and BHBA and a decrease in glucose and insulin. However, NEB is also associated with hormonal changes such as IGF-I, GH and luteinizing hormone (LH) (Beam & Butler 1998).

Five to 7 days postpartum, the first wave of follicular development occurs, driven by elevated follicle stimulating hormone (FSH) concentrations, regardless of NEB. The first following ovulation is a response to pulsatile LH secretion through preovulatory follicular growth and estradiol production (Butler 2003) (Figure 1.8).

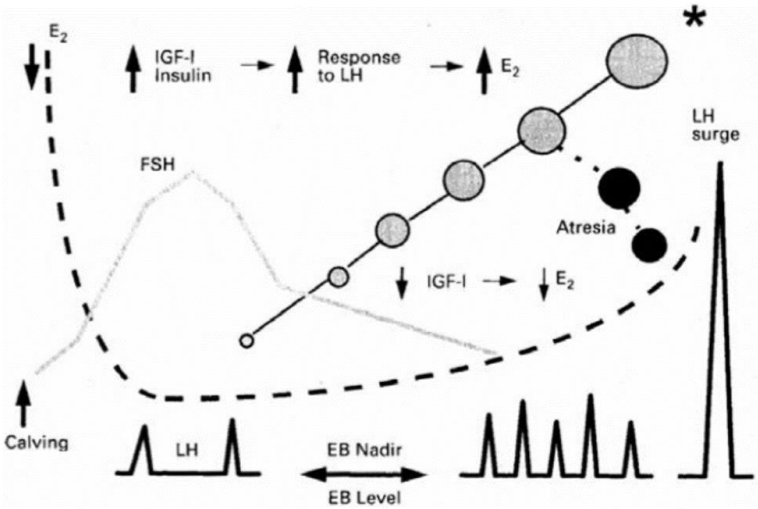


Figure 1.8. Dominant follicle development (circles) related to altering metabolic and reproductive hormones and NEB during first follicular wave postpartum. E_2: estradiol, EB: energy balance (Butler 2003) **.**

Due to NEB, that represents a physiological state of under nutrition, LH secretion and ovarian responsiveness to LH is reduced and the first ovulation is delayed to day 40-50 postpartum because of the development of a non-ovulatory dominant or a cystic follicle instead (Butler 2000). Upcoming follicles after NEB nadir have greater growth and diameter and an improved estradiol production than those before NEB nadir (Beam & Butler 1998).

Most IGF-I is derived as a reaction on GH binding to GH receptors (GHR) in the liver, with systemic IGF-I having a negative feedback to the pituitarian GH synthesis (Butler 2003). However, during NEB, the somatotropic axis (IGF-I, GH and GHR) is uncoupled because of a down-regulation in hepatic GHR, leading up to a reduction in IGF-I and an increase in GH (Wathes et al. 2007; Esposito et al. 2013). During the first 2 weeks postpartum, the IGF-I levels were 40 to 50% higher in dairy cows with ovulatory follicles as compared with those in which the dominant follicle would not ovulate (Butler 2003). The availability of IGF-I and insulin and the extent of NEB seemed important for the follicles to produce enough estradiol for granulosa cell proliferation and ovulation (Bossaert et al. 2008).

4.2. METABOLITES

The direct metabolites linked with the maladaptation to NEB (high NEFA and BHBA concentrations) also have a negative effect on follicle and oocyte development and quality (Leroy & De Kruif 2006). Dairy cows that were not pregnant after first insemination tended to have higher periparturient (3 weeks before until 9 weeks after parturition) BHBA concentrations in the blood than pregnant cows. Cows diagnosed with subclinical ketosis in the first week postpartum (blood serum BHBA ≥ 1 mmol/L) or in the second week postpartum (blood serum BHBA ≥ 1.4 mmol/L) were 20% less likely to become pregnant after first insemination. The possibility of a successful first AI decreased 50% when the cows had SCK in both week 1 and week 2 after parturition. (Walsh et al. 2007). However, not the elevated follicular fluid BHBA concentrations but the concomitant low glucose concentrations during subclinical ketosis seemed to hamper oocyte maturation (Leroy et al. 2006).

Elevated blood serum NEFA concentrations (≥ 0.7 mmol/L) in the first month after parturition delayed ovarian cyclicity of the cows and reduced the likelihood to be pregnant after the first AI (Ribeiro et al. 2013). Cows with blood serum NEFA higher than 0.72 mmol/L had a 16% less chance of being pregnant within 70 d postpartum (Ospina et al. 2010a), which might be partially linked to the toxicity of NEFA on granulosa cell growth and function as indicated from research *in vitro* (Vanholder et al. 2005).

Indeed, increased blood plasma NEFA concentrations in the first two weeks postpartum were accompanied by elevated NEFA concentrations in the follicular fluid early postpartum, although the concentration of NEFA was 40% lower in the follicular fluid (0.2-0.6 mmol/L) than in the serum (0.4-1.2 mmol/L). Despite some buffering capacity, C18:1 *cis*-9, C16:0 and C18:0 remained the most important fatty acids in the follicular fluid. Further, follicular fluid C16:0 and C18:0 had a negative impact on oocyte maturation, fertilization and cleavage rate and blastocyst yield (Leroy et al. 2005) and follicular fluid C18:1 *cis*-9 caused a decline in proliferation of granulosa cells (Jorritsma et al. 2004). Additionally, increased follicular fluid NEFA concentrations during oocyte maturation reduced the viability of the subsequent embryo as oocytes exposed to high follicular fluid NEFA

(0.425 mmol/L with 0.075 mmol/L C18:0 as most toxic fatty acid) formed less and inferior blastocysts (Van Hoeck et al. 2011).

Hence, increased NEFA concentration in the blood and thus in the follicular fluid hampered fertility of the high-performing dairy cow through a reduced oocyte developmental competence and viability of the subsequent embryo (Leroy et al. 2005; Van Hoeck et al. 2011).

5. BIOMARKERS

Changing concentrations of the circulating blood plasma NEFA and BHBA are commonly used indices of NEB. Even though some increase of these metabolites is normal due to the high energy demand and the reduced energy intake, an excessive elevation of blood plasma NEFA or BHBA can indicate poor adaptation to NEB (Ospina et al. 2010b). Indeed, a more negative energy balance is associated with a greater fat mobilization and thus a greater blood plasma NEFA concentration, while incomplete hepatic fatty acid oxidation results in the production of ketone bodies (acetoacetic acid, acetone and BHBA), resulting in a greater blood plasma BHBA concentration (Van Haelst et al. 2008). Even though blood plasma BHBA and NEFA concentrations are the 'golden standard' in the detection of subclinical ketosis (SCK) and a detrimental NEB and their analysis in diagnostic laboratories is quite accurate, they are costly, time consuming and invasive to the animals when repeated sampling is required (Townsend 2011).

Therefore, cow-side tests are currently available for blood, urine and milk samples. Cow-side tests for monitoring ketosis consists of detecting BHBA in blood or milk and acetoacetic acid and acetone in milk or urine (Duffield & Leblanc 2009). The tests are based on the elevated concentrations of ketone bodies in the blood plasma (Oetzel 2007). Cow-side test in the monitoring of NEFA are currently not in use (Townsend 2011).

Hence, milk fatty acids are of growing interest in the detection of elevated blood plasma NEFA, since release from body fat reserves is one of the 4 major pathways contributing to the milk fatty acids, besides the diet, de novo synthesis in the mammary gland and the formation in the rumen by biohydrogenation or bacterial degradation. Changes in NEB are related with shifts in the activity of these former pathways and so cause changes in the milk fat composition (Stoop et al. 2009). Indeed, NEB was associated with an increase of milk fat C18:1 *cis*-9 since it is the most important fatty acid component in adipose tissue (Gross et al. 2011), and a decrease in milk fat odd-chain C5:0 to C15:0, reflecting the reduced allocation of glucogenic components to milk fat synthesis (Stoop et al. 2009). During lactation, the proportion of milk saturated fatty acids such as C10:0, C12:0, C14:0 and C16:0 increased from week 1 up to week 12 postpartum, while the milk monounsaturated fatty acids with predominantly C18:1 *cis*-9 decreased as NEB became less severe (Gross et al. 2011). Since the milk fatty acid profile is closely related to the energy balance in dairy cows, milk fatty acids (especially the C18:1 *cis*-9 concentration) are interesting biomarkers for SCK and NEB (Van Haelst et al. 2008; Gross et al. 2011).

Chapter 2

MATERIALS AND METHODS

1. ANIMALS AND HOUSING

Milk and blood sampled and analysed within this master dissertation were taken on the dairy farm of Gut Hohen Luckow, Satow in Germany with approximately 2000 Holstein cows and an average milk production of 32 L per cow per day. The dry and fresh cows were housed in a free-ranging transition stable with slatted floor and cubicles. Animals stayed here from 2 months before parturition until about 14 days postpartum. During lactation, cows were milked three times daily for the first two weeks and twice or thrice for the remainder of lactation depending on milk production. On average 6 to 7 calves were born daily in the specific calving area, which was one of the most important reasons for the choice of this dairy farm. Further, the animals were very closely monitored in relation to e.g. illness and reproduction. The cows were fed a total mixed ration (TMR) including maize and alfalfa silage, maize and soybean meal and sugar beet pulp, without individual concentrate supplementation.

In the period of September 4th until 25th, 141 cows (40 primiparous and 101 multiparous cows) calved, of which veterinary or specific dietary treatments and other relevant data were recorded and which were sampled for blood and milk analyses.

Important to note was the use of propylene glycol (PG) the first 14 days postpartum. All cows received 600 mL propylene glycol after calving and on Monday, Wednesday and Friday, the animals were checked with a ketostick (Precision Xtra Ketone Test Strips, Abbot, North Chicago, United States) to measure the blood BHBA concentration. Dairy cows with a blood value higher than 1.2 mmol/L were drenched for 3 days with 600 mL propylene glycol. Only two cows included in the sampled sub-herd received this treatment (Table 2.1). During two weeks of sampling, there was a lack of test strips and not all cows were checked three times a week.

Table 2.1. Cows treated with propylene glycol for three days, the respective value of the Ketostick monitoring (resulting in the decision for treatment) and the number of days after calving when the Ketostick was positive.

Cow number	Value Ketostick (mmol/L)	days after calving
7514	1.5	2
7514	1.4	5
5617	1.2	4

2. MILK SAMPLING AND ANALYSIS

2.1. SAMPLING AND STORAGE OF THE MILK SAMPLES

Preliminary test

For logistic reasons, milk could not be sampled during the milking process (flow through) on the farm of Gut Hohen Luckow, but milk had to be stripped directly after the milking cluster was removed. To test whether the fatty acid profile would alter and if it would be still permissible to compare the strip sample results with other flow through results, a small scale experiment on 13 randomly selected early lactating Holstein cows from a dairy farm in Oostrozebeke, Belgium was conducted first. The milk sampling took place during the evening milking (18.00h – 19.30h). The flow through sample was taken by a sample-cup attached to the milk meter, from which 20-40 mL was transferred into the sample tube. Subsequently, when the milking cluster was pulled back, a strip sample of 20-40 mL was taken from the four teats before dipping the teats. Filtering, storage and milk fatty acid analysis of these milk samples were similar to the ones collected at Gut Hohen Luckow as explained further.

Sampling and storage at Gut Hohen Luckow

During the whole registration period in Germany, milk sampling for fatty acid analysis took place during the first milking run of the day (06.00h – 09.00h). When the milking cluster was pulled back, a strip sample of 20-40 mL was taken from the four teats before dipping the teats. The sample was filtered through a cheese cloth and stored at -20 °C.

