Improving adaptation to lactation

nutrition and management strategies for periparturient dairy cattle

Roselinde Goselink

Propositions

1. The stress of forcing dairy cows to exercise before calving is outweighed by its beneficial effect on the metabolic adaptation to lactation.
   (this thesis)

2. Low prepartum rumen fill of dairy cows is less detrimental than overfeeding energy during the same period.
   (this thesis)

3. Complex statistical techniques increase misuse of statistics to claim causal inference there where there is none.

4. The mental effect of exercise is larger than the physical effect.

5. The focus of veterinarians to define a problem slows down innovation by nutritionists.

6. Feeding ourselves at protein requirements has a large favourable effect on nitrogen pollution.

7. The concept of vegan water opens opportunities for dairy farmers to save the planet

Propositions belonging to the thesis, entitled

"Improving adaptation to lactation: nutrition and management strategies for periparturient dairy cattle"

Roselinde M. A. Goselink
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PREFACE

After completing my veterinary studies, I started my professional life in a research position at Wageningen Livestock Research in the department of Animal Nutrition. I was always interested in the connection between nutrition, physiology and health in dairy cattle, but I was not sure whether a research career could truly match my skills and passions. What I was sure about, was that committing to a PhD ("working four years on a single topic") would be extremely boring…

And here it is: my PhD thesis. It was not a four-year route, it was not really a single topic, and: not boring at all! This academic journey started in my early days at Wageningen Livestock Research with the first project assigned to me, entitled 'Zwangerschapsgym voor koeien' (Prenatal exercise in dairy cattle). This unconventional idea came from my colleague Frank Lenssinck who wanted to see how exercise could affect calving ease. Thinking along, this idea started my interest in the effect of physical exercise on dairy cow metabolism and health. That initial (crazy) pilot study we performed yielded some intriguing results regarding the energy and lipid metabolism of the cows involved. It was followed by a larger trial which is reported in Chapter 3 of this thesis.

Simultaneously I engaged in several other trials monitoring cows around calving, supporting their health by different strategies. With the inspiration of colleagues Ad van Vuuren, Gert van Duinkerken and Thomas Schonewille, the outline of this PhD thesis was shaped with a combination of four studies. It was a challenge to synthesize the wide variation of management and nutrition strategies into one thesis… but without such a challenge, it may have become dull!

Numerous people actively and passionately contributed along the way and I am grateful to each one of you. Highlighting some specific persons, I will start with my promotor Wouter Hendriks: even though I remember your first statement was "I have no interest in cows", thank you for giving me the opportunity to start this thesis, and for guiding me to the end, including the last hurdles! To Thomas and Gert, my co-promotors, your support during brainstorming sessions and manuscript reviews was invaluable, and you also kept reminding me now and then that I had a thesis to finish ;-) Here it is!

The data reported in this thesis has been collected with the help of many colleagues at our research farm De Waiboerhoeve / Dairy Campus. Thanks to Martin de Bree, Edwin Bleumer, Gerard de Bree, Jan Zandbergen, and many others, cows

were selected, checked, sampled, and where needed: convinced to exercise. Thank you all for keeping an eye on the cows!

Ad, Jan Dijkstra, André Bannink, Ariëtte van Knegsel, Jurgen van Baal, Eddy Weurding and Ronald Zom: thank you for your collaboration and co-authorship on one or more scientific articles with me. Ariëtte and Yvette de Haas, thank you for our “tea sessions”. Ad and Arie Klop, thank you for the moral support as my paranymphs towards this day.

The artistic touch on the cover was created by my mother – thank you once again, mom! And last but not least: a heartful thank you to my mom and dad for their support (and babysitting); and to my three unicorns Bart, Jolijn and Niek for always being there, making me laugh and dance!

Roselinde Goselink, Ambt Delden,
November 2023

ABSTRACT

A well-functioning liver is of great importance to dairy cattle, especially in the transition period where the cow is subjected to large changes in diet, management, and metabolic requirements. Improving the adaptation from the dry period to lactation can help to increase cow health and welfare. In this thesis, four nutrition and management strategies are investigated to improve the metabolic adaptive capacity of dairy cows during the transition period: 1) supporting hepatic metabolism; 2) increasing dry cow energy output; 3) reducing dry cow energy input; and 4) maintaining the lactating state prepartum. Each of the four strategies resulted in a reduction of lipid accumulation in the liver. Choline supplementation improved fatty acid processing and transport in the liver, but also hepatic carbohydrate metabolism, supporting the metabolic pathways required in early lactation. Cows with a high body condition score could benefit from physical exercise to increase energy output prepartum, stimulating the mobilization and processing of lipids before calving. Restricting energy intake prepartum by either restricting total intake or reducing the energy density of a dry cow diet prevents high liver lipid accumulation, without any health risks that could be related to low rumen fill. Maintaining lactation during the prepartum period by either continuous milking or shortening the dry period reduces dietary changes and improves the rumen absorption surface directly postpartum. Consequences of the improved liver health for either health or performance (milk yield, dry matter intake) were however not clear based on the studies in this thesis. This may be related to the relatively low number of animals and the high individual variation in liver fat content. Further improvement of our understanding of lipid mobilization and lipoprotein transport in dairy cows around calving is needed to understand the origin of these individual differences, to be able to identify cows ‘at risk’ and to customize support strategies.

CONTENTS

Summary		11
Samenvatting (Summary in Dutch)		15
Chapter 1	General introduction	21
Chapter 2	Effect of rumen-protected choline supplementation on liver and adipose gene expression during the transition period in dairy cattle	35
Chapter 3	Physical exercise prepartum to support metabolic adaptation in the transition period of dairy cattle: A proof of concept	67
Chapter 4	Restricting energy intake of dairy cows in the dry period with grass-based rations	93
Chapter 5	Dry period length affects rumen adaptation in dairy cattle precalving and during the first weeks after calving	115
Chapter 6	General discussion	143
Curriculum Vitae		174
Education certificate		175
Colophon		176

Summary

In the periparturient period, dairy cows are subjected to large changes: their diet, management, and metabolic requirements are quite different after calving compared to the last weeks of gestation. Directly after calving, the mammary gland requires increasing amounts of nutrients to support milk synthesis. In the initial weeks of lactation, the energy requirements for maintenance and milk production generally surpass dietary energy intake, resulting in a negative energy balance (**NEB**). Body reserves are mobilized in support of milk production, primarily by adipose tissue releasing non-esterified fatty acids (**NEFA**) into the blood. The excess of mobilized NEFA will be captured by the liver and metabolized through three pathways: 1) oxidation, to yield energy for hepatic cell metabolism; 2) partial oxidation, producing ketone bodies like β-hydroxybutyric acid (**BHB**) which can be used as an energy source in peripheral tissue; 3) re-esterification with glycerol to form triacylglycerides (**TG**). The liver exports TG to peripheral tissue by incorporating TG into very low density lipoprotein (**VLDL**) particles. These VLDL particles are released into the blood. However, if the processing capacity of the liver is insufficient relative to the influx of NEFA, TG accumulates, causing hepatic lipidosis. Liver TG accumulation beyond 70 mg/g wet weight is a risk factor for reduced performance, health disorders, and culling. A successful transition from the anabolic state of the prepartum period to the catabolic state of early lactation is therefore crucial for dairy cow health and lifespan. The adaptive capacity of the energy metabolism is essential, with the liver as the coordinating central organ.

The general objective of this thesis was to enhance the metabolic adaptive capacity of dairy cows during the transition period around calving, through four different nutrition and management strategies: 1) supporting hepatic metabolism; 2) increasing dry cow energy output; 3) reducing dry cow energy input; and 4) maintaining the lactating state prepartum. The effectiveness of each strategy was assessed by monitoring cow health, performance and liver function, by specifically analyzing the TG accumulation in the liver in the first weeks postpartum.

The first strategy aimed to support liver metabolism by increasing the availability of choline as one of the essential components of VLDL particles, required to export TG from the liver (Chapter 2). Sixteen multiparous cows were blocked and randomly assigned to one of two groups. The treatment group of 8 cows received rumen-protected choline (i.e. 14.4 g choline per day) from 3 weeks before until 6 weeks after calving. Liver and adipose tissue biopsies were taken

at 3 weeks before calving and in week 1, 3 and 5 of lactation to assess the mRNA abundance of key genes associated with lipid and energy metabolism. Choline supplementation demonstrated a reduction in hepatic TG accumulation. This was not related to an effect on gene expression in adipose tissue but to improved fatty acid transport and processing in the liver. Additionally, choline supplementation positively impacted hepatic carbohydrate metabolism. The results showed that choline facilitated the adaptation of metabolic pathways essential for early lactation by improving NEFA transport and processing, increasing VLDL synthesis and activating carbohydrate metabolism.

The second strategy aimed to support prepartum metabolic adaptation by increasing energy output before calving (Chapter 3). The hypothesis was that subjecting cows to a fixed amount of physical exercise until calving would prepare energy metabolism to the postpartum release of NEFA. The risk for extreme mobilization of adipose tissue is highest in cows with a high body condition score (**BCS**), primarily due to the larger potential for BCS loss compared with thin cows. Cows with a high BCS specifically could benefit from physical exercise, stimulating the mobilization and processing of lipids before calving. Therefore 16 cows with a high BCS (≥3.25) were blocked and randomly assigned to a control or exercise group; the same was done for 16 cows with a normal BCS (<3.25). Cows in the exercise group were walked twice daily for 45 min during the last 6 weeks of gestation, while the control group remained indoors. Liver biopsies were obtained from a subset of 16 cows to assess hepatic TG accumulation 2 weeks before calving and in weeks 1 and 2 postpartum. Exercise induced an NEB prepartum with elevated plasma NEFA and BHB concentrations, suggesting increased lipid mobilization and partial oxidation of NEFA. Postpartum, no differences in performance or health were observed between exercised and control cows. Exercise tended to reduce hepatic TG accumulation in cows with a high BCS, but this effect did not appear to be related to increased VLDL transport based on plasma lipoprotein concentrations.

The third strategy involved reducing the energy input before calving, to prepare cows for the postpartum period (Chapter 4). Previous studies have identified overfeeding in the dry period as a risk factor for metabolic disturbances in early lactation, often with experimental rations based on corn silage and alfalfa hay or silage. By restricting energy intake, catabolic processes could be initiated before calving, facilitating metabolic adaptation to lactation. In an experiment the effect of reducing prepartum energy intake under grass-rich, low-starch dietary conditions was validated. The reduction was achieved by comparing ad libitum feeding of a high-energy grass-based diet to either restricted intake of

the same ration, or to ad libitum feeding a low-energy ration. Liver biopsies were conducted 8 and 2 weeks before calving and 1, 3 and 5 weeks after parturition. Overfeeding the high-energy diet prepartum (at ~175% of energy requirements) resulted in an increased BCS and bodyweight at calving, along with high liver TG accumulation postpartum. Physically restricting feed intake in the prepartum period below maximal rumen capacity has been suggested to reduce postpartum feed intake. In the present study, restricting intake of a high-energy diet to a fixed amount did not limit feed intake in the first weeks postpartum compared to ad libitum feeding. Although postpartum feed intake or performance may not appear different, dairy cows should be fed at or just below their energy requirements during the dry period for a healthy start of the lactation.

The fourth strategy tested aimed to minimize the physiological differences between the pre- and postpartum periods by maintaining the lactating state, thereby reducing the need for adaptation (Chapter 5). By omitting the dry period, cows can receive a high energy (lactation) ration until calving. Shortening the dry period introduces two dietary changes – from lactation to the dry cow diet and vice versa – within a relatively short period. In an experiment the effect of dry period length on rumen adaptation and cow metabolic state during the transition period was assessed. Twelve pregnant, rumen-cannulated Holstein Friesian dairy cows at the end of their first lactation were assigned to one of three treatments: a conventional dry period of 60 days, a short dry period of 30 days, or no dry period at all. Rumen biopsies were taken from 3 locations in the rumen at 60, 40 and 10 days before calving and 3, 7, 14, 28 and 56 days after calving to evaluate papillae dimensions. Blood was sampled weekly from 3 weeks before until 8 weeks after calving, and liver biopsies were taken 2 weeks before calving, and in weeks 2 and 4 after parturition. Prepartum, cows with a short or no dry period showed greater feed intake and milk yield compared to cows with a conventional dry period. Postpartum, intake was higher for cows with a short dry period compared with cows with a conventional dry period. Plasma concentrations of NEFA and BHB, along with liver TG content, did not differ among dry period lengths. Rumen papillae surface area for cows managed for a 30- or 60-day dry period decreased towards calving, when the dry cow ration was fed. At 10 days prepartum, papillae surface area was greater for the no dry period treatment compared to both other treatments, and this difference

persisted 3 days postpartum. Cows managed for a short dry period showed faster increase in papillae dimensions after calving compared to cows managed for a conventional dry period. The faster rumen adaptation postpartum may be related to the increased intake during the first weeks postpartum, but did not result in an improved metabolic status or milk yield. Maintaining lactation during the prepartum period by either continuous milking or shortening the dry period safely reduces the period on a low energy diet and enhances the rumen absorption surface in the first weeks postpartum.

All four strategies presented in this thesis led to a reduction in hepatic TG levels, but the consequences of the improved liver health for animal health and performance outcomes (milk yield, dry matter intake) were not clear. This may be related to the relatively low number of animals and the high individual variation in liver fat content. Individual variation could be linked to differences in VLDL transport, but this is difficult to detect. First of all the quantification of plasma VLDL is a complex analytical process. Next to that, plasma VLDL concentration may not reflect the actual VLDL or TG export from the liver, as the VLDL turnover rate is strongly increased postpartum. The liver generally has a high capacity for lipid processing: in the first two weeks of lactation, lipid mobilization may easily range from 1 to 3 kg per day, while the amount of TG stored in the liver is around 0.4 kg up to 1.2 kg in cases of high liver TG accumulation.

The efficiency of lipid processing can vary significantly among individual cows, even under the same management. To optimally support cows with one or more strategies as practiced in this thesis, an early identification of cows susceptible to lipid related metabolic disorders is crucial. A high BCS at calving is considered a risk factor for metabolic disturbances postpartum, but results are inconsistent and may depend on other factors, such as the plane of energy fed in the prepartum period. The recent developments in data science provide opportunities to define cows at risk by the integration of existing and new individual cow parameters regarding performance, behavior, blood and milk biomarkers

Based on the four experiments presented in this thesis, there is a clear potential for nutritional and management strategies to support metabolic adaptation to lactation precalving, reducing the incidence of metabolic disturbances postpartum. Further improvement of our understanding of lipid mobilization and lipoprotein transport in dairy cows around calving is needed to understand the origin of individual differences, be able to identify cows ‘at risk’ and to customize support strategies to their specific needs.

Samenvatting (summary in dutch)

In de periode rondom afkalven worden melkkoeien blootgesteld aan een aantal grote veranderingen: hun rantsoen, management en energiestofwisseling zijn na het afkalven behoorlijk veranderd ten opzichte van de laatste weken van de dracht. Vanaf het moment van kalven vraagt de uier steeds grotere hoeveelheden voedingsstoffen om de melkproductie te ondersteunen. In de eerste weken van de lactatie is de energiebehoefte voor onderhoud en melkproductie daardoor doorgaans hoger dan de energieopname via het rantsoen, wat resulteert in een negatieve energiebalans (**NEB**). Lichaamsreserves worden gemobiliseerd ter ondersteuning van de melkproductie, voornamelijk via het vetweefsel dat grote hoeveelheden niet-veresterde vetzuren (**NEFA**) in de bloedsomloop afgeeft. De overmaat van gemobiliseerde NEFA wordt door de lever opgevangen en via drie routes verder verwerkt: 1) oxidatie, om energie te leveren voor de stofwisseling van levercellen; 2) gedeeltelijke oxidatie, waarbij ketonlichamen zoals β-hydroxyboterzuur (**BHB**) worden geproduceerd, die weer gebruikt kunnen worden als energiebron in perifere organen; 3) re-esterificatie met glycerol om triacylglycerides (**TG**) te vormen. De lever exporteert TG vervolgens naar perifeer weefsel door TG op te nemen in lipoproteïnedeeltjes die het transport verzorgen, en wel specifiek in lipoproteinen met een zeer lage dichtheid (**VLDL**). Deze VLDL-deeltjes worden afgegeven in het bloed. Als de verwerkingscapaciteit van de lever echter onvoldoende is in verhouding tot de instroom van NEFA hoopt TG zich op, waardoor leververvetting ontstaat. Ophoping van lever TG boven de 70 mg/g lever is een risicofactor voor verminderde prestaties, gezondheidsproblemen en zelfs afvoer. Een succesvolle overgang van de anabole toestand tijdens de periode vóór afkalven naar de katabole toestand van de vroege lactatie is cruciaal voor de gezondheid en levensduur van melkkoeien. Het aanpassingsvermogen van de energiestofwisseling is essentieel om deze periode goed door te komen, met de lever als het coördinerende centrale orgaan.

Het doel van dit proefschrift is het verbeteren van het aanpassingsvermogen van de stofwisseling van melkkoeien tijdens de transitieperiode rond afkalven, door middel van vier verschillende voedings- en managementstrategieën: 1) de ondersteuning van de leverstofwisseling; 2) het verhogen van het energieverbruik van droge koeien; 3) het verminderen van de energieopname van droge koeien; en 4) het handhaven van de metabole toestand van de lactatie tot aan het moment van kalven. De effectiviteit van iedere strategie werd beoordeeld door het monitoren van de gezondheid, prestaties en leverfunctie van de koe, en daarbij specifiek de TG stapeling in de lever in de eerste weken na afkalven te analyseren.

De eerste strategie was gericht op het ondersteunen van de leverstofwisseling door het verhogen van de beschikbaarheid van choline, één van de essentiële componenten van de VLDL-deeltjes, die nodig zijn om TG uit de lever te exporteren (Hoofdstuk 2). Zestien ouderekalfs koeien werden geblokt en willekeurig toegewezen aan één van de twee proefgroepen. De behandelgroep van 8 koeien kreeg pensbeschermde choline (i.e. 14,4 gram choline per dag) toegevoegd aan hun rantsoen vanaf 3 weken voor tot 6 weken na het afkalven. Lever- en vetweefselbiopten werden drie weken voor het afkalven genomen, en na kalven in week 1, 3 en 5 van de lactatie, om de genexpressie van de belangrijkste genen die geassocieerd zijn met de vet- en energiestofwisseling te beoordelen. Choline verstrekking resulteerde in een vermindering van de stapeling van TG in de lever. Dit hing niet samen met een veranderde genexpressie in het vetweefsel, maar met een verbeterd transport en verwerking van NEFA in de lever. Bovendien had het toevoegen van choline aan het rantsoen een positieve invloed op de koolhydraatstofwisseling in de lever. De resultaten toonden aan dat choline een bijdrage leverde in het aanpassen van verschillende stofwisselingsprocessen die essentieel zijn in de vroege lactatie, zoals het transport en de verwerking van NEFA, de VLDL-synthese en de koolhydraatstofwisseling.

De tweede strategie was gericht op het stimuleren van de stofwisseling vóór afkalven door het energieverbruik te verhogen (Hoofdstuk 3). De hypothese was dat de benodigde aanpassing van de energiestofwisseling voor de verhoogde afgifte van NEFA na afkalven ondersteund kan worden door koeien extra lichaamsbeweging te geven tot aan afkalven. Het risico op extreme mobilisatie van vetweefsel is het hoogst bij koeien met een hoge lichaamsconditie score (**BCS**), voornamelijk vanwege de grotere kans op BCS-verlies vergeleken met magere koeien. Koeien met een hoge BCS zouden baat kunnen hebben bij lichaamsbeweging om de mobilisatie en de verwerking van NEFA vóór het afkalven te stimuleren. Daarom werden 16 koeien met een hoge BCS (≥3,25) geblokt en willekeurig toegewezen aan een controle- of bewegingsgroep; hetzelfde werd gedaan voor 16 koeien met een normale BCS (<3,25). Koeien in de bewegingsgroep werden tijdens de laatste zes weken van de dracht tweemaal daags gedurende 45 minuten getraind in een stapmolen, terwijl de controlegroep binnen bleef. Er werden leverbiopten genomen van een subgroep van 16 koeien, 2 weken vóór het afkalven en in week 1 en 2 na kalven, om de stapeling van TG in de lever te beoordelen. De koeien in de bewegingsgroep hadden een NEB voor afkalven met verhoogde plasma NEFA- en BHB-concentraties, wat duidt op een verhoogde vetmobilisatie en een gedeeltelijke oxidatie van NEFA. In de eerste weken van de lactatie werden er geen verschillen in prestatie of gezondheid waargenomen tussen getrainde en controlekoeien. Bij koeien met een hoge

BCS was er een tendens dat beweging in de droogstand de stapeling van TG in de lever verminderde. Dit effect leek niet gerelateerd te zijn aan een verhoogd VLDL-transport, op basis van de lipoproteïne concentraties in plasma.

De derde strategie omvatte het verminderen van de energieopname vóór het afkalven, om koeien voor te bereiden op de postpartumperiode (hoofdstuk 4). Eerdere studies hebben aangetoond dat het overvoeren van koeien in de droogstand een risicofactor is voor stofwisselingsproblemen in de vroege lactatie, veelal met proefrantsoenen op basis van maïs en luzerne. Door de energieopname te beperken, kunnen katabole processen vóór het afkalven op gang worden gebracht, waardoor de aanpassing van de stofwisseling richting de lactatie wordt vergemakkelijkt. Voor deze strategie werd een experiment uitgevoerd om het effect van het verminderen van de energieopname vóór afkalven met grasrijke, zetmeelarme rantsoenen te valideren. Deze vermindering werd bereikt door het onbeperkt verstrekken van een energierijk, op gras gebaseerd rantsoen te vergelijken met een beperkte verstrekking van hetzelfde rantsoen, dan wel met het onbeperkt voeren van een energiearm rantsoen. Leverbiopten werden genomen rond 8 en 2 weken vóór het afkalven en 1, 3 en 5 weken na kalven. Het onbeperkt verstrekken van het energierijke rantsoen (op ~175% van de energiebehoefte) resulteerde in een verhoogde BCS en verhoogd lichaamsgewicht bij afkalven, met een verhoogde hoeveelheid TG in de lever in de eerste weken van de lactatie. Vaak wordt gesuggereerd dat het fysiek beperken van de voeropname voor afkalven onder de maximale opnamecapaciteit van de pens uiteindelijk de voeropname na afkalven vermindert. In het huidige experiment was de voeropname in de eerste weken na afkalven echter gelijk bij de beperkte gevoerde en de onbeperkt gevoerde dieren. Hoewel de voeropname of de prestaties na afkalven misschien vergelijkbaar lijken, zouden melkkoeien voor afkalven op of net onder hun energiebehoefte moeten worden gevoerd voor een gezonde start van de lactatie.

De vierde strategie had tot doel de fysiologische verschillen tussen de periode voor en de periode na afkalven te minimaliseren door de lactatie te handhaven, waardoor de noodzaak voor aanpassing van stofwisselingsprocessen wordt verminderd (Hoofdstuk 5). Door koeien door te melken tot aan afkalven kunnen ze een energierijker (lactatie)rantsoen krijgen dan wanneer ze droogstaan en geen melk produceren. Het verkorten van de droogstand brengt twee veranderingen in het rantsoen met zich mee – van lactatie- naar droogstandsrantsoen en vice versa – binnen een relatief korte periode. In een experiment werd het effect van de duur van de droogstand op de aanpassing van de pens en de energiestofwisseling tijdens de transitieperiode onderzocht. Twaalf drachtige

Holstein Friesian melkkoeien met penscanules werden aan het einde van hun eerste lactatie toegewezen aan een van de drie behandelingen: een conventionele droogstand van 60 dagen, een korte droogstand van 30 dagen, of helemaal geen droogstand. Er werden pensbiopten genomen op 3 locaties in de pens op 60, 40 en 10 dagen vóór het afkalven en 3, 7, 14, 28 en 56 dagen na het afkalven om de afmetingen van de papillen te beoordelen. Bloed werd wekelijks afgenomen van 3 weken vóór tot 8 weken na het afkalven; leverbiopten werden 2 weken vóór het afkalven genomen, en in week 2 en 4 na kalven. In de laatste fase van de dracht vertoonden koeien met een korte of geen droogstand een grotere voeropname en melkopbrengst vergeleken met koeien met een conventionele droogstand. Na afkalven was de voeropname hoger bij koeien met een korte droogstand vergeleken met koeien met een conventionele droogstand van 60 dagen. De plasmaconcentraties van NEFA en BHB, alsook het TG-gehalte in de lever, verschilden niet tussen de drie behandelingen. Het oppervlak van de penspapillen voor koeien die een droogstand van 30 of 60 dagen kregen, nam af richting het afkalven. Op 10 dagen voor kalven was het papilloppervlak groter voor de behandeling zonder droogstand vergeleken met beide andere behandelingen, en dit verschil bleef tot 3 dagen na kalven bestaan. Koeien met een verkorte droogstandslengte vertoonden na het afkalven een snellere groei van de papillen vergeleken met koeien die een conventionele droogstandslengte kregen. De snellere pensaanpassing na afkalven kan verband houden met de verhoogde voeropname tijdens de eerste weken van de lactatie, maar dit resulteerde niet in een verbeterde stofwisselingsstatus of melkproductie. Het in stand houden van de lactatie tijdens de dracht, door de koe te blijven melken of door de droogstand te verkorten, vermindert de periode met een energiearm rantsoen en verbetert het absorptieoppervlak van de penswand in de eerste weken na afkalven.

De vier in dit proefschrift gepresenteerde strategieën leidden tot een verlaging van de TG stapeling in de lever, maar de gevolgen van de verbeterde levergezondheid voor de diergezondheid en -prestaties (melkproductie, voeropname) waren niet duidelijk. Dit kan te maken hebben met het relatief lage aantal dieren en de grote individuele variatie tussen dieren voor wat betreft het vetgehalte in de lever. Deze individuele variatie zou in verband kunnen worden gebracht met verschillen in VLDL-transportcapaciteit, maar dit is moeilijk vast te stellen. Allereerst is de kwantificering van de concentratie VLDL in plasma een complex analytisch proces. Daarnaast weerspiegelt de VLDL concentratie in plasma mogelijk niet de werkelijke hoeveelheid VLDL- of TG-export uit de lever, aangezien ook het verbruik van VLDL in de eerste weken van de lactatie enorm toeneemt. De lever heeft een hoge capaciteit voor de verwerking van

vet: in de eerste twee weken van de lactatie wordt al snel 1 tot 3 kg vet per dag gemobiliseerd, terwijl de hoeveelheid TG die in de lever wordt opgeslagen slechts 0,4 kg tot (in geval van TG stapeling) 1,2 kg bedraagt.

De efficiëntie van de verwerking van NEFA kan aanzienlijk variëren tussen individuele koeien, zelfs onder hetzelfde management. Om koeien optimaal te ondersteunen met één of meer strategieën zoals toegepast in dit proefschrift, is een vroege identificatie van koeien die gevoelig zijn voor vet-gerelateerde stofwisselingsstoornissen cruciaal. Een hoge BCS bij het afkalven wordt beschouwd als een risicofactor voor stofwisselingsproblemen in de vroege lactatie, maar de resultaten zijn niet eenduidig en waarschijnlijk afhankelijk van andere factoren, zoals het energieniveau van het rantsoen voor kalven. De recente ontwikkelingen in de datawetenschappen bieden steeds meer mogelijkheden om koeien die een verhoogd risico lopen te identificeren, door zowel bestaande als nieuwe parameters te integreren, o.a. gericht op prestatiekenmerken, gedrag, biomarkers in bloed en biomarkers in melk.

Op basis van de vier experimenten die in dit proefschrift zijn gepresenteerd, hebben voedings- en managementstrategieën duidelijk potentie om de aanpassing van de stofwisseling aan de lactatie te ondersteunen en de incidentie van stofwisselingsstoornissen te verlagen. Onze kennis van vetmobilisatie en lipoproteïnetransport bij melkkoeien rondom het afkalven moet verder verbeterd worden om de oorsprong van individuele verschillen te begrijpen, om risicokoeien te kunnen identificeren, en om ondersteuningsstrategieën aangepast aan specifieke individuele behoeften aan te kunnen bieden.


1

Chapter 1

General introduction

R.M.A. Goselink

GENERAL INTRODUCTION

Liver function in dairy cows

The periparturient period has been mentioned to be the most important period for dairy cow health and lifespan, and optimal management of the transition period contributes to a sustainable dairy sector (Van Knegsel et al., 2014). The transition from the final phase of gestation to early lactation is turbulent. When comparing the nutrient requirements at 250 d of gestation with the d 4 of lactation, the demand for amino acids will double, glucose demand increases threefold and the demand for fatty acids fivefold (Bell, 1995). This transition requires effective metabolic adaptive mechanisms involving homeostatic and homeorhetic processes with the liver as the coordinating central organ.

As in all mammals, the liver directly receives the largest part of the intestinal blood flow to ensure a fast regulation of nutrient levels and the detoxification of unwanted (endogenous or exogenous) substances in the blood. The liver is also able to produce various metabolites through complex pathways, such as glucose from glucogenic precursors. Typically, gluconeogenesis is highly relevant for ruminants to ultimately support milk production. Furthermore, the liver is active in lipid processing and the production of plasma proteins such as acute phase proteins. The latter are essential to support an adequate immune response against infection.

A well-functioning liver is of great importance to dairy cattle, especially in early lactation. To highlight the relevance of the liver in the periparturient period, the main metabolic challenges around calving are described in the next paragraphs. After that, potential strategies are identified to support liver function, leading to the thesis' objectives described in the last paragraph.

Energy metabolism in dairy cows around calving

Around calving, the hormonally induced onset of milk production in dairy cows rapidly increases the demand for energy, protein and other nutrients. The mammary gland requires glucose, amino acids and fatty acids for milk synthesis to supply the offspring with lactose, milk protein and milk fat. During the first weeks after calving, the energy requirements for maintenance and milk production usually exceed the dietary intake of energy, thereby resulting in a so called "negative energy balance" (**NEB**) (see Figure 1).