2.2. ANALYSIS OF THE MILK SAMPLES

The frozen milk samples were transported to the Laboratory for Animal Nutrition and Animal Product Quality (Faculty of Bioscience Engineering, Ghent University, Belgium). The fatty acid profile was obtained after milk fat extraction (mini Roese-Göttlieb method, adapted from Chouinard et al. 1997), methylation (Stefanov et al. 2010) and gas chromatographic analysis (GC) of milk fatty acid methyl esters (MFAME) (Agilent Technologies 7890 A GC System equipped with a flame ionization detector). Samples were injected by split injection (split ratio1:50). The carrier gas was hydrogen with an inlet pressure of 246.38 kPa. Fatty acid peaks were identified based on their retention times. Separation of MFAME was realized with a Supelco column (SP-2560), Sigma-Aldrich) (75 m x 180 µm x 0.14 µm).

The temperature program ran from a starting temperature of 70 °C for 2 minutes, increasing 15 °C/min till 150 °C; from 150 °C till 165 °C by an increase of 1 °C/min, 165 °C was held for 12 minutes; from 165 °C till 170 °C, the temperature was increasing 2 °C/min with 170 °C held for 5 minutes and from 170 °C till 215 °C, an increase of 5 °C/min was applied, with the final temperature kept for 10 minutes. Fatty acid methyl esters were determined with Agilent ChemStation software (B.04.03) (Agilent Technologies, Inc. as governed by United States and International copyright laws. Edition 9/2010) and C13:0 (as triglyceride; Sigma, Bornem, Belgium) was used as internal standard.

The fatty acids were expressed as grams per 100 grams of MFAME. Fatty acid peaks were identified through mixtures of methyl ester standards (BR2 and BR3, Larodan Fine Chemicals AB, Malmö, Sweden; Supleco 37, Supleco Analytical, Bellefonte, PA; PUFA-3, Matreya LLC, Pleasant Gap, PA), based on retention times. Fatty acids were corrected for their respective theoretical relative response factors (Ackman & Sipos 1964; Wolff et al. 1995).

3. BLOOD SAMPLING AND ANALYSIS

3.1. SAMPLING AND STORAGE OF THE BLOOD SAMPLES

The fresh cows were fixed at the feed bunk every day after the first milking for a daily check-up which allowed blood sampling (08.00h-10.00h). Blood was obtained from the tail vein and collected in EDTA tubes (5-9mL). The blood samples were centrifuged within one hour after collection for 10 minutes at a relative centrifugal field of 3360 g (Sepatech labofuge 200, Heraeus, Hanau, Germany). Afterwards, 1.5 mL of blood plasma was transferred into a vial and stored in the freezer at -20°C.

3.2. ANALYSIS OF THE BLOOD SAMPLES

The frozen blood plasma samples were sent to the laboratory of DGZ Flanders, Torhout, Belgium. The plasma metabolites BHBA and NEFA were measured enzymatically using commercial kits as described by van Dorland et al. (2009), and spectrofotometrical measurements at 340 nm and 600 nm, respectively. The principle of the NEFA assay is as follows: In the presence of ATP, coenzyme A (CoA), and acetyl-CoA synthetase, forms acyl-CoA and the by-products AMP and pyrophosphate. Then, the product acyl-CoA is oxidized by acetylyl-CoA oxidase, which produces hydrogen peroxide. Peroxidase acts on the hydrogen peroxide in the presence of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline and 4-aminoantipyrine to form the final reaction product that is a purple quinone. The kinetic enzymatic method to measure the concentration of BHBA in plasma as follows: The method is based on the oxidation of BHBA to acetoacetate by the enzyme BHBA dehydrogenase. Concomitant with this oxidation the cofactor NAD+ is reduced to NADH and the associated change of absorbance can be directly correlated with the BHBA concentration.

4. DATA PROCESSING

4.1. DESCRIPTIVE STATISTICS

The milk fatty acid profile, blood plasma NEFA and BHBA and the reproductive indicator 'times bred' (TBRD) were used for several computations. For all calculations, data were split in day 3 and day 10 after parturition and all analyses were performed separately for the two days. Further, observations were split based on thresholds for blood plasma BHBA and NEFA (see 4.2. Blood plasma thresholds) and times bred (TBRD). Subsequently, means, standard error of the mean and p-values (one-way ANOVA with a Tukey ad hoc test) of BHBA, NEFA, C18:1 *cis*-9 and the C18:1 *cis*-9/C15:0 ratio were computed for each group by SPSS 22.0 (SPSS Inc., Chicago, USA).

4.2. BLOOD PLASMA THRESHOLDS

The observations were classified for "at risk" and "not at risk" based on thresholds for blood plasma BHBA and NEFA. For BHBA a threshold of 1.2 mmol/L was used, where values greater than or equal to 1.2 mmol/L were classified as "1", which stands for "suffering of subclinical ketosis (SCK)", while BHBA values lower than 1.2 mmol/L were classified as "0" or "not suffering of SCK" (Duffield et al. 1998). The blood plasma NEFA threshold in postpartum samples was determined at 0.6 mmol/L (Ospina et al. 2010b), where values greater than or equal to 0.6 mmol/L were classified as "1", which stands for "at risk of detrimental blood plasma NEFA" and NEFA values lower than 0.6 mmol/L were classified as "0" or "not at risk of detrimental blood plasma NEFA". Detrimental blood plasma NEFA (≥ 0.6 mmol/L) might result in metabolic disorders such as fatty liver and ketosis (Van Haelst et al. 2008). Additionally, detrimental blood plasma NEFA increases the risk for displaced abomasums, clinical ketosis, metritis and retained placenta (Ospina et al. 2010b). Furthermore, detrimental blood plasma NEFA concentrations seem to induce an impaired fertility (Leroy et al. 2005).

Hereafter, it was decided to continue with NEFA observations and not SCK cases because only 4.5% and 2% of the cows showed a blood plasma BHBA concentration greater than the BHBA threshold of 1.2 mmol/L at day 3 and day 10 after parturition, respectively.

4.3. VALIDATION C18:1 *CIS*-9 THRESHOLDS

Literature and former research performed at the department (Jorjong et al. 2014) suggested that milk fat C18:1 *cis*-9 particularly shows potential as early warning biomarker for elevated blood plasma NEFA (≥ 0.6 mmol/L) in dairy cows. Therefore, a threshold for C18:1 *cis*-9 was proposed to classify the observations of detrimental blood plasma NEFA concentration. However, milk C18:1 *cis*-9 concentrations depend on other factors besides blood plasma NEFA concentrations (e.g. diet, sampling day…). Hence, a "basal" farm-specific milk fat C18:1 *cis*-9 concentration was determined, which allowed the calculation of a 'subtracted' milk fat Δ C18:1 *cis*-9 value, which was used as benchmark instead of the absolute value of the C18:1 *cis*-9 concentration. The basal milk C18:1 *cis*-9 value was obtained by computing the mean of the 85% lowest milk fat C18:1 *cis*-9 concentrations.

The threshold value for milk Δ C18:1 *cis*-9, as obtained from a former experiment at the department (Jorjong et al. in preparation) equalled 4.11 g/100g milk fatty acid methyl ester (MFAME) and was used as benchmark to classify observations of the current dataset which allowed validation of this threshold based on the calculation by Excel (Microsoft 2013) of sensitivity, specificity and overall accuracy in classifying the observations of detrimental blood plasma NEFA (Table 2.2). The sensitivity of the test is defined as the proportion of cows with detrimental NEFA (≥ 0.6 mmol/L; "at risk") that will have a positive result and is a measure of how likely it is for the test to classify the "at risk" cows correctly. The specificity of the test is the proportion of cows without detrimental NEFA who will have a negative result and is a measure of how likely it is for the test to classify the "not at risk" cows correctly. The accuracy of the test is the whole proportion of the cows that is classified correctly (both "at risk" and "not at risk" cows) (Akobeng 2007).

Table 2.2. A 2x2 table with true positives, false positives, false negatives and true negatives for the potential of the milk fat C18:1 *cis*-9 threshold (ΔC18:1 *cis*-9) of 4.11 g/100g MFAME to diagnose cases of low or elevated blood plasma NEFA (< or ≥ 0.6 mmol/L) at day 3 and day 10 after parturition.

	NEFA ≥ 0.6 mmol/L	**NEFA < 0.6 mmol/L**
Δ C18:1 *cis*-9 ≥ 4.11 g/100g MFAME	True positive	False positive
Δ C18:1 *cis*-9 < 4.11 g/100g MFAME	False negative	True negative

$$Sensitivity = \frac{True\ positive}{True\ positive + False\ negative}$$

$$Specificity = \frac{True\ negative}{False\ positive + True\ negative}$$

$$Accuracy = \frac{True\ positive + True\ negative}{True\ positive + False\ positive + False\ negative + True\ negative}$$

4.4. CLASSIFICATION BASED ON HIGH INSEMINATION NUMBER (TBRD > 3)

Furthermore, the potential of this threshold of 4.11 milk fat ΔC18:1 *cis*-9 g/100g MFAME to classify the observations according the times bred (TBRD ≤ 3 and TBRD > 3) was calculated. Predictive values such as sensitivity, specificity and accuracy were computed again in Excel.

Table 2.3. A 2x2 table with true positives, false positives, false negatives and true negatives for the potential of the milk fat C18:1 *cis*-9 threshold (ΔC18:1 *cis*-9) of 4.11 g/100g MFAME to diagnose cases of low or high insemination number (TBRD ≤ 3 or > 3) at day 3 and day 10 after parturition.

	TBRD > 3	**TBRD ≤ 3**
Δ C18:1 *cis*-9 ≥ 4.11 g/100g MFAME	True positive	False positive
Δ C18:1 *cis*-9 < 4.11 g/100g MFAME	False negative	True negative

4.5. DISCRIMINANT FUNCTION

Discriminant analysis was performed to assess whether other milk fatty acids could be identified to optimise classification. Forty-five milk fatty acids as well as the milk fat C18:1 *cis*-9/C15:0 ratio as the anteisoC15:0/anteisoC17:0 ratio were subjected to a discriminant analysis performed by SPSS 22.0. Here, the most discriminating milk fatty acids for classification based on TBRD was conducted. Due to the large variation in range of milk fatty acids, standardized canonical discriminant coefficients were used to compare variables. Increased absolute values of these coefficients correspond to variables with increased discriminating competence. After this first exploratory linear discriminant analysis (default leave-one-out procedure in SPSS 22.0), aiming at identifying the most discriminating milk fatty acids or ratio of fatty acids, a second linear discriminant analysis was performed in which classification was based on one of the most discriminating milk fatty acids or ratio of milk fatty acids. The performance of the latter classification model was assessed through cross-validated discriminant analysis (default leave-one-out procedure) which allowed computation of the predictive values sensitivity, specificity and overall accuracy.

A new threshold for milk fat C18:1 *cis*-9 for the classification of observations of high insemination number (TBRD >3) was calculated by sorting the discriminant coefficients, resulting from the discriminant analysis performed based on the inclusion of only milk C18:1 *cis*-9. The discriminant coefficient score of the "last" observation which was classified as "0" and the discriminant coefficient score of the first observation classified as "1" were used to calculate the corresponding absolute C18:1 *cis*-9 value. From the mean of the two milk fat C18:1 *cis*-9 concentrations, the basal milk fat C18:1 *cis*-9 concentration (as defined in Chapter 2, 4.3. Validation C18:1 *cis-9* thresholds) was subtracted, resulting in a new milk fat Δ C18:1 *cis*-9 threshold.

4.6. EMPIRICAL CUMULATIVE PROBABILITY DISTRIBUTIONS FOR DIAGNOSIS OF RISK FOR LOW AND DETRIMENTAL BLOOD PLASMA NEFA

Further, a visual evaluation of the potential to distinct groups with elevated blood plasma NEFA was based on the cumulative distributions constructed for each of the three milk fatty acid parameters which were identified as the most discriminating based on the discriminant analysis: C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio.

Empirical cumulative probability distributions were generated, expressing the cumulative proportion of observations with a milk fat C18:1 *cis*-9 concentration (*a*) lower than or equal to an increasing value of C18:1 *cis*-9 (*A*) to the total number of detrimental blood plasma NEFA (≥ 0.6 mmol/L). The cumulative probability distribution of increasing milk fat C18:1 *cis*-9 for observations with elevated blood plasma NEFA (NEFA ≥ 0.6 mmol/L) was defined as $p_1(a)$, whereas the $p_2(a)$ was defined as the cumulative probability distribution of increasing milk fat C18:1 *cis*-9 concentration for cases not showing elevated blood plasma NEFA (low blood plasma NEFA). In these functions *a* is defined as the milk fat C18:1 *cis*-9. The curves were constructed through a stepwise increase of the milk fat C18:1 *cis*-9 "*A*" from the minimum to the maximum value observed in the study.

A same approach was followed for milk fat C18:1 *cis*-9/C15:0 (*c*) in milk. For milk fat anteisoC15:0 (*b*) a similar but 'reversed' curve was drafted. The cumulative proportion of observations with a milk fat anteisoC15:0 concentration higher than or equal to the increasing value of anteisoC15:0 (*B*) was expressed to the total number of detrimental blood plasma NEFA (≥ 0.6 mmol/L).

$$p_1(a) = \frac{Number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ for\ which\ a \leq A}{Total\ number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ in\ the\ study}$$

$$p_2(a) = \frac{Number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ for\ which\ a\ \leq A}{Total\ number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ in\ the\ study}$$

$$q_1(b) = \frac{Number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ for\ which\ b\ \geq\ B}{Total\ number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ in\ the\ study}$$

$$q_2(b) = \frac{Number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ for\ which\ b\ \geq\ B}{Total\ number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ in\ the\ study}$$

$$r_1(c) = \frac{Number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ for\ which\ c\ \leq\ C}{Total\ number\ of\ cases\ with\ elevated\ blood\ plasma\ NEFA\ in\ the\ study}$$

$$r_2(b) = \frac{Number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ for\ which\ c\ \leq\ C}{Total\ number\ of\ cases\ with\ low\ blood\ plasma\ NEFA\ in\ the\ study}$$

4.7. EMPIRICAL CUMULATIVE PROBABILITY DISTRIBUTIONS FOR DIAGNOSIS OF RISK FOR TBRD ≤ 3 AND TBRD > 3

Additionally, a visual evaluation of the potential to distinguish groups with high insemination number (TBRD > 3) was composed by the construction of the cumulative distributions for the milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio.

The cumulative proportion of observations with a milk fat C18:1 *cis*-9 concentration (*a*) lower than or equal to an increasing value of C18:1 *cis*-9 (*A*) were now in relation to the total number of high insemination number cases (TBRD > 3). The cumulative probability distribution of increasing milk fat C18:1 *cis*-9 for observations with high insemination number was defined as $s_1(a)$, whereas the $s_2(a)$ was defined as the cumulative probability distribution of increasing milk fat C18:1 *cis*-9 concentration for cases not showing high insemination number (TBRD ≤ 3). In these functions *a* is defined as the milk fat C18:1 *cis*-9. The curves were constructed through a stepwise increase of the milk fat C18:1 *cis*-9 "*A*" from the minimum to the maximum value observed in the study.

A same approach was followed again for milk fat C18:1 *cis*-9/C15:0 (*c*) in milk. For milk fat anteisoC15:0 (*b*), the cumulative proportion of observations with a milk fat anteisoC15:0 concentration higher than or equal to the increasing value of anteisoC15:0 (*B*) was expressed to the total number of TBRD > 3.

$$s_1(a) = \frac{Number\ of\ cases\ with\ TBRD > 3\ for\ which\ a \leq A}{Total\ number\ of\ cases\ with\ TBRD > 3\ in\ the\ study}$$

$$s_2(a) = \frac{Number\ of\ cases\ with\ TBRD \leq 3\ for\ which\ a\ \leq A}{Total\ number\ of\ cases\ with\ TBRD \leq 3\ in\ the\ study}$$

$$t_1(b) = \frac{Number\ of\ cases\ with\ TBRD > 3\ for\ which\ b\ \geq\ B}{Total\ number\ of\ cases\ with\ TBRD > 3\ in\ the\ study}$$

$$t_2(b) = \frac{Number\ of\ cases\ with\ TBRD \leq 3\ for\ which\ b\ \geq\ B}{Total\ number\ of\ cases\ with\ TBRD \leq 3\ in\ the\ study}$$

$$r_1(c) = \frac{Number\ of\ cases\ with\ TBRD > 3\ for\ which\ c\ \leq\ C}{Total\ number\ of\ cases\ with\ TBRD > 3\ in\ the\ study}$$

$$r_2(b) = \frac{Number\ of\ cases\ with\ TBRD \leq 3\ for\ which\ c\ \leq\ C}{Total\ number\ of\ cases\ with\ TBRD \leq 3\ in\ the\ study}$$

Logistic curve fitting

A logistic curve was fitted to the empirical cumulative probability distributions for both the diagnosis of low and detrimental blood plasma NEFA as well as for the diagnosis of risk for number of insemination number more than 3 and less or equal to 3 (TBRD).

$$y = \frac{\beta_2}{1 + \exp[-\beta_0 * (\mathrm{x} - \beta_1)]}$$

The parameters β_0 (slope) and β_1 (inflection point) were calculated by SPSS 22.0. The upper limit (β_2) equals 1.

Chapter 3

RESULTS

1. RECAPITULATION OF EXPERIMENTAL SET-UP AND OBJECTIVES

141 fresh cows were sampled on day 3 and day 10 after calving for milk and blood. The milk was analysed for milk fatty acids and the blood plasma for BHBA and NEFA. Further, the reproductive indicator Times Bred (TBRD) was registered. Data are presented and were treated in different ways. First, an overview of the dataset is given in 2. Descriptive statistics. Secondly, formerly proposed thresholds for milk C18:1 *cis*-9 to classify cases showing detrimental blood plasma NEFA (≥ 0.6 mmol/L) were tested on the new observations (3. Validation of the milk fat C18:1 cis-9 threshold). Thereafter, the potential of milk C18:1 *cis*-9 thresholds to classify the observations of high TBRD was computed (4. Classification based on high insemination number (TBRD > 3)). Further a discriminant analysis was performed to check whether other milk fatty acids or ratios would be of interest for the classification by TBRD (5. Discriminant function) From the results of the previous analysis, multiple empirical cumulative distributions were developed which were fitted to a logistic curve (6. Empirical cumulative distributions)

For all calculations, data were split in day 3 and day 10 after parturition and all analyses were performed separately for the two groups.

2. DESCRIPTIVE STATISTICS

Preliminary test

Since milk sampling during the milking process (flow through) was not possible at the farm of Gut Hohen Luckow and milk had to be stripped directly after the milking cluster was removed, a preliminary test was conducted whether the milk fatty acid profile differed between flow through sampling and strip sampling (Table 3.1).

Table 3.1. The mean values of the milk fatty acids are presented for the flow through (during milking process) and strip samples (after milking cluster was removed) and the p-value of one-way ANOVA is shown.

	Flow through sampling (g/100g MFAME)	Strip sampling (g/100g MFAME)	p-value
C4:0	3.48	3.47	0.896
C6:0	2.20	2.22	0.537
C8:0	1.26	1.28	0.366
C10:0	2.63	2.73	0.079
C10:1	0.272	0.277	0.636
C12:0	2.95	3.08	0.049
C14:0	10.4	10.5	0.601
iso15:0	0.200	0.183	0.0662
anteiso15:0	0.394	0.377	0.127
C14:1	0.869	0.879	0.642
C15:0	0.946	0.923	0.0936
iso16:0	0.221	0.202	0.0058
C16:0	27.2	28.6	0.0020
C16:1 *cis*-9	1.24	1.30	0.0131
anteiso17:0	0.395	0.365	0.0114
C17:0	0.685	0.635	0.0038
C18:0	10.3	9.49	0.0001
C18:1 *cis*-9	19.8	18.4	0.0001
C18:1 *cis*-9/C15:0	21.5	20.7	0.708

Most milk fatty acid parameters such as anteisoC15:0 and C18:1 *cis*-9/C15:0 did not differ between the two sampling methods (flow through vs. strip), some had the tendency to be higher (e.g. C10:0) or lower (e.g. C15:0) and others were significantly different (e.g. C18:1 *cis*-9).

However, the threshold (of milk C18:1 *cis*-9) conducted from flow through data, that was used in this master dissertation, was not an absolute milk fatty acid value, but was subtracted with a basal, farm specific value (see Chapter 2, 4.3. Validation C18:1 *cis-9* thresholds). Therefore, the assumption was made that this basal value would also level out the differences between sample methods.

Observations from Gut Hohen Luckow

Of all 133 observations of day 3 after parturition, only 5% showed a blood plasma concentration greater than 1.2 mmol/L (Table 3.2). However, 16% of the observations had a blood plasma NEFA concentration higher than 0.6 mmol/L (Table 3.3). For the 137 observations of day 10 after parturition, only 2% had blood plasma BHBA greater than 1.2 mmol/L (Table 3.2). Further, 18% of the samples were above 0.6 mmol/L NEFA (Table 3.3).

Table 3.2. Details of the observations at day 3 and day 10 after parturition split according the blood plasma BHBA threshold of 1.2 mmol/L. The mean values of blood plasma BHBA and NEFA as well as milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio are presented, SEM: standard error mean and the p-value of one-way ANOVA are shown.

BHBA	DAY 3				DAY 10			
	< 1.2 mmol/L	≥ 1.2 mmol/L	SEM	P-value	< 1.2 mmol/L	≥ 1.2 mmol/L	SEM	P-value
Number	126 (95%)	7 (5%)			134 (98%)	3 (2%)		
BHBA (mmol/L)	0.542	0.154	0.0260	< 0.001	0.584	4.05	0.0594	< 0.001
NEFA (mmol/L)	0.376	0.236	0.0282	< 0.001	0.394	0.977	0.0199	< 0.001
C18:1 *cis*-9 (g/100g MFAME)	23.9	4.75	0.440	< 0.001	24.6	32.3	0.31	< 0.001
AnteisoC15:0 (g/100g MFAME)	0.230	0.110	0.048	< 0.001	0.262	0.186	0.0045	0.012
C18:1 *cis*-9/C15:0 (g/100g MFAME)	32.9	14.9	1.60	< 0.001	32.9	70.8	1.18	< 0.001

MFAME = milk fatty acid methyl esters

Table 3.3. Details of the observations at day 3 and day 10 after parturition split according the blood plasma NEFA threshold of 0.6 mmol/L. The mean values of blood plasma BHBA and NEFA as well as milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio are presented, SEM: standard error mean and the p-value of one-way ANOVA are shown.