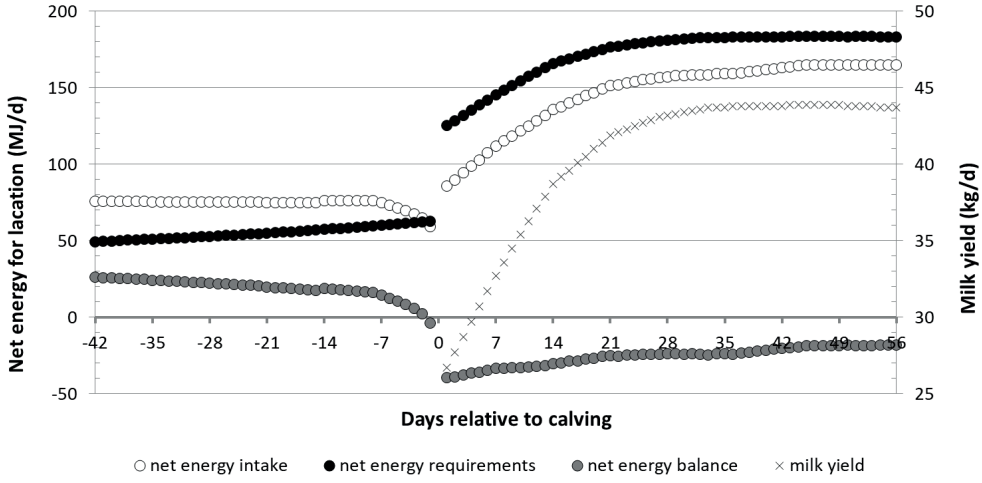

Figure 1. Average net energy intake, calculated net energy requirements, net energy balance and milk yield for multiparous dairy cows between 42 days before and 56 days after calving (unpublished *data from Dairy Campus research farm 2017, n = 60*).

To be able to accommodate NEB in early lactation cattle, adaptative processes take place in the periparturient period - well described in several reviews (Drackley, 1999; McNamara, 2015; Baumgard et al., 2017). There are two levels of metabolic regulation: homeostasis and homeorhesis. Homeostatic control comprises acute compensatory mechanisms to ensure physiological balance, as for example the regulation of plasma glucose concentration around its setpoint. In early lactation, the mammary gland demands a large amount of glucose for lactogenesis and homeostatic regulation will activate glycogenolysis and gluconeogenesis to support glucose production. Next to this acute compensatory mechanism, the concept of homeorhesis involves a slower regulation mechanism that can change homeostatic setpoints by orchestrating chronic adaptations in multiple tissues to support a dominant physiological state. In early lactation, homeorhetic adaptation will blunt insulin secretion and reduce the sensitivity of adipose and muscle tissue for insulin, all in support of lactation.

The mobilization of body reserves is also part of the homeorhetic regulation of early lactation. Adipose tissue is the main energy store, which may support over one-third of the milk produced in the first month after calving in terms of energy (Bauman and Currie, 1980). Activation of hormone-sensitive lipase in adipocytes will result in the hydrolysis of triacylglycerol (**TG**) and release of non-esterified fatty acids (**NEFA**) into the blood. Extramammary tissues adapt in using NEFA more prominently as an energy source instead of glucose, thereby sparing glucose to support lactogenesis. The excess of mobilized NEFA is metabolized by

the liver through three pathways. First of all, NEFA will be oxidized like in other tissue to yield energy for hepatic cell metabolism. Secondly, partial oxidation can take place producing ketone bodies like β-hydroxybutyric acid (**BHB**) which can also be used as an energy source in peripheral tissue. The production of BHB is especially relevant for specific tissue like the central nervous system that is not able to use lipids as an energy source. As BHB can pass the blood-brain barrier, it can deliver lipid-derived energy. Thirdly, NEFA are re-esterified in the liver with glycerol producing TG. The function of TG storage in the liver is not fully understood and may be simply determined by the quantitative influx of plasma NEFA and its corresponding export (Vernon, 2005). Export of TG from the liver to other tissues is handled by incorporating TG in very low density lipoprotein (**VLDL**) particles. These VLDL particles are released into the blood and transferred to peripheral tissues, delivering TG as a substrate to yield energy. In ruminants, however, TG export is quantitatively insufficient relative to the amounts of TG produced, resulting in hepatic lipidosis.

A successful transition through homeostatic and homeorhetic processes lays the foundation for a healthy and well-performing dairy cow. In some cases the adaptive capacity of an animal is not sufficient to manage the metabolic requirements of early lactation within the homeorhetic limits. This is most obvious in cases where metabolic adaptation is challenged by an (additional) external factor, e.g. clinical lameness hampering feed intake (Calderon and Cook, 2011) or a strong immune activation (Horst et al., 2021). Lipolysis may then evolve outside the span of control of adaptation mechanisms, resulting in high plasma NEFA, liver TG, and plasma BHB concentrations (Grummer, 1993; Drackley, 1999). High plasma NEFA, BHB and liver TG concentrations combined with low plasma glucose concentrations have been shown to correlate with a suppressed immune response (Ingvartsen and Mayes, 2013), increased risk for metabolic disease, a higher incidence of ketosis, abomasal displacements, milk fever and mastitis (Drackley, 1999) and a longer interval from parturition to first ovulation (Rukkwamsuk et al., 1999a).

Supporting cows in their adaptation to a new lactation

The transition from the dry period to early lactation is a very challenging period for the dairy cow, while at the same time a smooth and rapid transition is a prerequisite for an uncomplicated lactation. The definition of the transition period is not well described, but often referred to as the period starting 3 weeks before and ending 3 weeks after calving (Drackley, 1999). During this transition period, the energy metabolism needs to adapt from the anabolic state during

the dry period to the catabolic state in early lactation; a time-requiring process starting already in the dry period.

A consequence of a severe NEB and an insufficient capacity to adapt to the new lactation is the development of hepatic lipidosis (Bobe et al., 2004). Fatty liver and clinical ketosis were recognized as important production diseases in early lactation in the 1930s. The relevancy of the dry period in the etiology of these metabolic conditions was recognized in the second half of the 20th century (Schultz, 1968; Reid, 1973). From the 1980s onwards, research groups started to investigate the transition period and discovered that the physiological and metabolic state during this period has important consequences for cow health and performance in early lactation (Grummer, 1993; Drackley 1999). The complete prevention of NEB of dairy cattle in early lactation seems impossible as mobilization of fatty acids is a highly conserved response in the biology of mammals (Horst et al., 2021). Improving the adaptation from the dry period to lactation and reducing the impact of NEB can however help to increase cow health and welfare. Potential strategies can be defined in two categories: either through the reduction of excessive lipolysis and NEFA input to the liver by limiting NEB; or through an improvement of NEFA and TG processing and excretion from the liver to support homeostasis.

Limiting the negative energy balance

Feeding above energy requirement prepartum will result in greater NEB postpartum, a longer duration of NEB, higher serum NEFA, liver TG and serum BHB concentrations (Rukkwamsuk et al., 1999b; Holtenius et al., 2003; Nielsen et al., 2010). The energy requirement of a non-lactating, pregnant cow is more than twofold lower than the energy requirement of a lactating cow in the first weeks after calving. To prevent excessive feed intake during the dry period, energy intake needs to be restricted - which is often practiced by feeding a specific dry cow ration with a low energy density. During early lactation, cows are generally fed a highly digestible ration to provide ~6.8 MJ net energy for lactation (NE_L) per kg DM. At dry-off, cows are provided a low energy diet that should provide ~5.0 MJ NE_L per kg DM. This is realized by increasing the amount of less digestible, fiber-rich feeds such as wheat straw. At calving, the ration will change again to a highly digestible lactation ration. These dietary changes around the cessation of milking and at parturition will have implications for rumen functioning: the change in dietary ingredients and nutrients will affect microbiota composition, fermentation and volatile fatty acid production as well as the absorptive capacity of the rumen wall (Bannink et al., 2008). This will raise a dilemma when on the one hand trying to reduce dietary changes as a potential stressor in the

periparturient period in support of both rumen function and metabolic adaptation, while on the other hand overfeeding should be prevented during a period of low energy requirements.

Postpartum energy intake may be improved by preventing the feed intake depression frequently observed around calving. Increasing the energy density of the dry cow ration in the final weeks of gestation may, at least partially, compensates for this feed intake depression, thus improving energy balance and cow performance in early lactation (Vandehaar et al., 1999; Hayirli et al., 2002), although there is a discrepancy with other studies (Rabelo et al., 2005, Dann et al., 2006). To reduce energy output postpartum, a reduction of milk energy output is instrumental in early lactation, as realized by omitting or shortening the dry period (Rastani et al., 2005; Van Knegsel et al., 2014), feeding glucogenic rations instead of lipogenic rations (Van Knegsel et al., 2007) or reducing protein intake in early lactation (Whelan et al., 2014).

Supporting liver metabolism

To improve the processing of NEFA and TG within the liver and stimulate TG excretion via VLDL, several supplements can be of interest in early lactation. Propylene glycol and rumen-protected choline have the best potential to reduce the incidence of fatty liver and ketosis (Grummer, 2008). Propylene glycol is a substrate for propionate production in the rumen, providing extra glucose precursors, thereby reducing NEFA and BHB levels in early lactating cows (Nielsen and Ingvartsen, 2004). Choline is a precursor of phosphatidylcholine, an essential component of VLDL, and has proven to reduce liver TG when lactating cows were offered a restricted amount of feed (Cooke et al., 2007). Another method may be the intentional induction of NEB before calving to start adaptation of metabolic pathways before calving by either restricting energy intake (Dann et al., 2005; Loor et al., 2006) or increasing energy requirement by increasing physical activity without increasing energy intake (Anderson et al., 1979). Besides the direct effect of physical activity on the energy balance, exercise will also stimulate lipolysis of adipose tissue by itself, as described in humans (Tremblay et al., 1994). The effect of physical exercise has received little attention in dairy cattle research but showed to reduce plasma NEFA concentrations in lactating dairy cows by usage of NEFA as an energy source for muscle activity (Adewuyi et al., 2006). Alternatively, reducing dietary changes in the periparturient period to support rumen function may be a feasible strategy when combined with increasing prepartum energy requirement through continuous milking until calving (Rastani et al., 2005; Van Knegsel et al., 2014).

Objectives and thesis outline

The main objective of the research described in this PhD-thesis is *to find strategies to improve liver function and performance of dairy cows in early lactation by starting metabolic adaptation prepartum*. In this thesis, several options for nutrition and management strategies are investigated to improve the metabolic adaptive capacity of dairy cows during the transition period. The effect of starting metabolic adaptation prepartum on health and performance postpartum is investigated, focusing mainly on the liver as the central coordinator of energy metabolism. It was hypothesized that starting adaptation of the metabolic pathways in the liver in the transition period may reduce hepatic lipidosis in early lactation. Four different strategies are visited to test this hypothesis (Figure 2), further explained below.

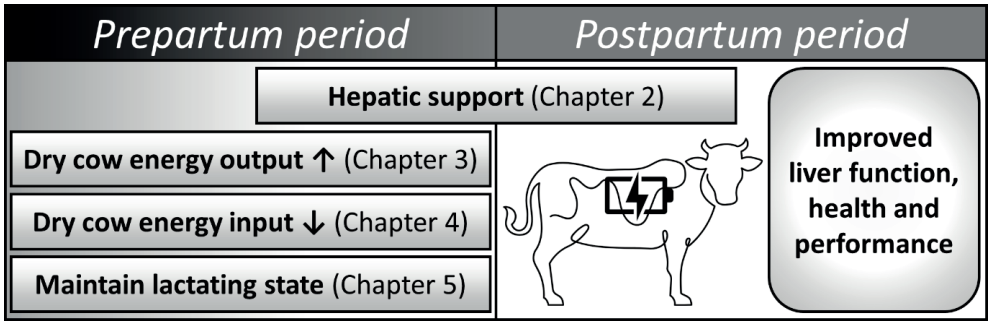

Figure 2. Schematic overview of the four strategies evaluated in this PhD-thesis, all aimed to support the adaptation of metabolic pathways in the liver in the periparturient period.

Hepatic support

For liver metabolism, important metabolic pathways during the transition period concern NEFA processing: beta-oxidation, esterification to TG and synthesis of VLDL. Specific nutrients such as choline may support liver metabolism in the periparturient period and reduce liver TG accumulation (Chapter 2). The underlying metabolic pathways for adaptation in the liver and the mode-of-action of choline in hepatic energy metabolism were studied at a cellular level by measuring expression of genes involved in lipid metabolism in liver and adipose tissue of dairy cows pre- and post-calving.

Increase dry cow energy output

As NEFA processing is a very important task for the liver to maintain homeostasis and support homeorhesis, adaptation to high NEFA concentration prepartum may help to support NEFA processing postpartum. This may be realized by increasing the energy requirement of dry cows before calving, which could be achieved by physical exercise in the dry period, stimulating NEFA release (Chapter 3).

Reduce dry cow energy input

In a subsequent experiment the restriction of energy intake prepartum was studied, as an alternative strategy to increasing energy output as evaluated in Chapter 3. In this study catabolic processes were started before calving, to prepare energy metabolism for the state of early lactation (Chapter 4).

Maintain lactating state

Finally, a strategy to reduce the physiological differences between the pre- and postpartum period was tested by maintaining the lactating state, reducing the need for adaptation (Chapter 5). Continuous milking or shortening the dry period can be management options to maintain digestive and metabolic pathways active throughout the transition period, aiming for an improved energy input directly postpartum.

The results of the four studies described in Chapter 2 to 5 are ultimately integrated in a general discussion (Chapter 6), bringing the knowledge from each of the separate studies together. The application of these strategies under practical circumstances is discussed, as well as the potential impact on cow health, lifespan and future challenges.

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2

Chapter 2

Effect of rumen-protected choline supplementation on liver and adipose gene expression during the transition period in dairy cattle

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ABSTRACT

We previously reported that supplementation of rumen-protected choline (**RPC**) reduces the hepatic triacylglycerol concentration in periparturient dairy cows during early lactation. Here, we investigated the effect of RPC on the transcript levels of lipid metabolism-related genes in liver and adipose tissue biopsies, taken at wk -3, wk 1, wk 3 and wk 6 after calving, in order to elucidate the mechanisms underlying this RPC-induced reduction of hepatic lipidosis.

Sixteen multiparous cows were blocked into 8 pairs and randomly allocated to either one of two treatments, with or without RPC. Treatments were applied from 3 wk before until 6 wk after calving. Both groups received a basal diet and concentrate mixture. One group received RPC supplementation, resulting in an intake of 14.4 g choline per day, while controls received an iso-energetic mixture of palm oil and additional soybean meal. Liver and adipose tissue biopsies were taken at wk -3, wk 1, wk 3 and wk 6 to determine the mRNA abundance of 18 key genes involved in liver and adipose tissue lipid and energy metabolism. Milk samples were collected in wk 1, 2, 3 and 6 postpartum for analysis of milk fatty acid composition.

The RPC-induced reduction in hepatic lipidosis could not be attributed to altered lipolysis in adipose tissue, since there was no treatment effect on the expression of peroxisome proliferator-activated receptor γ, lipoprotein lipase or fatty acid synthase in adipose tissue, nor on the milk fatty acid composition.

RPC supplementation increased the expression of fatty acid transport protein 5 and carnitine transporter SLC22A5 in the liver, suggesting an increase in the capacity of fatty acid uptake and intracellular transport, but there was no treatment effect on carnitine palmitoyl transferase 1A, transporting long chain fatty acids into mitochondria. In the same organ, RPC appeared to promote apolipoprotein B-containing lipoprotein assembly as shown by elevated microsomal triglyceride transfer protein expression and apolipoprotein B100 expression. Cows supplemented with RPC displayed elevated levels of glucose transporter 2 mRNA and a reduced peak in pyruvate carboxylase mRNA immediately after calving, showing supplementation also resulted in improved carbohydrate metabolism.