NEFA	DAY 3				DAY 10			
	< 0.6 mmol/L	≥ 0.6 mmol/L	SEM	P-value	< 1.2 mmol/L	≥ 1.2 mmol/L	SEM	P-value
Number	112 (84%)	21 (16%)			113 (82%)	24 (18%)		
BHBA (mmol/L)	0.531	0.964	0.0260	< 0.001	0.569	1.12	0.0294	< 0.001
NEFA (mmol/L)	0.309	1.02	0.0283	< 0.001	0.322	0.808	0.0199	< 0.001
C18:1 *cis*-9 (g/100g MFAME)	23.3	30.3	0.44	< 0.001	24.0	27.9	0.31	< 0.001
AnteisoC15:0 (g/100g MFAME)	0.237	0.155	0.0048	< 0.001	0.272	0.208	0.0045	< 0.001
C18:1 *cis*-9/C15:0 (g/100g MFAME)	30.2	63.5	1.60	< 0.001	30.8	47.2	1.18	< 0.001

MFAME = milk fatty acid methyl esters

From the 141 cows used for sampling, 36% were pregnant after the first insemination, 19% after the second and 12% after the third insemination. 21% was not yet pregnant after being bred three times (TBRD>3) but was successful after the 4^{th}, 5^{th}, 6^{th}, 7^{th} or even 8^{th} insemination or was not confirmed pregnant yet (Table 3.4). The remaining 12% cows died, were culled or sold.

Table 3.4. Details of the observations at day 3 after parturition split according the times bred (TBRD = 1, 2, 3 or >3). The mean values of blood plasma BHBA and NEFA as well as milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio are presented, SEM: standard error mean and the p-value of one-way ANOVA are shown.

TBRD – DAY 3	TBRD = 1	TBRD = 2	TBRD = 3	TBRD >3	SEM	P-value
Number	51 (36%)	27 (19%)	17 (12%)	29 (21%)		
BHBA (mmol/L)	0.622[a]	0.547[a]	0.578[a]	0.723[a]	0.0287	0.202
NEFA (mmol/L)	0.417[a]	0.387[a]	0.415[a]	0.560[a]	0.0301	0.200
C18:1 *cis*-9 (g/100g MFAME)	24.1[a]	24.1[a]	23.1[a]	26.6[a]	0.47	0.078
AnteisoC15:0 (g/100g MFAME)	0.231[b]	0.234[ab]	0.239[ab]	0.195[a]	0.0052	0.018
C18:1 *cis*-9/C15:0 (g/100g MFAME)	32.2[b]	33.0[ab]	35.5[ab]	45.7[a]	1.75	0.018

Values without a common superscript letter are significantly different (P < 0.05)
MFAME = milk fatty acid methyl esters

At day 3 after parturition, milk fat anteisoC15:0 and C18:1 *cis*-9/C15:0 of the cows that were successfully pregnant after the first insemination (TBRD = 1) differed from that of cows bred more than 3 times (TBRD >3), while also the milk fat C18:1 *cis*-9 content of the latter group tended to be higher (Table 3.4). At day 10 after parturition, blood plasma NEFA and milk fat anteisoC15:0 differed between TBRD = 1 and TBRD >3, while milk fat C18:1 *cis*-9 and C18:1 *cis*-9/C15:0 of the TBRD > 3 group were higher than for the other three groups (TBRD = 1, 2 and 3)(Table 3.5).

Table 3.5. Details of the observations at day 10 after parturition split according the times bred (TBRD = 1, 2, 3 or >3). The mean values of blood plasma BHBA and NEFA as well as milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio are presented, SEM: standard error mean and the p-value of one-way ANOVA are shown.

TBRD – DAY 10	TBRD = 1	TBRD = 2	TBRD = 3	TBRD >3	SEM	P-value
BHBA (mmol/L)	0.627[a]	0.567[a]	0.576[a]	0.982[a]	0.0663	0.112
NEFA (mmol/L)	0.377[b]	0.[343ab]	0.429[ab]	0.543[a]	0.0218	0.008
C18:1 *cis*-9 (g/100g MFAME)	24.2[b]	24.2[b]	23.6[b]	26.9[a]	0.33	0.003
AnteisoC15:0 (g/100g MFAME)	0.272[b]	0.264[ab]	0.276[ab]	0.235[a]	0.0050	0.024
C18:1 *cis*-9/C15:0 (g/100g MFAME)	31.9[b]	30.6[b]	29.1[b]	42.4[a]	1.29	0.002

Values without a common superscript letter are significantly different (P < 0.05)
MFAME = milk fatty acid methyl esters

Due to the low prevalence rate of subacute ketotic cases, the potential for its diagnosis based on milk biomarkers was not considered further in this master dissertation. Prevalence rate of 'detrimental NEFA" was acceptable: from all observations, 15 and 16% had a blood plasma NEFA concentration greater than 0.6 mmol/L at day 3 and day 10 after parturition, respectively. Secondly, 20% of the cows had been inseminated more than 3 times (TBRD >3).

3. VALIDATION OF THE MILK FAT C18:1 *CIS*-9 THRESHOLD

Former research at the Laboratory for Animal Nutrition and Animal Product Quality (Faculty of Bioscience Engineering, Ghent University, Belgium) proposed a threshold for milk fat C18:1 *cis*-9 (Δ C18:1 *cis*-9 = 4.11 g/100g MFAME) as an early warning for elevated blood plasma NEFA (≥0.6 mmol/L) (Jorjong et al. in preparation). However, milk C18:1 *cis*-9 concentrations depend on other factors besides blood plasma NEFA concentrations (e.g. diet, sampling day…). Hence, a "basal" farm-specific C18:1 *cis*-9 concentration was determined, which allowed the calculation of a 'subtracted' milk fat Δ C18:1 *cis*-9 value which was used as benchmark instead of the absolute value of the C18:1 *cis*-9 concentration. The basal C18:1 *cis*-9 value was obtained by computing the mean of the 85% lowest milk fat C18:1 *cis*-9 concentrations.

This milk fat Δ C18:1 *cis*-9 threshold was used to split both the day 3 as well as the day 10 after parturition dataset into two groups (≥ or < 4.11 g/100g MFAME) and the agreement of this grouping with classification based on the blood plasma NEFA threshold of 0.6 mmol/L was assessed, e.g. 15 observations from the group NEFA ≥ 0.6 mmol/L were higher than 4.11 g/100g MFAME Δ C18:1 *cis*-9 at day 3 after parturition and 6 lower (Table 3.6). From this, performance indicators for classification such as sensitivity, specificity and accuracy were computed (Table 3.6). E.g. the test correctly classified 71% and 77% of the cows at risk and not at risk, respectively, for detrimental blood plasma NEFA at day 3 after parturition. The overall accuracy of this test was 76%. A similar classification was done for the day 10 after parturition data.

Table 3.6. The 2x2 table with the number of true positives, false positives, false negatives and true negatives at day 3 and day 10 after parturition (with a total of 133 and 137 observations, respectively) in the potential of a milk fat C18:1 *cis*-9 threshold (Δ C18:1 *cis*-9) of 4.11 g/100g MFAME to diagnose cases of low or elevated blood plasma NEFA (< or ≥ 0.6 mmol/L). From this, the performance indicators sensitivity, specificity and accuracy at day 3 and day 10 of the milk fat C18:1 *cis*-9 threshold (Δ C18:1 *cis*-9: = 4.11 g/100g MFAME) were computed.

	DAY 3		DAY 10	
	NEFA ≥ 0.6 mmol/L	NEFA < 0.6 mmol/L	NEFA ≥ 0.6 mmol/L	NEFA < 0.6 mmol/L
Δ C18:1 *cis*-9 ≥ 4.11 g/100g MFAME	15	26	11	13
Δ C18:1 *cis*-9 < 4.11 g/100g MFAME	6	86	13	100
Sensitivity (%)	71		46	
Specificity (%)	77		88	
Accuracy (%)	76		81	

MFAME = milk fatty acid methyl esters

The proposed milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME to classify cows with detrimental blood plasma NEFA levels (≥0.6mmol/L) was validated with the new dataset. Classification based on this threshold showed a sensitivity of 71% and 46%, a specificity of 77% and 88% and overall accuracy of 76% and 81% when based on milk samples of day 3 and day 10 after parturition, respectively.

4. CLASSIFICATION BASED ON HIGH INSEMINATION NUMBER (TBRD > 3)

Furthermore, the potential of this threshold to classify the observations according to the times bred (TBRD ≤ 3 and TBRD > 3) was calculated. Observations were split into two groups based on the milk Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME and this classification was compared with classification based on TBRD > 3 or ≤ 3, to generate a 2x2 table e.g. 11 milk fat Δ C18:1 *cis*-9 observations from the group TBRD > 3 were higher than 4.11 g/100g MFAME at day 3 after parturition whereas 17 were lower (Table 3.7). From this, the classification performance indicators sensitivity, specificity and accuracy were computed (Table 3.7). E.g. 39% of the cows requiring more than 3 inseminations also showed a milk Δ C18:1 *cis*-9 value exceeding 4.11 g/100g MFAME at day 3 after parturition whereas 77% of the cows being successfully pregnant after the first, second or third insemination showed milk Δ C18:1 *cis*-9 values below this threshold. The overall accuracy of this test was 76%.

Table 3.7. The 2x2 table with the number of true positives, false positives, false negatives and true negatives at day 3 and day 10 after parturition (with a total of 118 and 122 observations, respectively) in the potential of a milk fat C18:1 *cis*-9 threshold (Δ C18:1 *cis*-9) of 4.11 g/100g to diagnose cases of low or high insemination number (≤ or > 3). From this, the performance indicators sensitivity, specificity and accuracy at day 3 and day 10 of the milk fat C18:1 *cis*-9 threshold (Δ C18:1 *cis*-9: = 4.11 g/100g) were computed.

	DAY 3		DAY 10	
	TBRD >3	**TBRD ≤ 3**	**TBRD >3**	**TBRD ≤ 3**
Δ C18:1 *cis*-9 ≥ 4.11 g/100g MFAME	11	21	11	11
Δ C18:1 *cis*-9 < 4.11 g/100g MFAME	17	69	18	82
Sensitivity (%)	39		38	
Specificity (%)	77		88	
Accuracy (%)	68		76	

MFAME = milk fatty acid methyl esters

The milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME did not allow to accurately identify cases requiring more than 3 inseminations as only 39% or 38% of the cases were correctly classified based on observations at day 3 and day 10 after parturition.

5. DISCRIMINANT FUNCTION

The results from the milk fat Δ C18:1 *cis*-9 threshold to classify the observations according the times bred were poor, which might be related to the fact that the threshold was formerly developed for the classification of detrimental blood plasma NEFA since reproductive data were not available. Therefore, an assessment whether other milk fatty acids could be identified to optimise classification was performed.

The most discriminating milk fatty acid variables to classify the observations of high insemination number (TBRD > 3) were identified by the standardized canonical discriminant function coefficients, which revealed milk anteisoC15:0 to be the most discriminating variable for the classification of TBRD > 3 at day 3 after parturition and the ratio of milk C18:1 *cis*-9 and C15:0 at day 10 after parturition. Subsequently, cross-validation results for grouping based on these single variables (milk anteisoC15:0, C18:1 *cis*-9/C15:0 and C18:1 *cis*-9) resulted in classification sensitivities, specificities and accuracies as summarized in Table 3.8. At day 3 after parturition, the milk fat anteisoC15:0 had an overall accuracy of 64%, and more specifically a correct classification of 57% of the cows with TBRD > 3 and 67% of the cows with TBRD ≤ 3. The milk fat C18:1 *cis*-9/C15:0 ratio at day 10 after parturition had an accuracy of 68% overall, and 48% and 74% of high and lower TBRD cows, respectively, were correctly classified.

A new threshold for milk Δ C18:1 *cis*-9 for the classification of observations of high insemination number (TBRD >3) was calculated from the discriminant analysis performed based on the inclusion of only C18:1 *cis*-9. This resulted in a milk Δ C18:1 *cis*-9 threshold of 2.29 g/100g MFAME at day 3 after parturition and 1.83 g/100g MFAME at day 10 after parturition.

Table 3.8. The classification performance indicators sensitivity, specificity and accuracy calculated for milk fatty acid C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio on day 3 and day 10 after parturition in the classification of high insemination number (TBRD >3) through cross-validated discriminant analysis (default leave-one-out procedure in SPSS 22.0) .

	C18:1 *cis*-9		AnteisoC15:0		C18:1 *cis*-9/C15:0	
	DAY 3	DAY 10	DAY 3	DAY 10	DAY 3	DAY 10
Sensitivity (%)	57	55	57	52	36	48
Specificity (%)	60	68	67	67	78	74
Accuracy (%)	59	65	64	63	68	68

The most discriminating variables for the classification of high TBRD cows (TBRD >3) were anteisoC15:0 (day 3) and the C18:1 *cis*-9/C15:0 ratio (day 10). Nevertheless, early identification of risk for excessive number of inseminations remains poor.

6. EMPIRICAL CUMULATIVE DISTRIBUTIONS

Besides classification performance characteristics based on discriminant functions, a visual evaluation of the potential to distinguish groups with elevated blood plasma NEFA and an excessive number of inseminations can be done by means of the construction of the cumulative distributions for each of the three milk fatty acid parameters which were identified as the most discriminating based on the discriminant analysis: C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio.

6.1. Empirical cumulative probability distributions for diagnosis of risk for low and detrimental blood plasma NEFA

The cumulative proportion of cases at risk of detrimental blood NEFA to the total number of detrimental NEFA cases was plotted against the milk fat C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio for both day 3 and day 10 after parturition.

C18:1 *cis*-9

The cumulative proportion of observations at day 3 after parturition with a milk fat C18:1 *cis*-9 concentration (*a*) lower than or equal to an increasing value of C18:1 *cis*-9 ("*A*" ranging from 15 to 36 in steps of 0.25) to the total number of detrimental blood plasma NEFA (≥ 0.6 mmol/L) was plotted against the milk fat C18:1 *cis*-9 concentration to which a logistic curve was fitted ($p_1(a)$; Figure 3.1). This logistic curve is characterized by the slope β_0 (0.318) and the inflection point β_1 that equalled 31.0 which indicates that 50% of the observations with a blood plasma NEFA concentration of 0.6 mmol/L or more were associated with a milk fat C18:1 *cis*-9 concentration of at least 31 g/100g MFAME. Similarly, a logistic curve was fitted for low blood NEFA cases, $p_2(a)$, which is characterized by β_0 (0.385) and β_1 (23.4).

Further, the milk C18:1 *cis*-9 concentration was determined which corresponded to 90% of the "healthy cases" (NEFA < 0.6mmol/L) (i.e. 90% of the cases with NEFA < 0.6 mmol/L showed a milk C18:1 *cis*-9 concentration below this threshold). A high true positive rate is wanted because farmers indicate to have little confidence in management systems that generate a high rate of false alarms. At day 3 after parturition, this C18:1 *cis*-9 concentration was 29.1 g/100g MFAME. However 35% of the "sick cases" (NEFA ≥ 0.6 mmol/L) also showed a milk C18:1 *cis*-9 concentration lower than 29.1 g/100g MFAME, resulting in a false negative rate of 35% (Figure 3.1).

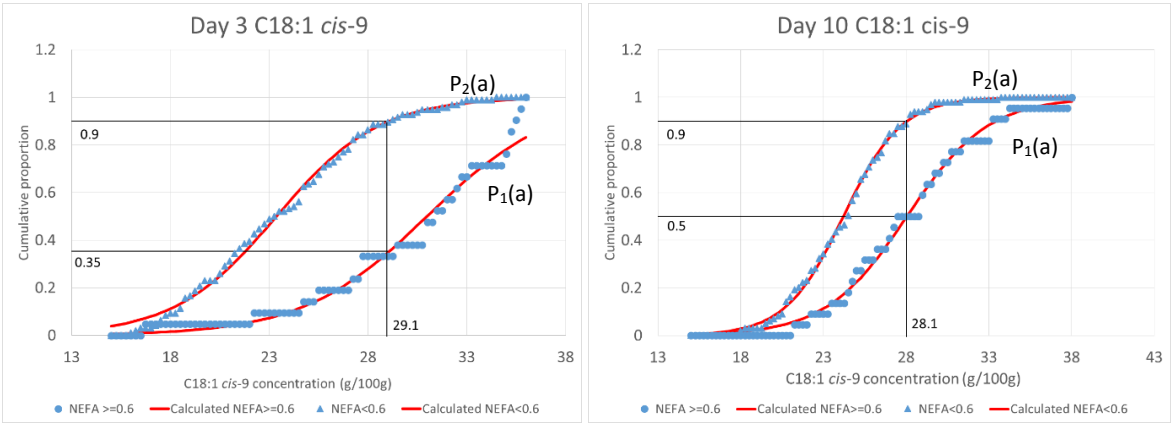


Figure 3.1. The empirical cumulative probability distributions for milk C18:1 *cis*-9 on day 3 and day 10 after parturition, respectively. A logistic curve, $p_1(a)$, representing the cumulative proportion of observations with milk fat C18:1 *cis*-9 (a) ≤ A in case of detrimental blood plasma NEFA (≥0.6 mmol/L; blue circles) to the total number of cases with detrimental blood NEFA. The logistic curve is characterized by its slope (β_0=0.318 (day 3) or 0.408 (day 10)) and inflection point (β_1=31.0 (day 3) or 28.0 (day 10)). Similarly, cases of low blood NEFA (blue triangles) showing a milk fat C18:1 *cis*-9 (a) ≤ A were presented relative to the total low blood NEFA cases, $p_2(a)$, with β_0 and β_1 of respectively 0.385 and 23.4 for day 3 and 0.571 and 24.2 for day 10.

The same was done for day 10 after parturition (Figure 3.1). The logistic curve $p_1(a)$, representing the cumulative proportion of cases with detrimental blood plasma NEFA had a slope β_0 of 0.408 and an inflection point β_1 of 28.0. Further, $p_2(a)$, the logistic curve of the low blood NEFA observations, is characterized by β_0 (0.571) and β_1 (24.202). Here, the milk C18:1 *cis*-9 concentration corresponding to 90% of the "healthy cases" was 28.1 g/100g MFAME, with a false negative rate of 50% (Figure 3.1).

AnteisoC15:0

In a similar way, the empirical cumulative probability distributions were generated for the milk fat anteisoC15:0 (*b*). 90% of the "healthy cases" (NEFA < 0.6 mmol/L) had a milk anteisoC15:0 concentration greater than 0.182 g/100g MFAME at day 3 after parturition, while 29% of the "sick animals" (NEFA ≥ 0.6 mmol/L) also showed a milk anteisoC15:0 higher than 0.182 g/100g MFAME, which corresponded to the false negative rate (Figure 3.2).

Further, the milk anteisoC15:0 concentration corresponding to 90% of the "healthy cows" at day 10 after parturition equalled 0.218 g/100g MFAME and had a false negative rate of 39% (Figure 3.2).

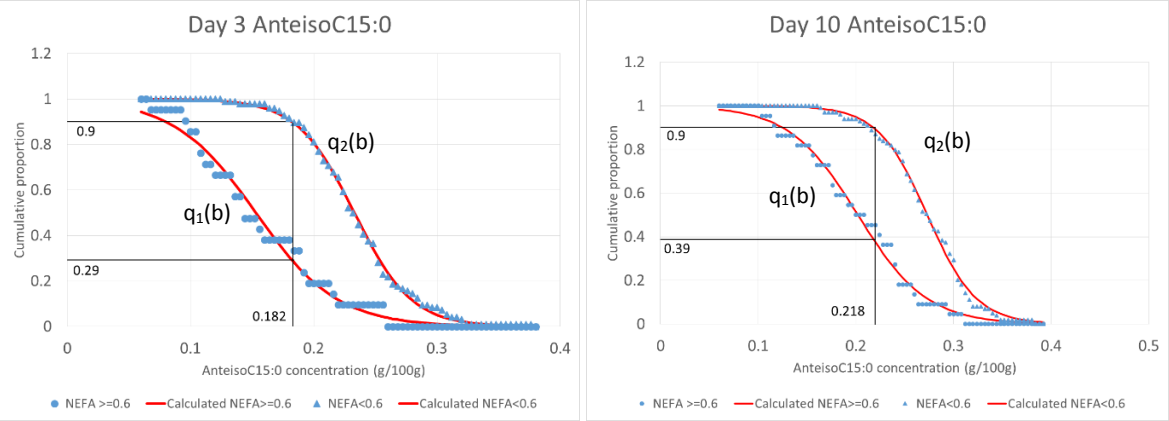


Figure 3.2. The empirical cumulative probability distributions for milk anteisoC15:0 on day 3 and day 10 after parturition, respectively. A logistic curve, q1(b), representing the cumulative proportion of observations with milk fat anteisoC15:0 (b) ≤ B in case of detrimental blood plasma NEFA (≥0.6 mmol/L; blue circles) to the total number of cases with detrimental blood NEFA. The logistic curve is characterized by its slope (β0=-30.1 (day 3) or -28.4 (day 10)) and inflection point (β1=0.153 (day 3) or 0.202 (day 10)). Similarly, cases of low blood NEFA (blue triangles) showing a milk fat anteisoC15:0 (b) ≤ B were presented relative to the total low blood NEFA cases, q2(b), with β0 and β1 of respectively -43.4 and 0.233 for day 3 and -39.9 and 0.273 for day 10.

C18:1 *cis*-9/C15:0

Subsequently, empirical cumulative probability distributions were plotted for the milk C18:1 *cis*-9/C15:0 ratio (*c*). 90% of the cows with a blood plasma NEFA lower than 0.6 mmol/L at day 3 and day 10 after parturition had a C18:1 *cis*-9/C15:0 ratio lower than 42.1 and 41.3, respectively. The false negative rate corresponded to 22% at day 3 after parturition and 41% at day 10 after parturition (Figure 3.3).