The results from this study suggest that RPC supplementation reduces liver TAG by improved FA processing and very-low-density lipoprotein synthesis, and RPC also benefits hepatic carbohydrate metabolism.

INTRODUCTION

The transition to lactation is supported by hormone-induced adaptations in fat metabolism in all mammals, including dairy cows (Friggens et al., 2004). These homeorhetic processes are accompanied by an increased release of fatty acids (**FA**) from adipose tissue, elevating blood level of NEFA. Furthermore, lipolysis is sustained during early lactation as long as energy intake cannot compensate for the increased energy demand of lactation (McNamara, 1991; Grummer, 2008). Aside from being utilized by the mammary gland, part of the circulatory NEFA are taken up by the liver, where they can be metabolized through one of three major pathways: 1) direct production of energy via oxidation of NEFA in mitochondria or peroxisomes; 2) production of ketone bodies (i.e. acetoacetate, acetone, BHBA) through partial oxidation; or 3) reesterification into triacylglycerol (**TAG**) which can then either be sequestered in internal stores or be released into the circulation as, TAG-rich, very-low-density lipoproteins (**VLDL**) (Drackley et al., 2006; Grummer, 2008). In ruminants however, VLDL secretion is relatively low, which predisposes the animals to hepatic lipidosis and ketosis (Kleppe et al., 1988).

Synthesis of VLDL requires TAG, phospholipids, cholesterol esters, microsomal triglyceride transfer protein (**MTTP**) and apolipoproteins such as apolipoprotein B100 (Bernabucci et al., 2004). Choline, a quasi-vitamin with various functions, is incorporated into phosphatidylcholine, the major phospholipid of VLDL. When phosphatidylcholine is limiting, choline supplementation may improve the rate of VLDL synthesis and thereby prevent excessive TAG accumulation in the liver (Grummer, 2008). In contrast to humans and rodents, choline availability in ruminants is hampered by the loss of dietary choline by extensive microbial degradation (Sharma and Erdman, 1989), which means that supplements should be industrially protected against ruminal degradation. Indeed, we and others have previously demonstrated that rumen-protected choline (**RPC**) supplementation to dairy cattle reduces fat accumulation in the liver (Cooke et al., 2007; Zom et al., 2011), increases milk production (Elek et al., 2008) and milk protein production (Zom et al., 2011). Yet, the underlying molecular mechanisms for the beneficial effects of RPC in periparturient dairy cattle are not fully understood.

In the present study, we propose a model for the action of RPC on FA processing by the bovine liver during early lactation, based on the temporal gene-expression profiles of 18 key energy metabolism-related enzymes in liver and adipose tissue, assessed by quantitative real-time PCR (**qPCR**) and the FA composition of milk to assess adipose mobilization. This model may be helpful in defining new

strategies of RPC supplementation to reduce the incidence of hepatic lipidosis and ketosis in dairy cattle.

MATERIAL AND METHODS

Animals and Treatments

All experimental protocols and interventions were approved by the Ethical Committee on Animal Experiments of the Animal Sciences Group of Wageningen UR. The experiment was carried out between 5 January and 26 April 2009 as a complete randomized block designed structure comprising 16 Holstein-Friesian cows (7 second parity, 5 third parity and 4 older cows), within a larger performance trial described by Zom et al. (2011). Cows were paired in 8 blocks on the basis of similarity in parity, expected date of calving and milk performance in the previous lactation (in order of priority).

Cows were housed in a cubicle shed and were kept in separate dry cow and lactating cow groups. Four wk before the expected date of calving, cows were moved to the pre-calving group. On the day of calving, cows were separated from the dry cow group and housed in a straw bedded calving pen. After calving, the cows were moved to the post-calving group. Cows were milked twice daily at 6 AM and 5 PM in a milking parlor

Cows within each block were randomly allocated to either control (**CON**) or choline (**CHOL**) treatment group. Cows in group CHOL received daily 60 g of an RPC source (ReaShure™, Balchem Encapsulates, Slate Hill, New York, USA), that was mixed with 540 g of soybean meal (**SBM**). Since ReaShure™ contained 24% choline, each CHOL cow received 14.4 g choline per day. Cows in group CON did not receive any choline supplementation, but were supplemented with a mixture of SBM and palm oil (582 and 18 g/day, respectively) to supply equal protein, energy and crude fat levels. The experimental treatments started 3 wk before the expected calving date (wk -3) and lasted until 6 wk after calving (wk 6).

Diets and Feeding Management

From 4 wk before calving until calving, cows received *ad libitum* the pre-calving feed mixture (Table 1) supplemented with a close-up compound concentrate. The daily concentrate allowance was increased gradually from zero at d-21 to 0.9 kg of DM on the expected day of calving.

After calving, cows received *ad libitum* the post-calving feed mixture (Table 1), supplemented with an early-lactation compound concentrate. The daily allowance of this concentrate was increased with 0.45 kg of DM/day from 0.9 kg of DM/day on day 0 (i.e. calving) up to 8.1 kg of DM/day on d17. The maximum level of concentrate was maintained from d17 until the end of the experimental period at d43. Concentrate ingredients and chemical composition of all feeds are described by Zom et al. (2011).

Table 1. Ingredients of the feed mixtures and amounts of supplement fed

Ingredient	**Pre-calving treatment**		**Post-calving treatment**	
	CON	**CHOL**	**CON**	**CHOL**
Ingredients feed mixture (% of DM)				
Wilted grass silage	29.8	29.8	52.3	52.3
Corn silage	29.6	29.6	34.7	34.7
Grass seed straw			6.2	6.2
Wheat straw	32.3	32.3		
Soybean meal solvent extract	7.0	7.0	3.1	3.1
Soybean meal formaldehyde treated	-	-	3.0	3.0
Vitamin and mineral premix	0.8	0.8	0.5	0.5
Magnesium oxide	0.5	0.5	-	-
Salt	-	-	0.2	0.2
Concentrate dispensers (kg cow/day)				
Soybean meal with palm oil	0.6		0.6	
Soybean meal with choline		0.6		0.6
Pre-lactation compound feed	1.0	1.0		
Lactation compound feed			9.0	9.0

The compound concentrates as well as the CHOL or CON supplement were fed individually using three transponder-controlled concentrate dispensers. The feed mixtures were supplied in feed weighing troughs with transponder-controlled access gates (Insentec, Marknesse, The Netherlands) which were continuously accessible for each cow, except during milking and when refusals were removed and fresh feed was supplied. Daily, between 10:30 and 11:00 AM feed refusals were removed from the troughs and a fresh feed mixture was supplied. To ensure *ad libitum* intake of the feed mixture, the refusal weight was at least 10% of the fresh weight at offer. The cows had unrestricted access to fresh drinking water.

Tissue sampling

Liver and adipose tissue biopsies were taken on Mondays of wk -3, just before the experimental treatment was started, and on Mondays of wk 1, wk 3 and

wk 6 post-calving. Liver tissue was harvested by percutaneous needle biopsy as described by Zom et al. (2011).

Subsequently, adipose tissue was sampled from the tail region between the ischium (pin bone) and coccygeal vertebrae. First the area was clipped, scrubbed with antiseptic solution and disinfected. A local anesthetic was injected subcutaneously (5 ml of lidocaine-HCL 2% with adrenaline, Eurovet, Bladel, The Netherlands). An incision was made and 3 samples of approximately 5 g of adipose tissue were dissected. The incisions were sutured with staples and protected with a film dressing spray (Opsite, Smit & Nephew, Hoofddorp, Netherlands). All dissected liver and adipose tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Quantitative PCR

For gene expression measurements, frozen samples of liver and adipose tissue were ground under liquid nitrogen and total RNA was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands), following the manufacturer's instructions. To eliminate DNA contamination, the isolated RNA was subjected to an on-column DNAse treatment (Nucleospin RNA II kit; Machery-Nagel, Düren, Germany). Reverse transcription of 1 mg of total RNA was performed in a 20-ml reaction using Superscript III reverse transcriptase (Invitrogen), dNTPs (Roche) and random hexamer primers (Roche, Almere, The Netherlands) for 1 h at 50°C according to the manufacturer's protocol (Invitrogen). The primers used are presented in Table 2. Templates were amplified after a preincubation for 10 min at 95 °C, followed by amplification for 40 cycles (10 s at 95 °C, 5 s at 60 °C, 5 s at 72 °C) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) by using the Sensimix SYBR Low Rox kit (Bioline, London, UK). All reactions revealed a single product as determined by melting curve analysis. Quantitative mRNA measurement was performed by establishing a linear calibration curve using serial dilutions of cDNA for corresponding genes. We measured the transcript levels of the following key enzymes related to FA and energy metabolism in the liver: FA transport proteins 2 and 5 (**FATP2 and FATP5**), FA binding protein 1 (**FABP1**), glucose transporter 2 (**GLUT2**), carnitine palmitoyltransferase 1A (**CPT1A**), organic cation-carnitine transporter (**SLC22A5**), glycerol-3-phosphate O-acetyltransferase 1 (**GPAT1**), MTTP, apoprotein B100 (**APOB100**), peroxisome proliferator activated receptor (**PPAR-α and PPAR-δ**), pyruvate carboxykinase (**PC**), pyruvate dehydrogenase kinase isotype 4 (**PDK4**), and mitochondrial as well as cytosolic phosphoenolpyruvate carboxykinase (**PEPCK-m** and **PEPCK-c**). In addition, the expression of three key enzymes was analyzed in adipose tissue: FA synthase (**FASN**), lipoprotein lipase (**LPL**) and peroxisome proliferator activated

receptor (**PPAR-γ**). Gene expression of housekeeping genes 18S ribosomal RNA and b-actin (**ACTB**) were also analysed as internal standard. Variation in 18S ribosomal RNA expression was much higher than for ACTB in the collected liver biopsies (unpublished results). Absolute expression levels of genes of interest were therefore normalized using their corresponding values of ACTB.

Milk FA composition

For milk FA analysis, samples of each cow were collected during two AM milkings of consecutive days in wk 1, 2, 3 and 6 postpartum. Both AM milkings were pooled to one composite sample per cow per week. Milk samples were analyzed for FA composition as described previously by Mach et al. (2011).

Table 2. Sequences of the primers used for quantitative PCR in liver and adipose tissue

Housekeeping gene		
ACTB	5'-GCCCTGAGGCTCTCTTCCA-3'	5'-CGGATGTCGACGTCACACTT-3'
Hepatic tissue		
PPARα	5'-GGATGTCCCATAACGCGATT-3'	5'-GGTCATGCTCACACGTAAGGATT-3'
PPARδ	5'-TGTGGCAGCCTCAATATGGA-3'	5'-GACGGAAGAAGCCCTTGCA-3'
FATP2	5'-ATTGGTGCGGTTGGAAGAGT-3'	5'-TCTCGAATGGGTTCATCTTTCTC-3'
FATP5	5'-GCTTGTCCTTGGAGTCCTCAGT-3'	5'-GCCGACAGTCATCCCAGAAG-3'
FABP1	5'-CAAGTCCAGACCCAGGAGAACT-3'	5'-TTTCCGACACCCCCTTGATA-3'
GPAT1	5'-GTTGCCAGCTATACTTCCCTCAA-3'	5'-TCGGCGGGATTCATCTGTT-3'
MTTP	5'-ACCTGTGCTCCTTCATCTAATTCAT-3'	5'-GCTAGCCAGGCCTCTCTTGA-3'
APOB100	5'-GCGGTCACCTCCCTCTTGCC-3'	5'-GGCCGAGGGCTCTGGGATCA-3'
SLC22A5	5'-GTTTTTCGTGGGTGTGCTGAT-3'	5'-TGTCTGCATGCCCATCGT-3'
CPT1A	5'-TGCCTCTACGTGGTGTCCAA-3'	5'-CTGGCTGGTGGATAATCTCCAA-3'
GLUT2	5'-GCGGCTCAGCAATTTTCTG-3'	5'-TGCATAAACAGGTTGGCTGATT-3'
PC	5'-GGCCGCATCGAGGTGTTCCG-3'	5'-GGGTGGTCCTTGCCGTGAGC-3'
PDK4	5'-AGAGGAGGTGGTGTTCCCCTGA-3'	5'-AAACCAGCCAGCGGAGCATTCC-3'
PEPCK-m	5'-CCGCCTTCCCCAGTGCTTGTG-3'	5'-TGGCCCGGAGTCGACCATCAC-3'
PEPCK-c	5'-CCAACGTGGCCGAGACCAGTG-3'	5'-TGGGCACAAGGCTCCCCATCC-3'
Adipose tissue		
LPL	5'-CACTTCAACCACAGCAGCAAAA-3'	5'-TGTACAAGGCAGCCACGAGTT-3'
PPARγ	5'-GAGCCCTTCGCTGTCACAGT-3'	5'-CGGAGCTGATCCCAAAGTTG-3'
FASN	5'-GGCATACCTCCAGTCCAGGTT-3'	5'-GTGGTTTTTGGAAAGGTCAAATTT-3'

Statistical Analysis

For qPCR results, the ratios between the absolute mRNA concentration of the relevant genes and housekeeping gene ACTB were calculated at each time point

(wk -3, 1, 3 and 6). For milk FA composition, individual FA levels were expressed relatively to the total amount of FA in milk samples at each time point (wk 1, 2, 3 and 6). Mixed model analysis with repeated measurements was performed using the REML procedure in Genstat 12th edition (2009). Treatment days and DIM were included as fixed effects in the model (both linear and quadratic). Cow, block, experimental week and the cow by week interaction were included as random effects in the model. The model structure was (Zom et al., 2011):

$$\log (Y)_{ijk} = \beta_0 + \beta_1 DIM_i + \beta_2 DIM_i^2 + \beta_3 TD_j + \beta_4 TD_j^2 + \varepsilon_{ijk,}$$

Where log Y = the log transformed ratios of each of the analyzed genes vs. ACTB

β_0 = the intercept
β_1 = the fixed effect for DIM_i
β_2 = the fixed quadratic effect of DIM_i^2
β_3 = the effect of treatment day TD_j (Control: TD = 0)
β_4 = the quadratic effect of treatment day TD_j^2 (Control: TD = 0).
ε_{ijk} = residual variance

Differences were declared significant at $P < 0.05$. Quadratic effect of DIM and effect of TD and TD^2 were removed from the model if parameters were non-significant ($P>0.05$).

RESULTS AND DISCUSSION

We have reported that the level of liver TAG was significantly reduced in the first wk after calving for CHOL compared to the CON group (P<0.05), indicating that RPC supplementation during the periparturient period reduces hepatic TAG accumulation (Zom et al., 2011). Moreover, DMI and milk protein concentration for the CHOL group were significantly higher than for the CON group at the start of lactation (P<0.05), but this effect gradually decreased as lactation progressed (Zom et al., 2011). In the present study, we aimed to develop understanding on the molecular mechanisms through which RPC affects hepatic FA metabolism by analyzing the role of 18 enzymes in liver and adipose tissue as depicted in Figures 1 and 2, respectively.

Effects in Liver

Transcription factors. Genes belonging to the PPAR family of ligand-activated nuclear transcription factors can be activated by NEFA and their derivatives,

thereby controlling the expression of several genes involved in lipid metabolism. Three isoforms of PPAR are known: PPARα, PPARδ and PPARγ (Alaynick, 2008), of which PPARg is essentially expressed in adipose tissue, where it regulates adipogenesis. Isoform PPARα is mainly involved in expression of genes involved in lipid oxidation, ketogenesis and gluconeogenesis in the liver. PPARβ/δ activation in rodents controls carbohydrate catabolism and fatty acid synthesis in the liver (Takahashi et al., 2007).