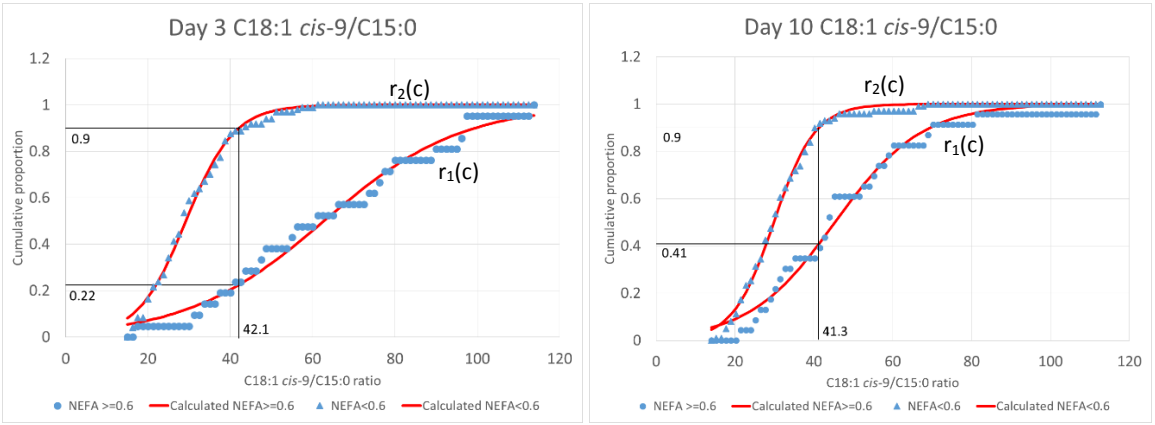


Figure 3.3. The empirical cumulative probability distributions for the milk C18:1 *cis*-9/C15:0 ratio on day 3 and day 10 after parturition, respectively. A logistic curve, $r_1(c)$, representing the cumulative proportion of observations with milk fat C18:1 *cis*-9/C15:0 (c) ≤ C in case of detrimental blood plasma NEFA (≥0.6 mmol/L; blue circles) to the total number of cases with detrimental blood NEFA. The logistic curve is characterized by its slope (β_0=0.060 (day 3) or 0.091 (day 10)) and inflection point (β_1=62.7 (day 3) or 45.4 (day 10)). Similarly, cases of low blood NEFA (blue triangles) showing a milk fat C18:1 *cis*-9/C15:0 (c) ≤ C were presented relative to the total low blood NEFA cases, $r_2(c)$, with β_0 and β_1 of respectively 0.171 and 29.2 for day 3 and 0.192 and 29.9 for day 10.

Cumulative probability distributions for diagnosis of risk for low and detrimental blood plasma NEFA were constructed and fitted with a logistic curve for two milk fatty acids and a milk fatty acid ratio (C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio). After calculating the threshold associated with a maximum of 10% false positives, the curve of the cases with detrimental blood NEFA allowed to assess the false negative rate as summarized in Table 3.9. Milk fatty acid characteristics as mentioned before seem more performant in diagnosing high blood NEFA cases at day 3 than day 10 after parturition.

Table 3.9. Overview of the results after constructing the cumulative probability distributions for the diagnosis of risk for low and detrimental blood plasma NEFA at both day 3 and day 10. The threshold value is defined as the value where 90% of the observations with a blood NEFA lower than 0.6 mmol/L is lower than the threshold for milk C18:1 *cis*-9 or C18:1 *cis*-9/C15:0 and higher than the threshold for milk anteisoC15:0. The false negative rate is the percentage of observations with a blood NEFA higher than or equal to 0.6 mmol/L that is lower than the threshold for milk C18:1 *cis*-9 and C18:1 *cis*-9/C15:0 ratio and higher than the threshold for milk anteisoC15:0.

NEFA	DAY 3		DAY 10	
	Threshold	False negative rate (%)	Threshold	False negative rate (%)
C18:1 *cis*-9	29.1 g/100g MFAME	35	28.1 g/100g MFAME	50
AnteisoC15:0	0.182 g/100g MFAME	29	0.218 g/100g MFAME	39
C18:1 *cis*-9/C15:0	42.1	22	41.3	41

MFAME = milk fatty acid methyl esters

6.2. EMPIRICAL CUMULATIVE PROBABILITY DISTRIBUTIONS FOR DIAGNOSIS OF RISK FOR TBRD ≤ 3 AND TBRD > 3

Following the visual evaluation of the potential to distinct groups with elevated blood plasma NEFA, the potential to distinct the groups with high insemination number (TBRD >3) was evaluated for each of the three milk fatty acid parameters: C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio.

C18:1 *cis*-9

The cumulative proportion of observations from day 3 after parturition with a milk fat C18:1 *cis*-9 concentration (*a*) lower than or equal to an increasing value of C18:1 *cis*-9 ("*A*" ranging from 15 to 36 in steps of 0.25) to the total number of high insemination number (TBRD >3) was plotted against the milk fat C18:1 *cis*-9 concentration to which a logistic curve was fitted ($s_1(a)$). Similarly, a logistic curve was fitted for cases with less than or equal to 3 inseminations (TBRD ≤ 3; $s_2(a)$).

The milk C18:1 *cis*-9 concentration was determined which corresponded to 90% of the "not at risk cases" (TBRD ≤ 3) (i.e. 90% of the cases with TBRD ≤ 3 showed a milk C18:1 *cis*-9 concentration below this threshold). At day 3 after parturition, this C18:1 *cis*-9 concentration was 30.3 g/100g MFAME. However 75% of the "at risk cases" (TBRD > 3) also showed a milk C18:1 *cis*-9 concentration lower than 30.3 g/100g MFAME, which corresponded to the false negative rate (Figure 3.4).
At day 10 after parturition, the milk C18:1 *cis*-9 concentration corresponding to 90% of the "not at risk cases" was 28.2 g/100g MFAME, with a false negative rate of 65% (Figure 3.4).

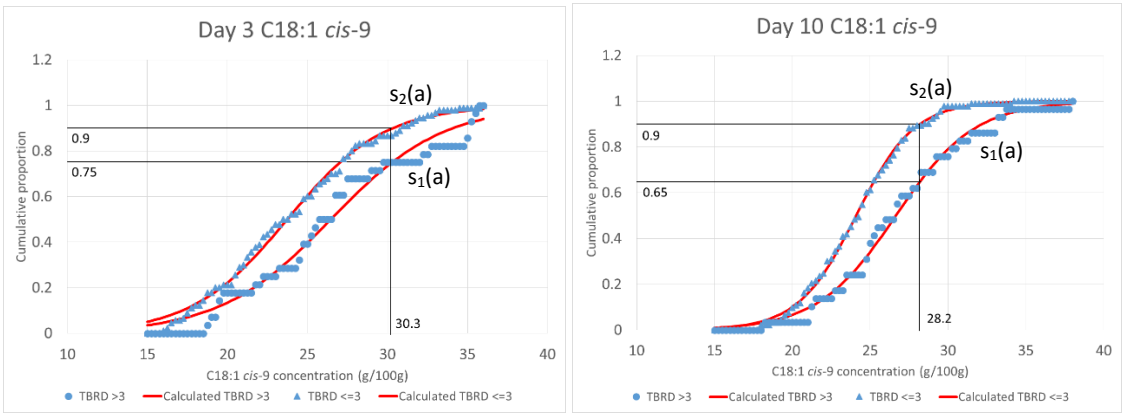


Figure 3.4. The empirical cumulative probability distributions for milk C18:1 *cis*-9 on day 3 and day 10 after parturition, respectively. A logistic curve, $s_1(a)$, representing the cumulative proportion of observations with milk fat C18:1 *cis*-9 (a) ≤ A in case of high insemination number (TBRD >3; blue circles) to the total number of observations with high insemination number. The logistic curve is characterized by its slope (β0=0.288 (day 3) or 0.397 (day 10)) and inflection point (β1=26.5 (day 3) or 26.7 (day 10)). Similarly, cases of 3 or less inseminations (TBRD ≤ 3; blue triangles) showing a milk fat C18:1 *cis*-9 (a) ≤ A were presented relative to the total number of cases with 3 or less inseminations, $s_2(a)$, with β0 and β1 of respectively 0.334 and 23.8 for day 3 and 0.536 and 24.1 for day 10.

AnteisoC15:0

In a similar way, the empirical cumulative probability distributions were generated for the milk fat anteisoC15:0 (*b*). 90% of the "not at risk cases" (TBRD ≤ 3) had a milk anteisoC15:0 concentration greater than 0.171 g/100g MFAME at day 3 after parturition, while 69% of the "at risk animals" (TBRD >3) also showed a milk anteisoC15:0 higher than 0.171 g/100g MFAME, which corresponded to the false negative rate (Figure 3.5).

Further, the milk anteisoC15:0 concentration corresponding to 90% of the "not at risk cows" at day 10 after parturition equalled 0.214 g/100g MFAME and had a false negative rate of 63% (Figure 3.5).

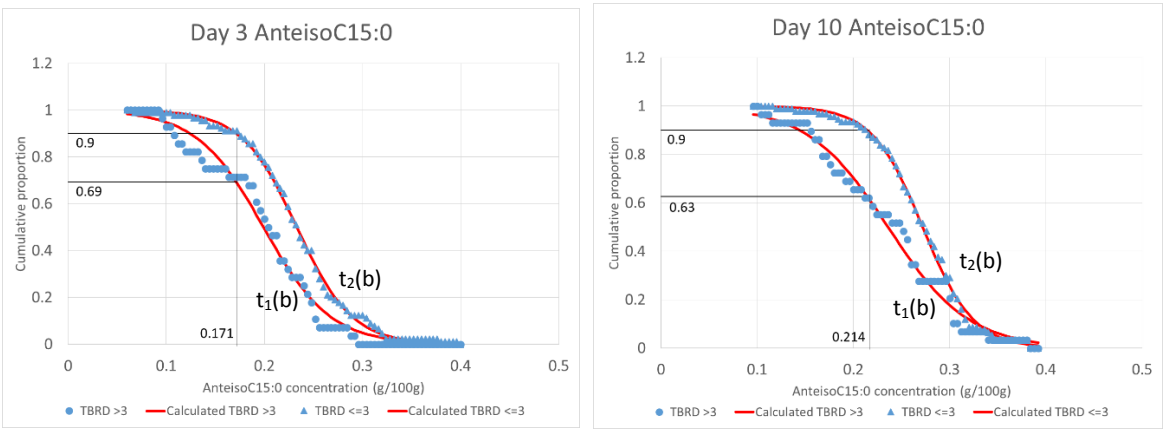


Figure 3.5. The empirical cumulative probability distributions for milk anteisoC15:0 on day 3 and day 10 after parturition, respectively. A logistic curve, *$t_1(b)$*, representing the cumulative proportion of observations with milk fat anteisoC15:0 (b) ≤ B in case of high insemination number (TBRD >3; blue circles) to the total number of observations with high insemination number. The logistic curve is characterized by its slope (β_0=-30.1 (day 3) or -28.4 (day 10)) and inflection point (β_1=0.153 (day 3) or 0.202 (day 10)). Similarly, cases of 3 or less inseminations (TBRD ≤ 3; blue triangles) showing a milk fat anteisoC15:0 (b) ≤ B were presented relative to the total number of cases with 3 or less inseminations, *$t_2(b)$*, with β_0 and β_1 of respectively -43.4 and 0.233 for day 3 and -39.9 and 0.273 for day 10.

C18:1 *cis*-9/C15:0

Finally, empirical cumulative probability distributions were plotted for the milk C18:1 *cis*-9/C15:0 ratio (*c*). 90% of the cows with a low insemination number (TBRD ≤ 3) at day 3 and day 10 after parturition had a milk C18:1 *cis*-9/C15:0 ratio lower than 47.2 and 42.6, respectively. The false negative rate corresponded to 60% at day 3 after parturition and 57% at day 10 after parturition (Figure 3.6).

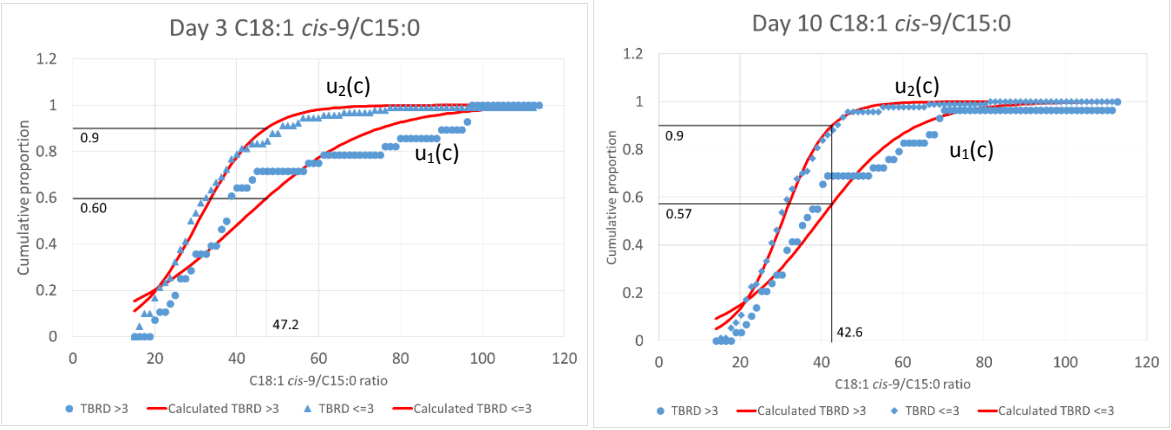


Figure 3.6. The empirical cumulative probability distributions for the milk C18:1 *cis*-9/C15:0 ratio on day 3 and day 10 after parturition, respectively. A logistic curve, $u_1(c)$, representing the cumulative proportion of observations with milk fat C18:1 *cis*-9/C15:0 (c) ≤ C in case of high insemination number (TBRD >3; blue circles) to the total number of observations with high insemination number. The logistic curve is characterized by its slope (β_0=0.065 (day 3) or 0.090 (day 10)) and inflection point (β_1=41.2 (day 3) or 39.3 (day 10)). Similarly, cases of 3 or less inseminations (TBRD ≤ 3; blue triangles) showing a milk fat C18:1 *cis*-9/C15:0 (c) ≤ C were presented relative to the total number of cases with 3 or less inseminations, $u_2(c)$, with β_0 and β_1 of respectively 0.133 and 30.7 for day 3 and 0.180 and 30.4 for day 10.

Cumulative probability distributions for diagnosis of risk to require 3 or less inseminations vs. more than 3 inseminations were constructed and fitted to a logistic curve for two milk fatty acids and a milk fatty acid ratio (C18:1 *cis*-9, anteisoC15:0 and the C18:1 *cis*-9/C15:0 ratio) measured 3 to 10 days after parturition. After calculating the threshold associated with a maximum of 10% false positives, the curve of the cases with high insemination number (TBRD >3) allowed to assess the false negative as summarized in Table 3.10.

Table 3.10. Summarizing table of the results after constructing the cumulative probability distributions for the diagnosis of risk for low and high insemination number on both day 3 and day 10. The threshold value is defined as the value where 90% of the observations with an insemination number lower than or equal to 3 is lower than the threshold for milk C18:1 *cis*-9 and C18:1 *cis*-9/C15:0 and higher than the threshold for milk anteisoC15:0. The false negative rate is the percentage of observations with an insemination number higher than 3 that is lower than the threshold for milk C18:1 *cis*-9 and C18:1 *cis*-9/C15:0 ratio and higher than the threshold for milk anteisoC15:0.

TBRD	DAY 3		DAY 10	
	Threshold	False positive rate (%)	Threshold	False positive rate (%)
C18:1 *cis*-9	30.3 g/100g MFAME	75	28.2 g/100g MFAME	65
AnteisoC15:0	0.171 g/100g MFAME	69	0.214 g/100g MFAME	63
C18:1 *cis*-9/C15:0	47.2	60	42.6	57

MFAME = milk fatty acid methyl esters

Chapter 4

DISCUSSION

The transition period, defined as 3 weeks before to 3 weeks after parturition, is a critical period for the dairy cow, since the cow experiences the stress of parturition, starts lactation and recommences a reproductive cycle (Drackley 1999). At the onset of lactation, the high milk producing dairy cow encounters a steep increase in energy and protein requirements, concomitant with a reduced feed intake, which is commonly inadequate to meet the maintenance and production demands. Therefore, the high yielding dairy cow enters a state of negative energy balance (NEB).

Changes in NEB cause shifts in the milk fat composition (Stoop et al. 2009) which makes concentrations of specific milk fatty acids interesting biomarkers for NEB and thus elevated blood plasma NEFA concentrations (Gross et al. 2011) (indicated as (1) in Figure 4.1) . Further, since NEB and blood plasma NEFA are related to impaired fertility through a reduced oocyte developmental competence and viability of the subsequent embryo (Figure 4.1 (2)) (Leroy et al. 2005; Van Hoeck et al. 2011), this master dissertation examined the potential of milk fatty acid parameters to diagnose reproductive problems (Figure 4.1 (4)), here assessed through the number of artificial inseminations (Figure 4.1 (3)).

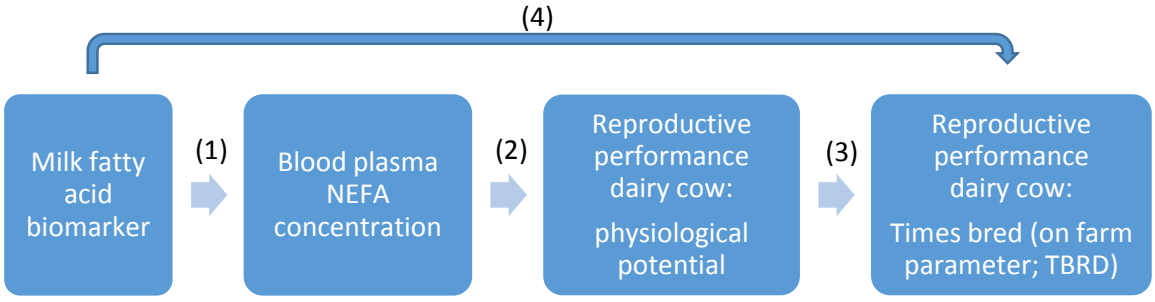


Figure 4.1. Summarizing scheme of the master dissertation objective.

1. VALIDATION OF THE MILK FAT C18:1 *CIS*-9 THRESHOLD

During a period of negative energy balance (NEB), NEFA in blood plasma are increased and the fatty acid supply to the mammary gland is altered. Indeed, NEFA released from lipolysis are mainly C16:0, C18:0 and C18:1 *cis*-9, with a further possible conversion of C18:0 to C18:1 *cis*-9 in the mammary gland through the action of Δ9-desaturase (Hostens et al. 2012). Therefore, milk fat C18:1 *cis*-9 is a suitable indicator of the energy balance in dairy cows (Gross et al. 2011) since it is found related to the blood plasma BHBA and NEFA concentrations (Van Haelst et al. 2008; Jorjong et al. 2014). This explains the significantly higher milk C18:1 *cis*-9 concentration at both day 3 and day 10 after parturition of the cows with detrimental blood plasma NEFA (≥ 0.6 mmol/L) compared with cows having blood plasma NEFA lower than 0.6 mmol/L ($p < 0.001$). The threshold value for milk fat Δ C18:1 *cis*-9, as obtained from a former experiment at the department (Jorjong et al. in preparation) equalled 4.11 g/100g milk fatty acid methyl esters (MFAME) and was used as benchmark to classify observations of detrimental vs. non-detrimental blood plasma NEFA of the current dataset.

In the former research, a sensitivity of 55%, specificity of 89% and overall accuracy of 80% was observed when applying this threshold value to a completely new dataset (Jorjong et al. in preparation). When validating this threshold on the current dataset, similar results were obtained, especially at day 10 after parturition (sensitivity, specificity and accuracy of 46%, 88% and 81%, respectively). At day 3 after parturition, the sensitivity of the threshold was remarkably higher (71%), but a somewhat lower specificity (77%) and accuracy (76%) were observed (Table 3.6). Hence, the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME maintained its potential in the diagnosis of detrimental blood plasma NEFA early in lactation.

As a comparison, cow-side tests in milk samples are already available for the diagnosis of elevated blood BHBA (ketosis defined as blood serum BHBA ≥ 1.4 mmol/L). E.g. KetoCheck powder (Great States Animal Health, St. Joseph, US), which measures concentrations of acetoacetic acid in milk, was shown to be 41% sensitive and 99% specific and KetoTest (Sanwa Kagaku Kenyusho Co. Ltd., Nagoya, Japan), which measures BHBA concentrations in milk had sensitivity and specificity ranges of 27-59% and 76-99%, respectively. Cow-side tests in the monitoring of NEFA are currently not in use (Townsend 2011).

2. CLASSIFICATION BASED ON HIGH INSEMINATION NUMBER (TBRD > 3)

The milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME proved to be an interesting benchmark to classify observations of detrimental blood plasma NEFA. Further, elevated blood plasma NEFA concentrations delayed ovarian cyclicity and reduced the likelihood to be pregnant after the first artificial insemination (AI) (Ribeiro et al. 2013) which might be partially linked to the toxicity of NEFA on granulose cell growth and function (Vanholder et al. 2005).

Therefore, the idea rose that C18:1 *cis*-9 could be used as biomarker for reproductive performance. Indeed, significant differences in milk fat C18:1 *cis*-9 concentrations at day 10 after parturition between the groups with TBRD lower than or equal to 3 and the group TBRD > 3 were observed, so the first results seemed to indicate elevated milk fat C18:1 *cis*-9 for extreme cases. Subsequently, the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME was examined as a diagnostic tool for high number of inseminations (TBRD >3). However, with a sensitivity, specificity and overall accuracy of 39%, 77% and 68%, respectively, at day 3 after parturition and 38%, 88% and 76%, respectively, at day 10 after parturition, the performance indicators of the milk fat Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME to classify TBRD >3 were disappointing (Table 3.7).

3. THREE FATTY ACID PARAMETERS IN THE CLASSIFICATION OF TBRD AND NEFA

Therefore, a discriminant analysis was performed to identify the most discriminating milk fatty acid parameters in the classification of high insemination number (TBRD > 3), which revealed milk anteisoC15:0 to be the most discriminating variable for the classification of TBRD > at day 3 after parturition and the milk C18:1 *cis*-9/C15:0 ratio at day 10 after parturition. This might have been expected given the origin of milk C18:1 *cis*-9, C15:0 and anteisoC15:0. While C18:1 *cis*-9 is an indicator of elevated lipolysis, C15:0 is positively related to glucogenic precursor supply as it is formed through elongation by bacteria in the rumen or in the udder with propionate as the main precursor. Additionally, increased milk fat anteisoC15:0 is related to a higher proportion amylolytic rumen bacteria, which are correlated with a quicker rumen fermentation and thus greater flux of volatile fatty acids into the blood, resulting in a rise of blood insulin and reduction in lipolysis (Fievez et al. 2012). Milk anteisoC15:0 and C18:1 *cis*-9/C15:0 ratio of the cows with detrimental blood plasma NEFA (≥ 0.6 mmol/L) were significantly lower and higher, respectively, as compared with the cows with blood plasma NEFA lower than 0.6 mmol/L at day 3 ($p < 0.001$) and day 10 after parturition ($p = 0.012$). Further, significant differences were found between the milk anteisoC15:0 and C18:1 *cis*-9/C15:0 concentrations at day 3 after parturition of the cows with TBRD = 1 and those with TBRD > 3. At day 10 after parturition, milk anteisoC15:0 differed significantly between TBRD = 1 and TBRD > 3 cows, while the milk C18:1 *cis*-9/C15:0 ratio was significantly lower for all cows with TBRD ≤ 3 as compared with those with more than 3 inseminations.