As presented in Table 3 and Figure 3, the hepatic mRNA abundance of PPARα increased significantly with increasing DIM, but was unaffected by RPC treatment. The expression of PPARa in ruminant liver is mainly induced by plasma NEFA levels, as shown in dairy cows during the periparturient period (Loor et al., 2005) and during fasting-induced ketosis (Loor et al., 2007). Van Dorland et al. (2009), however, did not observe a periparturient rise in PPARα mRNA and they ascribed this to the relatively low plasma NEFA concentration observed in their study (average peak concentration approximately 0.3 to 0.5 mmol/l in week 2 postpartum). In our experiment NEFA levels were equal in both treatment groups, which is consistent with equal PPARα concentrations for both treatment groups.

For PPARδ a significant effect of DIM as well as RPC treatment was observed, with increasing PPARδ mRNA abundance for cows in group CHOL and decreasing mRNA levels in group CON (Table 3 and Figure 3). In a study with dairy cows, PPARδ mRNA expression was increased in severely feed restricted, ketotic cows vs. healthy cows; the ketotic cows showed increased liver TAG, plasma NEFA and BHBA concentrations (Loor et al., 2007). PPARδ expression showed to be increased by FA; activation of PPARδ is involved in regulatory loops for lipid oxidation and lipid transport (Loor et al., 2007). Recent in vitro studies confirmed that specific monounsaturated fatty acids, including oleic acid, activate PPARδ expression (Brown et al., 2011). In rodents, PPARδ activation improves glucose utilization and lipoprotein metabolism, and exerts an anti-inflammatory effect in the liver (Sanderson et al., 2010). Although there may be differences in PPARδ activation pathways for each different species, taken together with the results of Loor et al. (2007) in dairy cattle, these results confirm that PPARδ activation is needed for optimal FA processing at increased FA influxes in the liver around parturition.

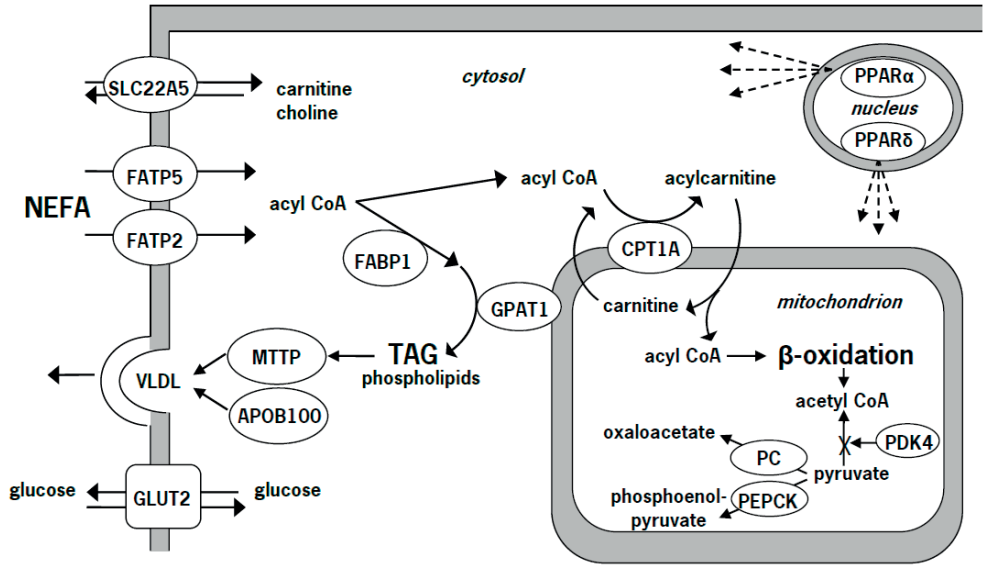

Figure 1. Model of NEFA uptake and -metabolism in hepatocytes. Cellular uptake of NEFA by hepatocytes is facilitated by FATP2 and FATP5, which are predominantly localized to the plasma membrane. Cytoplasmic FA are then either directed toward TAG synthesis by the action of GPAT1 and storage in lipid droplets, or toward mitochondria or peroxisomes (in the case of branched and very-long chain fatty acids) for b-oxidation. Excretion of stored TAG is facilitated by MTTP in the endoplasmic reticulum, followed by VLDL secretion. The nuclear receptors PPARa and PPARd modulate gene expression to regulate FA metabolism in liver.
PPARα = peroxisome proliferator-activated receptor alpha, PPARδ = peroxisome proliferator-activated receptor delta, FATP2 = fatty acid transport protein 2 (or SLC27A2), FATP5 = fatty acid transport protein 5 (or SLC27A5), FABP1 = fatty acid binding protein 1, GPAT1 = glycerol-3-phosphate acyltransferase 1 (or GPAM), MTTP = microsomal triglyceride transfer protein, APOB100 = apolipoprotein B100, SLC22A5 = organic cation transporter (also known as OCTN2), CPT1A = carnitine palmitoyltransferase 1A, GLUT2 = glucose transporter 2 (or SLC2A2), PC = pyruvate carboxylase, PDK4 = pyruvate dehydrogenase kinase isotype 4, PEPCK = phosphoenolcarboxykinase.

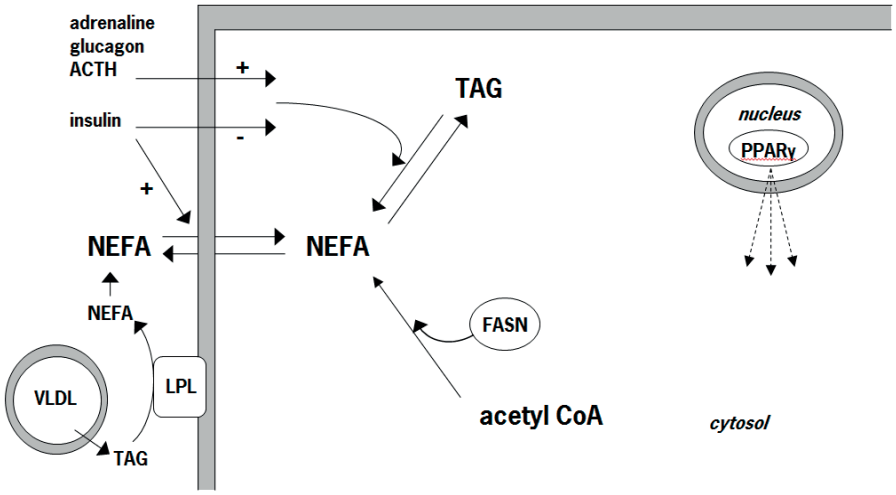

Figure 2. Model of NEFA metabolism in adipocytes. The balance between lipogenesis and lipolysis in adipocytes is determined by the nutritional state and is regulated by endocrine factors including ACTH, glucagon, adrenalin and insulin. Under conditions of a positive energy balance, NEFA released from lipoproteins (e.g. VLDL and chylomicrons by the catalytic activity of LPL) enter the adipocytes through both passive diffusion and active transport. Intracellular FA are first converted to acyl-CoA and then reassembled into TAG, which can be stored in lipid droplets. Alternatively, NEFA can be synthesized de novo by FASN, using acetyl-CoA as a substrate. During a state of negative energy balance, the hydrolysis of TAG to NEFA in adipocytes prevails. Subsequently NEFA are released into the circulation to supply energy to other organs. The nuclear receptor PPARγ is known to target genes involved in FA metabolism and adipocyte differentiation.
PPARγ = peroxisome proliferator-activated receptor gamma, LPL = lipoprotein lipase, FASN = fatty acid synthase.

Table 3. The effect of choline supplementation on gene expression in liver and adipose tissue expressed as the relative ratio with housekeeping gene β-actin (ACTB) mRNA concentration, analyzed using the statistical model: $\log(Y)_{ijk} = \beta_0 + \beta_1 DIM_i + \beta_2 DIM^2_i + \beta_3 TD_j + \beta_4 TD^2_j + \varepsilon_{ijk}$[1]. Parameters are declared significant at P-value < 0.05; non-significant parameters for DIM^2, TD and TD^2 are released from the model (n.s.).

			Factor		**Effect of choline treatment day**	
		Constant	**DIM**	**DIM^2**	**TD**	**TD^2**
Item	Parameter[2]	β_0	β_1	β_2	β_3	β_4
Hepatic gene expression						
PPARα	Estimate	-3.945	0.005012	-	-	-
	SED	0.1865	0.002386	-	-	-
	P-value		0.049	n.s.	n.s.	n.s.
PPARδ	Estimate	-5.600	-0.009806	0.000267	0.01087	-
	SED	0.0920	0.002933	0.000111	0.00252	-
	P-value		0.002	0.020	0.001	n.s.
FATP2	Estimate	-5.668	0.01599	-	-	-
	SED	0.611	0.00974	-	-	-
	P-value		0.110	n.s.	n.s.	n.s.
FATP5	Estimate	-1.548	-0.001378	-	0.006014	-
	SED	0.067	0.002398	-	0.002757	-
	P-value		0.568	n.s.	0.037	n.s.
FABP1	Estimate	-0.2982	0.01437	-	-	-
	SED	0.1695	0.00472	-	-	-
	P-value		0.004	n.s.	n.s.	n.s.
GPAT1	Estimate	-4.154	0.000223	0.000240	-	-
	SED	0.107	0.002768	0.000111	-	-
	P-value		0.936	0.035	n.s.	n.s.
MTTP	Estimate	-2.230	0.002364	-0.000266	0.008928	-
	SED	0.077	0.002516	0.000088	0.002418	-
	P-value		0.352	0.004	0.001	n.s.

Table 3 Continued

			Factor		Effect of choline treatment day	
		Constant	**DIM**	**DIM²**	**TD**	**TD²**
Item	Parameter[2]	$ß_0$	$ß_1$	$ß_2$	$ß_3$	$ß_4$
APOB100	Estimate	2.0550	-0.000520	-	-0.01138	0.000281
	SED	0.0576	0.001681	-	0.00563	0.000104
	P-value		0.757	n.s.	0.049	0.010
CPT1A	Estimate	-2.443	0.005181	-	-	-
	SED	0.108	0.002045	-	-	-
	P-value		0.018	n.s.	n.s.	n.s.
SLC22A5	Estimate	-5.549	0.006284	-0.000633	-0.01920	0.000492
	SED	0.077	0.002211	0.000100	0.00826	0.000163
	P-value		0.007	<0.001	0.026	0.004
GLUT2	Estimate	-2.554	-0.001470	-	0.008617	-
	SED	0.087	0.002751	-	0.003038	-
	P-value		0.597	n.s.	0.012	n.s.
PC	Estimate	-0.3258	0.01744	-0.00089	-0.02759	0.000554
	SED	0.10072	0.00282	0.00012	0.01070	0.000206
	P-value		<0.001	<0.001	0.013	0.010
PDK4	Estimate	-0.7914	0.01049	-	-	-
	SED	0.1012	0.00267	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
PEPCK-m	Estimate	-0.1454	0.002077	-	-	-
	SED	0.0404	0.001526	-	-	-
	P-value		0.186	n.s.	n.s.	n.s.
PEPCK-c	Estimate	2.221	0.009403	-	-	-
	SED	0.060	0.002213	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.

Table 3 Continued

			Factor		Effect of choline treatment day	
		Constant	**DIM**	**DIM²**	**TD**	**TD²**
Item	Parameter[2]	ß$_0$	ß$_1$	ß$_2$	ß$_3$	ß$_4$
Adipose gene expression						
PPARγ	Estimate	-3.611	-0.03161	-	-	-
	SED	0.141	0.00536	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
FASN	Estimate	-3.235	-0.08156	0.001985	-	-
	SED	0.240	0.00676	0.000240	-	-
	P-value		<0.001	<0.001	n.s.	n.s.
LPL	Estimate	-2.250	-0.04588	0.000791	-	-
	SED	0.167	0.00496	0.000196	-	-
	P-value		<0.001	<0.001	n.s.	n.s.

[1]log Y = the log-transformed gene expression ratios; ß$_0$ = the intercept; ß$_1$ = the fixed effect for DIM$_i$; ß$_2$ = the fixed quadratic effect of DIM$_i^2$; ß$_3$ = the effect of treatment day TD$_j$ (Control: TD = 0); ß$_4$ = the quadratic effect of treatment day TD$_j^2$ (Control: TD = 0); and ε$_{ijk}$ = the residual variance.
[2]SED = standard error of difference

Fatty acid transport. The integral membrane proteins FATP2 and FATP5 function as hepatic transporters for long chain FA, activating them to acyl-CoA (Kazantzis and Stahl, 2012). While FATP2 is predominantly a plasma membrane transporter transporting FA into the cytosol (Figure 1), a minor fraction is localized in liver peroxisomes, allowing peroxisomal oxidation of branched and very-long chain FA (Falcon et al., 2010). We did not observe a significant effect of DIM for FATP2 mRNA level; neither did RPC treatment change the levels of expression (Table 3 and Figure 3).

For FATP5, there was a significant effect of treatment on mRNA transcript (Table 3). The mRNA abundance of this transporter increased in CHOL cows postpartum (Figure 3), suggesting that RPC facilitates the import of FA into the liver after calving. Since the stimulatory effect on FATP5 expression was paralleled by an increased PPARd expression (Figure 3), it would be interesting to investigate in further studies whether this stimulatory effect could be attributed to the mediatory action of PPARδ.

Similarly to PPARa expression, the mRNA abundance of FABP1 increased significantly with DIM but was unaffected by RPC treatment (Figure 3). FABP1, also known as liver-type fatty acid-binding protein, is a cytosolic protein that binds and transports NEFA between membranes. In addition, FABP1 can physically interact with PPARa, explaining its partially nuclear localization (Wolfrum et al, 2001; Schroeder et al., 2008) and activation of transcription by PPARa (Kersten et al., 2010). This lack of a treatment effect on FABP1 transcription thus confirms that the effect of choline supplementation on liver metabolism is not implemented through PPARa activation.

Esterification and TAG transport. Once taken up by the hepatocyte, NEFA can be esterified into TAG either for delivery into blood as VLDL or storage as cytosolic lipid droplets (Figure 1). The first, rate limiting step in TAG synthesis is the attachment of activated NEFA (i.e. acyl-coA) at the sn-1 position of glycerol-3-phosphate, a process catalyzed by GPAT1 (Takeuchi and Reue, 2009).

We found a significant effect of DIM on GPAT1 mRNA expression, with increasing expression postpartum (Table 3 and Figure 3). Loor et al. (2005; 2006) described a comparable expression pattern of GPAT1 around parturition, with lowest levels at calving. In our study, lowest levels of GPAT1 in wk 1 (Figure 3) coincided with peak hepatic TAG concentration in wk 1 (Zom et al., 2011) and increased levels of GPAT1 were found when TAG concentration returned to normal levels in wk 6. Loor et al. (2006) suggested that GPAT1 expression may not be limiting for periparturient TAG accumulation. Also in our trial, the reduction of TAG accumulation by RPC supplementation was not effectuated through changes in GPAM1 expression.