However, the use of these milk fatty acids as single classifiers of cows with high insemination number (TBRD > 3) was still inadequate with sensitivities ranging from 36% to 57%, specificities from 60% to 78% and overall accuracies between 59% and 68% (Table 3.8), probably due to the large variation within each of the groups (e.g. milk C18:1 *cis*-9/C15:0 had a relative standard deviation of 47.0% for TBRD ≤ 3 and 55.4% for TBRD > 3 at day 3 after parturition).

Therefore, a visual evaluation of the potential of these three milk fatty acid parameters (C18:1 *cis*-9, anteisoC15:0 and C18:1 *cis*-9/c15:0) to distinguish on the one had groups with detrimental blood plasma NEFA (≥ 0.6 mmol/L) and on the other hand groups with high insemination number (TBRD > 3) was drafted by means of the construction of the cumulative distributions. After determining the threshold of milk C18:1 *cis*-9, anteisoC15:0 and C18:1 *cis*-9/C15:0 corresponding to 90% of the

cases with a blood NEFA < 0.6 mmol/L, promising false negative rates of 22% to 35% at day 3 after parturition and 39% to 50% at day 10 after parturition were found. Hereby, the potential of milk fatty acid parameters to diagnose detrimental blood plasma NEFA was reaffirmed.

On the contrary, the distances between the empirical cumulative probability distributions of TBRD ≤ 3 and TBRD > 3 were small, reflected by the minor differences between the β_1 values (e.g. 23.8 g/100g MFAME (TBRD ≤ 3) and 26.5 g/100g MFAME (TBRD > 3) for C18:1 *cis*-9 at day 3 after parturition). Therefore, the false negative rates (ranging from 57% to 75%) corresponding to a false positive rate of 10% were disappointing again.

4. LIMITED POTENTIAL OF MILK FATTY ACIDS AS BIOMARKERS OF ELEVATED NUMBER OF INSEMINATIONS

Since specific milk fatty acids are interesting biomarkers for NEB and thus elevated blood plasma NEFA concentrations (Gross et al. 2011) (indicated as (1) in Figure 4.2) and NEB and blood plasma NEFA are related to impaired fertility through a reduced oocyte developmental competence and viability of the subsequent embryo (Figure 4.2 (2)) (Leroy et al. 2005; Van Hoeck et al. 2011), this master dissertation examined the potential of milk fatty acid parameters to diagnose reproductive problems (Figure 4.2 (4)), here assessed through the number of artificial inseminations (Figure 4.2 (3)).

However, the results related to the potential of milk fatty acids, measured early in lactation, to diagnose observations at risk for excessive number of inseminations (Figure 4.2 (4)) were poor. Hence, the use of a blood plasma NEFA biomarker to classify high insemination numbers (Figure 4.2 (5)) as well as the reproductive performance indicator TBRD (Figure 4.2 (3)) could be questioned.

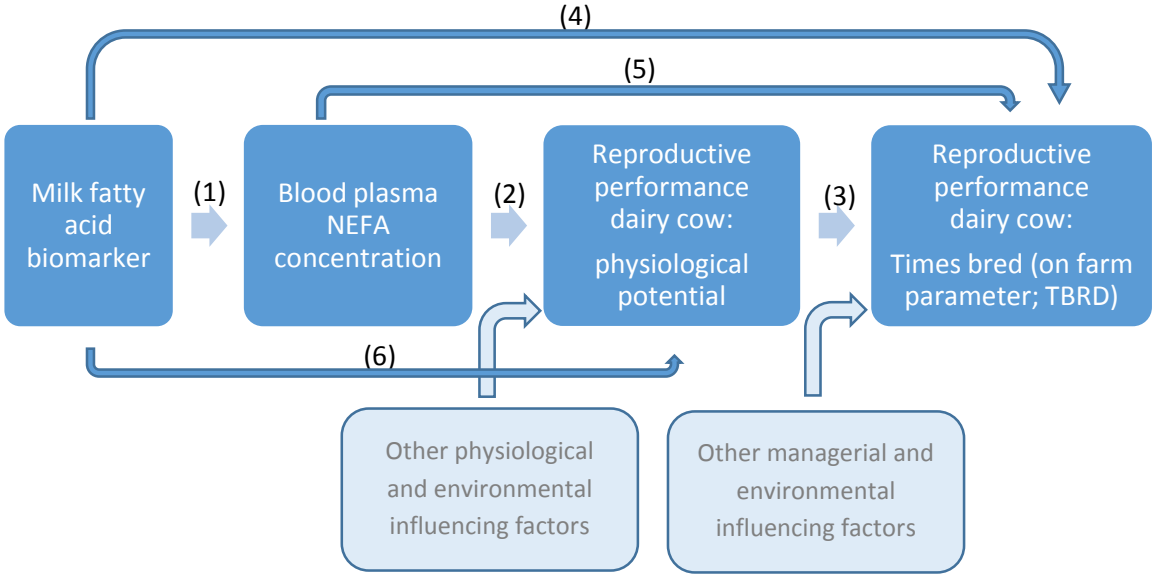


Figure 4.2. Summarizing scheme of the master dissertation objective with inclusion of hampering effects and discussion points.

4.1. A BLOOD PLASMA NEFA BIOMARKER FOR DIAGNOSIS OF HIGH INSEMINATION NUMBER

When using blood plasma NEFA biomarkers to diagnose excessive number of inseminations (TBRD > 3), there needs to be a close relationship between blood plasma NEFA and TBRD to start with. Therefore, a visual evaluation of the potential of blood plasma NEFA to distinguish groups with TBRD ≤ 3 and TBRD > 3 was drafted (Figure 4.3).

The cumulative proportion of cases with a blood plasma NEFA content (*d*) lower than or equal to an increasing value of NEFA ("*D*") to the total number of high insemination number (TBRD > 3) were plotted against the blood plasma NEFA content ($v_1(d)$) and similarly for the cases of low insemination number ($v_2(d)$). The blood plasma NEFA concentration that corresponded to 90% of the "not at risk cases" (TBRD ≤ 3), incorrectly classified 60 to 62% of the "at risk animals" as "not at risk", which corresponded to the unacceptably high false negative rates.

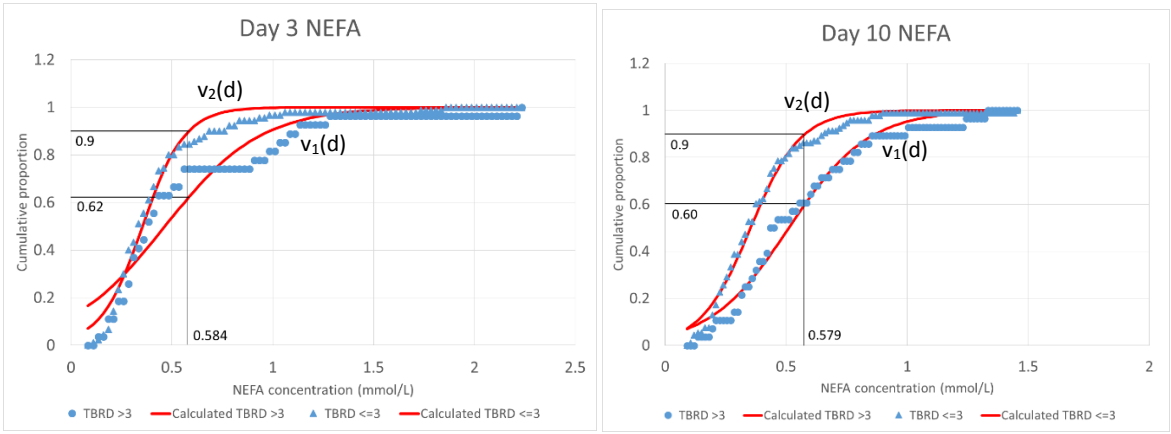


Figure 4.3. The empirical cumulative probability distributions for blood plasma NEFA on day 3 and day 10 after parturition, respectively. A logistic curve $v_1(d)$, representing the cumulative proportion of observations with a blood plasma NEFA (d) ≤ D in case of high insemination number (TBRD > 3; blue circles) to the total number of high insemination number. The logistic curve is characterized by its slope (β_0= 4.24 (day 3) or 6.13 (day 10)) and inflection point (β_1= 0.466 (day 3) or 0.511 (day 10)). Similarly, cases of low insemination number (TBRD ≤ 3; blue triangles) showing blood plasma NEFA (d) ≤ D were presented relative to the total low insemination number cases, $v_2(d)$, with β_0 and β_1 of respectively 9.59 and 0.355 for day 3 and 9.74 and 0.353 for day 10.

Elevated blood plasma NEFA can hamper the reproductive performance, but several other factors influence fertility and are not necessarily correlated with the metabolic energy status of the cow e.g. claw problems, diseases, dystocia (obstructed labour), lactation number and twin birth (Löf et al. 2014), next to management aspects such as heat detection and insemination timing (see further).

It is confirmed that milk fatty acid biomarkers can be of meaningful interest in the early warning of detrimental blood plasma NEFA (Figure 4.2 (1)), which might potentially result in impaired physiological fertility. However, milk fat biomarkers (Figure 4.2 (4)) and blood plasma NEFA (Figure 4.2 (5)) measured early in lactation, are insufficiently related to the on farm parameter TBRD as indicated above. Indeed, better results were obtained when NEB indicators were linked with

physiological characteristics such as first luteal activity, while no distinct effects were shown for the on farm parameter days open (von Leesen et al. 2014). Therefore, it could be of interest to further investigate the relationship between milk fatty acid parameters measured early in lactation and the physiological potential of the cow for reproductive performance, e.g. ovarian cyclicity (Figure 4.2 (5)).

4.2. OTHER PARAMETERS INFLUENCING TBRD

The reproductive indicator used in this study was TBRD, the times the cow received artificial insemination. The advantage of this indicator is the convenience of registration and the ease of interpretation. However, TBRD might be misleading, since these are not physiological but technical data. Indeed, the success of pregnancy is not only determined by the physiological potential of the cow for reproductive performance such as oocyte development and quality but also management factors such as heat detection, time of insemination and inseminator, environmental factors (e.g. stable and weather) and sperm quality (López-Gatius 2012).

The herd manager went once or twice per day through the several groups to check whether there were animals sick, with claw problems or in heat. The cows were painted on top of their tail to detect the cows in heat (paint mark rubbed off by mounting cow), however up to 60% of ovulations may be accompanied by no physical signs such as standing to be mounted (Saint-Dizier & Chastant-Maillard 2012), whereby many cows are not heat detected properly. Further, with one observation per day, only 50% of the cows are truly seen in heat, which makes insemination timing difficult, since this is best 12 hours after first mount standing (Nebel et al. 2000). Subsequently, the inseminator and location of semen disposition can alter the chance of pregnancy with 25% and the quality of the semen and the originating bull was associated with a possible 4.7 fold increase in the pregnancy rate (López-Gatius 2012). Therefore, TBRD might be a biased indicator for reproductive performance, but indicates the importance of the ongoing progress to improve management aspects such as heat detection and the timing and method of the artificial insemination.

5. CONCLUSION

A formerly determined milk fatty acid Δ C18:1 *cis*-9 threshold of 4.11 g/100g MFAME can be a meaningful biomarker in the diagnosis of elevated blood plasma NEFA at day 3 and day 10 after parturition. However, this or other milk fatty acids seem to be insufficient as biomarkers for diagnosis of high insemination number (TBRD > 3). Therefore it can be of interest to investigate further the relationship between specific milk fatty acid parameters (e.g. C18:1 *cis*-9, anteisoC15:0 and C18:1 *cis*-9/C15:0 ratio) and physiological reproductive characteristics such as ovarian cyclicity and oocyte development and quality.

Chapter 5

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