Expression of MTTP was significantly affected by DIM and treatment (Figure 3). In CON cows the MTTP mRNA level was increased significantly at wk 1, and decreased gradually below antepartum level in wk 6. Our results are consistent with earlier work demonstrating that MTTP mRNA levels in the bovine liver are highest around parturition

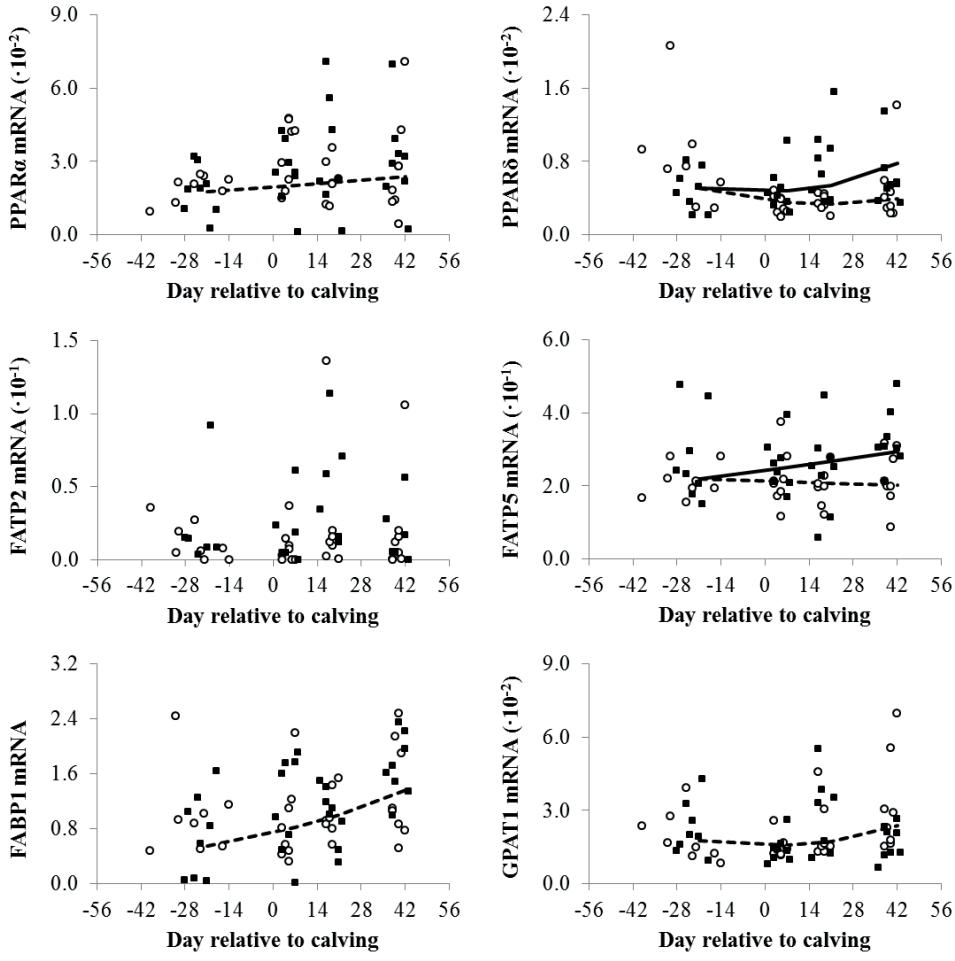

Figure 3. Effect of choline supplementation on the expression of 15 energy metabolism related genes in hepatic tissue of periparturient dairy cows. Indicated mRNA levels are expressed as a ratio to housekeeping gene β-actin (ACTB). Open symbols = cows in control group (CON); closed symbols = cows in choline-supplemented group (CHOL); broken line = modeled effect of DIM (Table 3); solid line = modeled effect of treatment days (Table 3; P<0.05).

PPARα = peroxisome proliferator-activated receptor alpha, PPARδ = peroxisome proliferator-activated receptor delta, FATP2 = fatty acid transport protein 2 (or SLC27A2), FATP5 = fatty acid transport protein 5 (or SLC27A5), FABP1 = fatty acid binding protein 1, GPAT1 = glycerol-3-phosphate acyltransferase 1 (or GPAM), MTTP = microsomal triglyceride transfer protein, APOB100 = apolipoprotein B100, SLC22A5 = organic cation transporter (also known as OCTN2), CPT1A = carnitine palmitoyltransferase 1A, GLUT2 = glucose transporter 2 (or SLC2A2), PC = pyruvate carboxylase, PDK4 = pyruvate dehydrogenase kinase isotype 4, PEPCK = phosphoenolcarboxykinase.

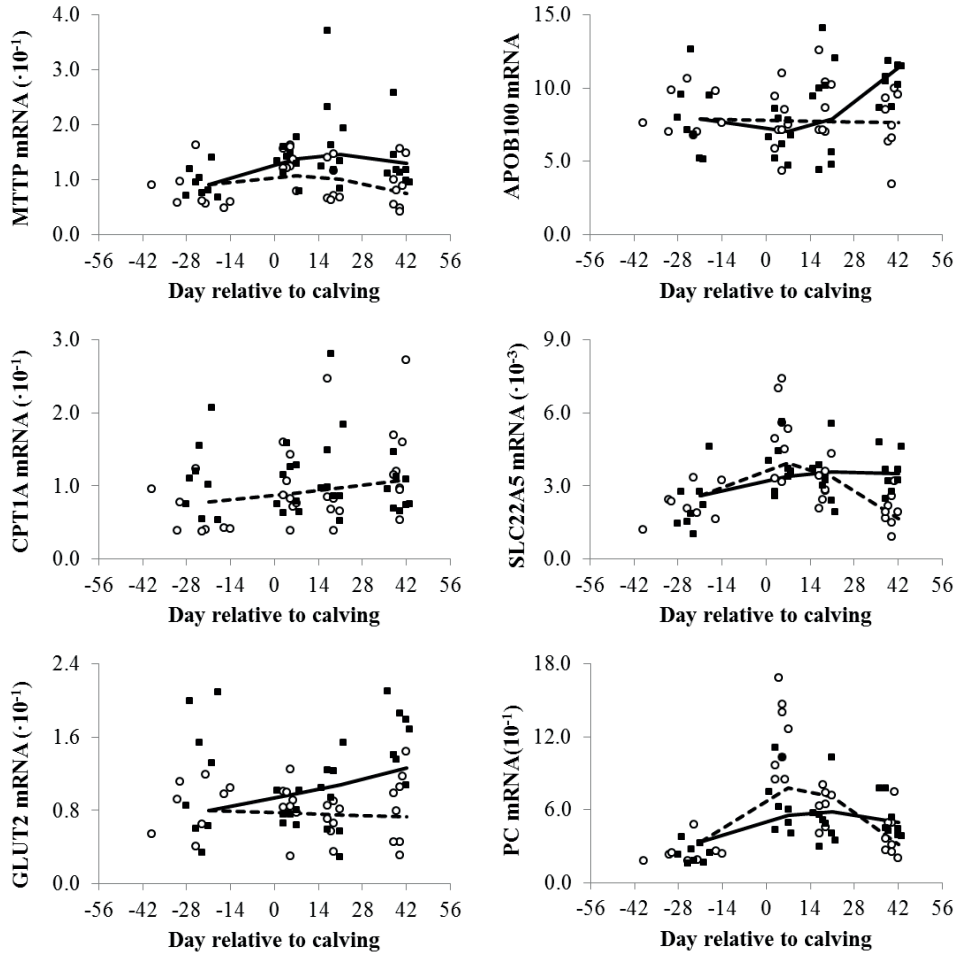

Figure 3 (Continued). Effect of choline supplementation on the expression of 15 energy metabolism related genes in hepatic tissue of periparturient dairy cows. Indicated mRNA levels are expressed as a ratio to housekeeping gene β-actin (ACTB). Open symbols = cows in control group (CON); closed symbols = cows in choline-supplemented group (CHOL); broken line = modeled effect of DIM (Table 3); solid line = modeled effect of treatment days (Table 3; P<0.05).

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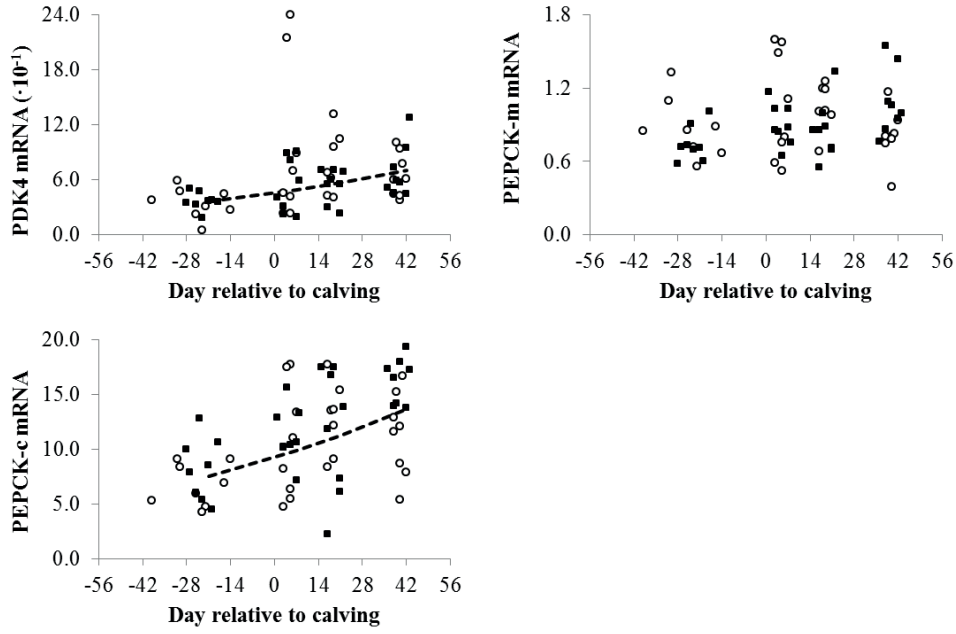

Figure 3 (Continued). Effect of choline supplementation on the expression of 15 energy metabolism related genes in hepatic tissue of periparturient dairy cows. Indicated mRNA levels are expressed as a ratio to housekeeping gene β-actin (ACTB). Open symbols = cows in control group (CON); closed symbols = cows in choline-supplemented group (CHOL); broken line = modeled effect of DIM (Table 3); solid line = modeled effect of treatment days (Table 3; P<0.05).
PPARα = peroxisome proliferator-activated receptor alpha, PPARδ = peroxisome proliferator-activated receptor delta, FATP2 = fatty acid transport protein 2 (or SLC27A2), FATP5 = fatty acid transport protein 5 (or SLC27A5), FABP1 = fatty acid binding protein 1, GPAT1 = glycerol-3-phosphate acyltransferase 1 (or GPAM), MTTP = microsomal triglyceride transfer protein, APOB100 = apolipoprotein B100, SLC22A5 = organic cation transporter (also known as OCTN2), CPT1A = carnitine palmitoyltransferase 1A, GLUT2 = glucose transporter 2 (or SLC2A2), PC = pyruvate carboxylase, PDK4 = pyruvate dehydrogenase kinase isotype 4, PEPCK = phosphoenolcarboxykinase.

(Bernabucci et al., 2004). In response to RPC supplementation, however, this increase at parturition was sustained during the first 6 wk (Figure 3). As MTTP promotes VLDL assembly in the endoplasmic reticulum (Wetterau et al., 1997), this result suggests that RPC increases VLDL assembly and thus TAG export from the liver, allowing hepatocytes to cope with the elevated NEFA influx during early lactation.

For APOB100, mRNA levels were also significantly affected by treatment (Figure 3). Cows in group CON did not show a change in APOB100 mRNA, while CHOL cows had increased levels of APOB100 in week 6 postpartum. We did not find a pronounced decrease in APOB100 immediately postpartum as described by

Bernabucci et al. (2004), but the increased mRNA levels of APOB100 and MTTP with RPC treatment suggest that improved VLDL synthesis is a main route of reduced TAG concentration in early lactation with RPC *supplementation*.

Fatty acid buffering and mitochondrial transport. We observed a significant effect of DIM on the CPT1A mRNA level (Figure 3). It has been well established that CPT1A is responsible for the regulation of mitochondrial b-oxidation in liver. In previous studies, CPT1A expression levels in dairy cattle in early lactation were variable (Loor et al., 2005, 2006; Selberg et al., 2005; Van Dorland et al., 2009). The hepatic CPT1A gene is a known target of PPARα in mouse and human (Kersten et al., 2010) as well as in dairy cattle (Loor et al., 2005; Van Dorland et al., 2009) and this relationship between the expression levels of PPARα and CPT1A is also found in our study with increasing DIM. Treatment had no effect on PPARα expression (as mentioned above), nor on CPT1A mRNA abundance. This suggests that RPC supplementation did not affect FA transport into mitochondria.

We found that SLC22A5 mRNA abundance was significantly affected by DIM and treatment, with increased expression around parturition (Figure 3). This is the first study in which hepatic SLC22A5 expression is explicitly analyzed in periparturient dairy cattle. As a plasma membrane transporter, SLC22A5 facilitates the import of carnitine and other organic cations into the hepatocyte (Koepsell et al., 2007; Figure 1). Carnitine is an obligatory cofactor for β-oxidation of fatty acids, enabling the transport of long-chain fatty acids across the inner mitochondrial membrane as acylcarnitine esters (Ramsay and Arduini, 1993). The increased expression of SLC22A5 around parturition therefore suggests a need for organic cation transport into hepatocytes postpartum. In rats and pigs, increased lipolysis due to fasting led to an increased hepatic transcription of SLC22A5, facilitating carnitine transport into liver cells, buffering cytosolic FA and supporting oxidation of the increased flux of FA (Ringseis et al., 2009). The results of Grum et al. (1996) confirm that the same pathway is also active in dairy cattle, showing increased carnitine concentration in liver around calving when DMI was low. Choline has been shown to have a dual effect on carnitine availability: it can support carnitine synthesis in the liver by functioning as a methyl donor in a methylation step (Bremer, 1983) and it can also directly stimulate the transport of dietary carnitine into liver cells as found in rats (Carter and Frenkel, 1978). Choline-induced SLC22A5 expression may have resulted in an increased intracellular carnitine concentration in RPC supplemented cows. Increased carnitine availability has been found to reduce liver TAG accumulation in dairy cattle through a stimulation of FA oxidation, as well as an improved gluconeogenesis (Carlson et al., 2007). Serum BHBA concentrations were

found to be higher with carnitine supplementation, as a result of increased NEFA oxidation (Carlson et al., 2007). In our study plasma BHBA concentrations were not significantly different between treatment groups (Zom et al., 2011), suggesting that a potential increase in FA oxidation by RPC supplementation was not substantial.

Pyruvate metabolism and glucose transport. We analyzed mRNA expression of GLUT2, the bidirectional glucose transporter in the plasma membrane (Zhao et al., 1993), to evaluate the glucogenic status of the liver. As shown in Figure 3 and Table 3 there was no significant effect of DIM on GLUT2 mRNA abundance, as was also found by Hammon et al. (2009), but RPC increased GLUT2 expression postpartum. Since the GLUT2 is a glucose-sensitive gene in the liver (Leturque et al., 2005), increased GLUT2 activity in group CHOL could be a result of higher glucose production in the liver, indicating improved carbohydrate metabolism. Increased DMI maThe increase in glucose output will support the energy demand of other organs, such as the mammary gland. However, RPC had no significant influence on milk yield nor lactose yield (Zom et al., 2011).

Additionally, mRNA expression of four genes involved in gluconeogenesis was analyzed. PC was significantly increased in week 1 postpartum, and decreased again in the first 6 weeks of lactation. In cows supplemented with RPC this periparturient increase was significantly lower. PC is important in gluconeogenesis and formation of oxaloacetate, and expression is upregulated around parturition in cows with lower energy status or high liver fat content (Loor et al., 2007; Hammon et al., 2009; Castro et al., 2012). The reduced expression in CHOL cows relatively to CON cows suggests that RPC supplementation improved energy status and reduced the need for PC.

There was no effect of treatment on PEPCK-m or PEPCK-c, but PEPCK-c increased significantly with days in milk as also found by Hammond et al. (2009) when looking at cows with relatively high compared to low liver fat concentration. The same effect of an increase in PC but not in PEPCK-m or PEPCK-c expression was found after sever feed restriction in mid-lactating dairy cattle (Velez and Donkin, 2005). These results confirm the improved energy status of CHOL cows.

PDK4 is involved in the inactivation of pyruvate dehydrogenase and forms a regulating link between fat and carbohydrate metabolism in mammals: expression of PDK4 is increased in starvation and with increased intracellular lipid concentration, thereby favoring the oxidation of long chain FA over pyruvate as an energy substrate (Holness and Sugden, 2003; Connaughton et

al., 2010). PDK4 mRNA expression is induced by glucocorticoids and reduced by insulin (Connaughton et al., 2010). In dairy cow, PDK4 expression was increased in endometrial tissue of animals in severe negative energy balance (Wathes et al., 2011). To our knowledge, expression has not been analyzed in dairy cow hepatic tissue before. In our study, there was an increase in PDK4 expression with DIM, suggesting increasing utilization of FA over carbohydrate energy sources, but we did not find a significant effect of treatment.

Effects in adipose tissue

During the dry period, adipose tissue metabolism is in an anabolic state, with high PPARγ expression, altering gene expression to direct adipose cells towards lipogenesis and enhanced glucose uptake (Walczak and Tontonoz, 2002). Around parturition, lipolysis in adipose tissue is favored over lipogenesis, to mobilize FA for lactogenesis in the mammary gland.

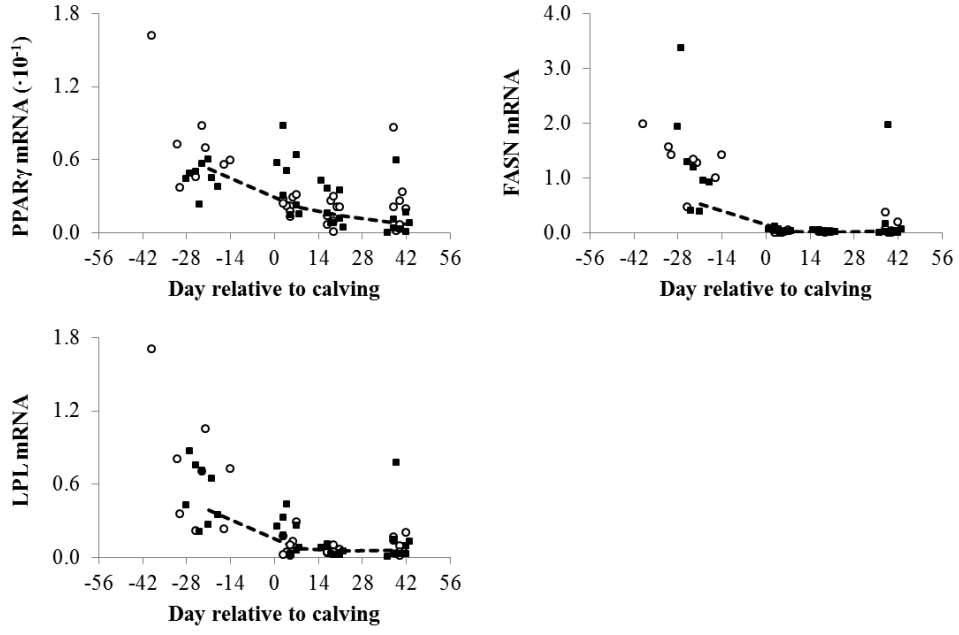

Figure 4. Quantitative PCR analysis of the expression of 3 fat metabolism-related genes in adipose tissue of periparturient dairy cows. Indicated mRNA levels are expressed as a ratio to housekeeping gene β-actin (ACTB). Open symbols = cows in control group (CON); closed symbols = cows in choline-supplemented group (CHOL); broken line = modeled effect of DIM (Table 3; $P<0.05$). PPARγ = peroxisome proliferator-activated receptor gamma, LPL = lipoprotein lipase, FASN = fatty acid synthase.

To evaluate whether RPC supplementation had an effect on lipogenesis, we analyzed the transcript levels of the following three genes in adipose tissue:

PPARγ, FASN and LPL. PPARγ is the transcription factor for lipogenesis in adipocytes, known to target the FASN and LPL genes (Thering et al., 2009). FASN catalyzes FA synthesis, while LPL promotes the cellular uptake of FA from VLDL in the bloodstream. For each of these genes expressed during our trial, there was a significant effect of DIM with the highest gene expression level before calving followed by a sharp decrease postpartum (Table 3 and Figure 4) as expected. After calving, catabolic processes result in lipolysis, sharply decreasing the expression of these genes. Above this, there was no significant effect of treatment (Table 3 and Figure 4), indicating that RPC supplementation had no effect on FA metabolism in adipose tissue when considering the three genes evaluated.

Milk fat

The effect of RPC on the amount of FA mobilization from adipose tissue can be evaluated indirectly by considering the FA composition of milk. Small and medium chain FA (<C16:0) as well as part of C16:0 FA in milk fat originate from *de novo* synthesis in the mammary gland, whereas longer chain FA originate from dietary fat or mobilization from adipose tissue (Mansbridge and Blake, 1997). Reduced mobilization from adipose tissue will decrease the relative proportion of long chain FA compared with small and medium chain FA in milk. Results for FA composition are presented in Table 4.

As expected, the milk FA composition was significantly affected by DIM. The proportion of small and medium chain FA originating from de novo synthesis in the mammary gland increased, whereas long chain FA originating from adipose tissue (C18:0 and C18:1 in milk) decreased ($P<0.001$) with time postpartum.

There was no significant effect of treatment on milk FA originating from de novo synthesis, implying that choline supplementation did not affect the balance between de novo synthesis of FA and the mobilization of FA from adipose tissue. This corresponds with the lack of treatment effect for body condition score and plasma NEFA concentration as published previously (Zom et al., 2011). There is therefore no indication that RPC affected the amount of lipolysis in adipose tissue, in agreement with the absence of an effect of RPC on the tested lipogenic genes in adipose tissue (Table 3 and Figure 4). Two specific FA with a relatively small contribution to the total amount of milk FA, C18:2 cis-9, trans-11 (rumenic acid) and C18:1 trans-9, were significantly affected by treatment days. Both FA specifically originate from incomplete biohydrogenation of dietary PUFA in the rumen. Their contribution to milk FA was increased for group CHOL. On both treatments, cows received equal amounts of concentrate during the trial (Table

1), but voluntary DMI of the roughage feed mixture was increased during the first weeks of lactation for group CHOL (Zom et al., 2011). This feed mixture consisted mainly of 52% wilted grass silage and 35% corn silage (Table 1). The increased intake of the forage mixture increased the amount of dietary PUFA ingested and may also have caused changes in factors affecting rumen biohydrogenation of PUFA (e.g. rumen pH and rumen transit time), resulting in increased proportions of rumenic acid in milk fat (Griinari et al., 1998).

Table 4. The effect of choline supplementation on the proportion of fatty acids (g/100 g FA) in the AM milk during the first 6 weeks of lactation, analyzed using the statistical model: $\log(Y)_{ijk} = \beta_0 + \beta_1 DIM_i + \beta_2 DIM^2_i + \beta_3 TD_j + \beta_4 TD^2_j + \varepsilon_{ijk}$[1]. Parameters are declared significant at P-value < 0.05; non-significant parameters for DIM^2, TD and TD^2 are released from the model (n.s.).

			Factor		**Effect of choline treatment day**	
		Constant	DIM	DIM^2	TD	TD^2
Item	Parameter[2]	β_0	β_1	β_2	β_3	β_4
C4:0	Estimate	0.7631	-0.004970	-	-	-
	SED	0.0654	0.002187	-	-	-
	P-value		0.030	n.s.	n.s.	n.s.
C6:0	Estimate	0.7273	0.001427	-	-	-
	SED	0.0274	0.000904	-	-	-
	P-value		0.120	n.s.	n.s.	n.s.
C8:0	Estimate	0.2439	0.005388	-	-	-
	SED	0.0429	0.001299	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C10:0	Estimate	0.9367	0.007902	-	-	-
	SED	0.0536	0.001588	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C12:0	Estimate	1.007	0.01005	-	-	-
	SED	0.058	0.00176	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C14:0	Estimate	2.220	0.006283	-	-	-
	SED	0.032	0.001034	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C14:1	Estimate	-0.8872	0.03436	-0.000450	-	-
	SED	0.0784	0.00680	0.000128	-	-
	P-value		<0.001	<0.001	n.s.	n.s.

Table 4 Continued

			Factor		**Effect of choline treatment day**	
		Constant	DIM	DIM^2	TD	TD^2
Item	Parameter[2]	$ß_0$	$ß_1$	$ß_2$	$ß_3$	$ß_4$
C15:0	Estimate	-0.5163	0.02737	-0.000430	-	-
	SED	0.0618	0.00610	0.000117	-	-
	P-value		<0.001	<0.001	n.s.	n.s.
C16:0	Estimate	3.398	0.000999	-	-	-
	SED	0.014	0.000392	-	-	-
	P-value		0.016	n.s.	n.s.	n.s.
C16:1	Estimate	0.7889	-0.006032	-	-	-
	SED	0.0567	0.001219	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C17:0	Estimate	-0.3135	-0.01332	0.0001391	-	-
	SED	0.0326	0.00327	0.0000634	-	-
	P-value		<0.001	0.032	n.s.	n.s.
C17:1	Estimate	-1.091	-0.01357	-	-	-
	SED	0.057	0.00154	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.
C18:0	Estimate	2.665	-0.01834	0.000261	-	-
	SED	0.050	0.00527	0.000106	-	-
	P-value		<0.001	0.016	n.s.	n.s.
C18:1 trans-9	Estimate	-2.064	0.003509	-	0.003501	-
	SED	0.048	0.001850	-	0.001105	-
	P-value		0.066	n.s.	0.010	n.s.
C18:1 cis-9	Estimate	3.144	-0.004906	-	-	-
	SED	0.037	0.000898	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.

Table 4 Continued

			Factor		Effect of choline treatment day	
		Constant	DIM	DIM^2	TD	TD^2
Item	Parameter[2]	$ß_0$	$ß_1$	$ß_2$	$ß_3$	$ß_4$
C18:2 cis-9, cis-12	Estimate	0.3084	0.01278	-0.000210	-	-
	SED	0.0379	0.00327	0.000061	-	-
	P-value		<0.001	0.001	n.s.	n.s.
C18:2 cis-9, trans-11	Estimate	-1.482	0.03834	-0.000653	0.008553	-0.000139
	SED	0.071	0.00635	0.000125	0.004074	0.000069
	P-value		<0.001	<0.001	0.046	0.049
C18:3	Estimate	-0.7563	-0.01356	0.000237	-	-
	SED	0.0548	0.00531	0.000102	-	-
	P-value		0.013	0.024	n.s.	n.s.
% of FA originating from de novo synthesis (vs. diet + mobilization)	Estimate	3.661	0.003904	-	-	-
	SED	0.022	0.000647	-	-	-
	P-value		<0.001	n.s.	n.s.	n.s.

[1]log Y = the log-transformed FA (in g/100 g total FA); $ß_0$ = the intercept; $ß_1$ = the fixed effect for DIM_i; $ß_2$ = the fixed quadratic effect of DIM_i^2; $ß_3$ = the effect of treatment day TD_j (Control: TD = 0); $ß_4$ = the quadratic effect of treatment day TD_j^2 (Control: TD = 0); and ε_{ijk} = the residual variance.
[2]SED = standard error of difference

CONCLUSIONS

The beneficial effect of RPC on hepatic lipidosis during the first weeks postpartum, as observed by Zom et al. (2011), could not be attributed to a difference in the amount of adipose tissue lipolysis, since there was no treatment effect on 1) gene expression in adipose tissue; 2) NEFA concentration in blood (Zom et al., 2011); nor 3) milk FA composition.

Our results indicate that the RPC-reduced hepatic TAG concentration is most likely attributed to a combination of 1) improved buffering and transport of intracellular NEFA by increased carnitine concentration, as evidenced by the

increase in PPARδ, FATP5 and SLC22A5 expression; 2) improved excretion by VLDL transport, shown by increased MTTP and APOB100 mRNA expression; and 3) improved carbohydrate metabolism, shown by increased GLUT2 expression postpartum and reduced PC peak expression levels immediately after calving. Overall RPC supplementation improved hepatic energy metabolism. Choline may have been the rate limiting nutrient in VLDL assembly, resulting in TAG accumulation in unsupplemented cows during the first weeks postpartum.

Finally, we hypothesize that the beneficial effects of RPC on liver function are extended beyond the period of hepatic lipidosis, because mRNA abundance of genes highly relevant in energy metabolism (SLC22A5, MTTP, APOB100, GLUT2) were still affected in wk 6, when DMI between groups was equal and hepatic TAG concentration had already returned to normal levels.

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3

Chapter 3

Physical exercise prepartum to support metabolic adaptation in the transition period of dairy cattle: A proof of concept

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ABSTRACT

In dairy cattle the hormonal changes around calving induce large metabolic changes to support milk production. Mobilization of adipose reserves is one of the changes involved, imposing a metabolic load on the liver. We hypothesized that the risk for excessive lipolysis and hepatic lipidosis postpartum can be reduced by starting fat mobilization and processing during the prepartum period by physical exercise, especially in cows with a high body condition score (**BCS**). As a proof of concept, 32 pregnant Holstein-Friesian dairy cows were selected for a 2 × 2 experimental design. Sixteen cows had a BCS < 3.25 (group **LOW**) and 16 cows a BCS ≥ 3.25 (group **HIGH**). Cows within each group were randomly allocated to one of two treatments: group **STEP** was walked twice daily for 45 min during the dry period while group **CON** remained indoors. Treatment was stopped at calving and cows were monitored until 6 wk after calving. Liver biopsies were taken in a subset of 16 cows to determine liver triglyceride (**TG**) concentration. We found that calculated energy balance was more negative for group STEP prepartum, resulting in higher plasma non-esterified fatty acids and β–hydroxybutyrate concentrations. During the first 6 weeks postpartum neither dry matter intake nor milk yield were affected by exercise. As expected, the cows in group HIGH had increased liver TG concentrations postpartum relative to group LOW with increased plasma non-esterified fatty acids directly after calving. Exercise during the dry period mitigated postpartal liver TG accumulation, but this did not seem to be related to increased plasma lipoprotein transport. We conclude that substantial physical activity prepartum can induce lipolysis and lipid utilization, thereby starting an early adaptation to lactation. This may be instrumental to reduce the risk for excessive liver TG accumulation postpartum, especially in cows with a high BCS at dry-off.

INTRODUCTION

Hormone-induced adaptations in fat metabolism lead to increased fat mobilization in dairy cows around calving, thereby leading to an increased release of fatty acids from adipose tissue elevating blood non-esterified fatty acids (**NEFA**) concentrations (Friggens, Andersen, Larsen, Aaes, & Dewhurst, 2004). This release can be considered an evolutionary advantage to provide energy for milk synthesis for the survival of the offspring. At the same time however, the risk for hepatic lipidosis is increased (Van Knegsel *et al.*, 2014).

Important risk factors associated with hepatic lipidosis are overfeeding during the dry period and a high body condition score (**BCS**) at calving (Van Den Top *et al.*, 1995; Roche *et al.*, 2015). It is generally accepted that a high BCS (> 3.5) at calving causes excessive mobilization of body fat postpartum, thereby increasing the risk for metabolic diseases such as ketosis and fatty liver (Gillund, Reksen, Gröhn, & Karlberg, 2001; Roche *et al.*, 2015). Avoiding excess energy intake during the dry period is therefore an important strategy to prevent excessive fat mobilization postpartum (Janovick, Boisclair, & Drackley, 2011; Roche *et al.*, 2015). An alternative strategy may be to trigger fat mobilization and early adaptation of metabolic processes needed postpartum by inducing a mild negative energy balance (**NEB**) before calving. This may be achieved by increasing physical activity during the dry period as a method to increase energy output and stimulate energy metabolism. Besides the net energy cost of exercise, training will also stimulate lipolysis of adipose tissue while enhancing muscular development and muscle enzyme activity, as described in humans (Tremblay, Simoneau, & Bouchard, 1994). The outcome of previous research indicates that prepartal exercise of 1.6 up to 9.7 km/d reduces weight gain in multiparous cows, while feed intake and postpartal milk yield remain unaffected; nutrient intake and calculated energy balance were however not available (Anderson, Lamb, & Walters, 1979; Lamb, Anderson, & Walters, 1981). Walking 0.5 to 3 km/d did improve general health of tied dairy cows compared to non-exercised, tied dairy cows; especially regarding disease incidence in the first two weeks after calving (Gustafson, 1993). Forcing dry cows to exercise for 1.25 to 1.5 h/d at a speed of 3.25 km/h resulted in improved physical fitness, as determined by a lower heart rate, lower plasma lactate and an improved ability to maintain their systemic acid-base balance at a given workload (Davidson & Beede, 2009).

In this study we aimed to induce prepartal lipolysis using increased physical activity in dairy cows with normal versus high BCS as a proof of concept. We

hypothesize that especially in cows with a high BCS at dry-off, prepartal physical exercise prevents excessive fat accumulation in the liver after calving.

MATERIALS AND METHODS

Animals and treatments

All experimental protocols and interventions were approved by the Ethical Committee on Animal Experiments of the Animal Sciences Group of Wageningen University & Research, Wageningen, The Netherlands. The experiment was conducted between April and October 2012 and comprised 32 pregnant Holstein-Friesian cows selected at our 450-cow research dairy farm in Lelystad, The Netherlands. The animals were selected based on parity (no heifers), expected calving date and BCS. The herd's mean BCS during the last trimester of gestation was 3.2 which was used as cut-off level to define our study groups: 16 cows were selected with a BCS < 3.25 (group **LOW**) and 16 cows a BCS ≥ 3.25 (group **HIGH**) between 6-8 wk before the expected calving date. The 32 selected cows were then blocked (two LOW and two HIGH cows per block) by parity, previous 305 day milk production and expected calving date. Within each block, the two LOW cows were randomly allocated to either control (**CON**) or exercise (**STEP**) group and the same was done for the two HIGH cows.

The experiment started at dry-off, 41 d (SD ± 7 d) before calving. The cows allocated to STEP received physical exercise during the entire dry period by walking two times per day (at 0730 and 1600 h) for 45 min in a mechanical horse walker at a speed of 3.4 km/h and an ambient temperature varying between 7 and 27°C. Exercise was stopped as soon as pelvic ligaments were weakened before calving (on average 1 d prior), and all cows were monitored for 6 wk after calving.

Cows were housed in a cubicle shed with 1 cubicle available per cow and were kept in separate dry cow and post-calving groups with approximately 5 m^2 walking area per cow, without any access to either a pasture or outdoor paddock. At the first signs of calving, cows were separated from the dry cow group and housed in a straw bedded calving pen (20m^2) where the animals had unrestricted access to the dry cow diet and fresh water. Directly after calving, the cow was moved to the post-calving group.

Rations and feeding management

Fresh feed mixtures were provided daily between 1030 and 1100 h. From 6 wk before until calving, cows received a dry cow feed mixture supplemented with 1.0 kg compound concentrate from 2 wk before calving (Tables 1 and 2). During the prepartal period, daily energy intake was restricted to maximally 60 MJ of NE_L/cow for both CON and STEP to prevent excessive gain of body fat (CVB, 2012).

Maximum energy intake was estimated to be ~ 110% of the mean (32 cows) energy requirement for maintenance and gestation during the last six weeks of gestation and did not include the energy requirement for exercise. Cows in group CON did not have access to the feeding bins during the periods cows in group STEP were outside exercising, to prevent a difference between the two treatment groups in the time available for eating.

After calving, all cows were fed the lactation feed mixture *ad libitum* (Tables 1 and 2). The cows were fed two compound concentrates: one supplied at milking (0.5 kg per milking, at 0600 and 1700 h) and one in the barn (gradually increased with 0.5 kg/d from 1.0 kg/d on the day of calving up to 8.5 kg/d on d 17). The maximum level of concentrate was maintained from d 17 until the end of the experimental period at d 42.

Throughout the entire study, the cows had access to troughs with individual transponder-controlled access gates (Roughage Intake Control system, Hokofarm Group, Marknesse, The Netherlands). The troughs were equipped with an electronic balance and allowed automatic registration of individual feed intakes. Except during exercise sessions (from 0730 to 0830 h and from 1600 to 1700 h) and feeding time (from 1030 to 1100 h), cows had continuous access to the feed troughs. Overall, the amount of feed mixture that was supplied to the cows was 110% of actual intake. The compound concentrates were fed individually, using transponder-controlled concentrate dispensers which made the individual allowance available in equal portions over six 4-h periods. All cows had unrestricted access to fresh water, except cows in group STEP during their physical training sessions in the horse walker.

Table 1 Ingredients of the feed mixtures and amounts of concentrate fed during pre and post-calving period of dairy cows

	Dry cow ration	**Lactation ration**
Feed mixture (% of DM)		
Wilted grass silage	46.7	51.9
Corn silage	15.8	33.3
Chopped rapeseed straw	2.8	3.1
Chopped wheat straw	23.6	-
Rapeseed extract	9.9	3.1
Soybean extract	-	10.1
Vitamin and mineral premix	1.0	1.0
Magnesium oxide	0.2	-
Sodium bicarbonate	-	0.6
Concentrate (kg/cow/d)		
Barn[†]	0 to 1.0	1.0 to 8.5
Milking parlour[‡]	-	1.0

[†] Compound concentrate (based on ground corn, palm kernel expeller, rapeseed solvent extract, soy hulls and soybean solvent extract) with 6.7 MJ NE_L and 180 g CP per kg; pre-calving gradually increasing from 0 kg/d at d -14 to 1.0 kg/d on the expected day of calving, and post-calving gradually increasing from 1.0 kg/d at d 0 to 8.5 kg/d at d 17 and maintained at 8.5 kg/d until the end of the trial.
[‡] Compound concentrate (based on ground corn, palm kernel expeller, citrus pulp, rapeseed solvent extract and rapeseed formaldehyde treated) with 6.6 MJ NE_L and 165 g CP per kg.

Table 2 Chemical composition and feeding value of dry period feed mixture, lactation feed mixture and concentrate (g/kg of dry matter).

	Dry cow mixture	Lactation mixture	Concentrate
Chemical composition			
DM (g/kg of product)	526	458	878
Crude protein	117	149	206
Crude fat	31	31	38
NDF	544	430	298
ADF	318	246	166
Starch	54	110	220
Sugars	55	62	85
Ash	86	85	73
Feeding value			
DVE[†]	44	71	123
OEB[‡]	18	29	28
FOM[§]	452	509	539
NE_L (MJ/kg of DM)	5.25	6.20	7.62

[†] Intestinal digestible protein (Tamminga et al., 1994).
[‡] Rumen degraded protein balance (Tamminga et al., 1994).
[§] Fermentable organic matter.

Feed sampling and feed analysis

Feed mixtures were sampled daily for DM analysis. The DM content was determined after oven drying at 104°C during 36 h. Each separate ingredient of the feed mixtures as well as the compound concentrates were sampled weekly and stored at –20°C. For grass and maize silage, samples of 5-6 consecutive weeks were pooled into 1 composite sample; for concentrates and wheat straw, all samples were pooled per feedstuff. The pooled samples were used for chemical analysis and determination of the feeding values at the MasterLab Laboratory (MasterLab, Boxmeer, the Netherlands). Briefly, the crude fat content was determined gravimetrically as the ether extract (ISO 6492; ISO, 1999) and crude ash content after incineration at 550°C (ISO 5984; ISO, 2002). Content of NDF and ADF were determined according to Van Soest, Robertson, & Lewis (1991) and expressed without residual ash. Crude protein was calculated as 6.25 × N-Kjeldahl (ISO 5983; ISO, 2005) and sugar concentrations were determined as described by Van Vuuren, Van der Koelen, Valk & De Visser (1993). Starch was released by heating in a boiling water bath in the presence of 2 N HCl (Cone, 1991) after which starch concentration was determined using the amyloglucosidase method (ISO 15914; ISO, 2004). The NE_L and intestinal digestible protein (DVE) were calculated according to the guidelines from the Central Bureau for Livestock Feeding (CVB, 2012).

Milk yield and milk composition

Cows were milked twice daily at 0600 and 1700 h with milk weights recorded automatically at each milking. Weekly, milk samples of each cow were taken at 4 consecutive milkings (2 morning milkings and 2 afternoon milkings). Both morning milk samples were pooled to 1 composite sample; afternoon milk samples were processed likewise. The composite morning and afternoon milk samples were analyzed for fat, protein, and lactose concentrations by Qlip (Zutphen, the Netherlands) using a Foss MilkoScan infrared automatic analyzer (Foss Electric, Hillerød, Denmark). Weighted means were calculated from the recorded morning and afternoon milk weights and the analyses of the composite morning and afternoon milk samples.

Body weight, body condition score and energy balance

The precalving BW was recorded weekly between 0800 and 1000 h before fresh feed was supplied. The postcalving BW was automatically recorded at the same weighing scale, twice per day at entry into the milking parlor. Cows were scored weekly for body condition from 1 (thin) to 5 (fat) with 0.25 point increments according to Ferguson, Galligan, & Thomsen (1994). Energy balance was calculated as described by Van Knegsel *et al.* (2007) as the difference between net

energy for lactation (**NE_L**) supplied with feed and the NE_L required for gestation, maintenance and milk production. Calculations were based on the stage of gestation, average feed intake, milk yield, milk composition and body weight results of that week. The additional metabolizable energy (**ME**) requirement for forced walking at 3.4 km/h is on average 0.52 kcal ME/kg/km (Hall & Brody, 1934) and as such for the present trial, energy required was estimated to be 4.6 MJ NE/d for cows of approximately 700 kg BW walking 5 km per day (ME : NE_L equivalent to 1 : 0.6).

Blood collection and analysis

Blood samples were taken twice prepartum, at 6 wk (30-48 d) and 2 wk (3-20 d) before calving and postpartum on d 3, 7, 10, 14, 21 and 42. Blood samples were collected from the tail vein (vena cauda) using lithium-heparin-coated tubes (Vacuette, type 455084, Greiner Bio-One, Frickenhausen, Germany) for analyses of beta-hydroxybutyrate (**BHB**); and in EDTA-coated tubes (Vacuette, type 455036, Greiner Bio-One) for analysis of NEFA, triglycerides (**TG**), high density lipoprotein (**HDL**) cholesterol and total cholesterol. Immediately after collection, blood samples were placed in ice water and centrifuged at 1,500 × *g* for 10 min within 1 h after collection. Subsequently, 1 mL of blood plasma was transferred to a vial and stored at −20°C until analysis.

NEFA were determined using a colorimetric assay and BHB by an enzymatic method (both kits from Randox Laboratories Ltd, Crumlin, United Kingdom). Plasma total cholesterol and TG were measured using a commercial enzymatic dry chemistry kit (Ortho Clinical Diagnostics, Raritan, NJ). After precipitation of low density lipoprotein (**LDL**) and very low density lipoprotein (**VLDL**) with sodium phosphotungstate magnesium chloride, HDL cholesterol was determined using a commercial enzymatic kit (Roche Diagnostics, Indianapolis, IN). Finally, LDL-cholesterol was calculated as follows (all measures in mg/dL):

LDL-cholesterol = total cholesterol – HDL-cholesterol – TG / 5

where 5 is the ratio between TG and VLDL cholesterol (Friedewald, Levy, & Fredrickson, 1972).

Liver biopsies and analysis

Liver biopsies were taken from 16 cows (from 4 randomly selected blocks) in wk −2 (6-23 d before calving), wk 1, and wk 2 relative to calving according to the method described by Zom *et al.* (2011). Briefly, a skin incision was made at the 11th intercostal space after local anesthesia and a biopsy needle was inserted to

collect ~2 g of liver tissue for analysis. Samples were immediately frozen in liquid N and stored at −80°C until analysis. Before analyses, liver samples were thawed and adhered water was removed using paper tissues. The concentrations of liver TG were determined using enzymatic hydrolysis of triglycerides with lipase into glycerol and fatty acids using the Triglycerides LiquiColor Mono test kit (Instruchemie BV) by photometric analysis at 550 nm (HumaLyzer 3000, Human Diagnostics).

Calculations and statistical analysis

Daily feed intake, milk yield, BW and energy balance were averaged per cow per week relative to calving. Liver biopsies were available for a subset of 16 cows (from 4 randomly chosen blocks) in wk -2, wk 1 and wk 2 relative to calving; liver TG content was determined relative to wet weight and expressed as a relative value compared to the TG content in week -2, where the value of week -2 was set at 100%.

A mixed model analysis with repeated measures was performed using the REML procedure in Genstat 19th edition (2018). For the dry period and lactation period separately, each parameter was modelled over time for each individual cow using a random coefficient regression model, i.e.

$$Y_{ijkl} = \mu + \underline{\varepsilon}_i + C_j + T_k + W_l + C_j \times T_k + C_j \times W_l + T_k \times W_l + C_j \times T_k \times W_l + C_j \times T_k + \underline{\varepsilon}_{i \cdot l}$$

where Y_{ijkl} = dependent variable; μ = overall mean; $\underline{\varepsilon}_i$ = random effect of cow (where $\varepsilon_i \sim N(0,\sigma^2_i)$; C_j = effect of BCS class (LOW or HIGH); T_k = effect of treatment (CON or STEP); W_l = effect of time (prepartum -6 to -1; postpartum 1 to 6); $C_j \times T_k$ = interaction between BCS class and treatment; $C_j \times W_l$ = interaction between BCS class and time; $T_k \times W_l$ = interaction between treatment and time; $C_j \times T_k \times W_l$ = interaction between BCS class, treatment and time; and $\underline{\varepsilon}_{i \cdot l}$ = residual error (or cow × time). The necessity of an auto-regressive function as well as heterogeneity were tested and judged by the difference in deviance of each model with the change in degrees of freedom in a chi-squared distribution. Auto-regressive function improved model deviance of all parameters; heterogeneity was not always relevant but for most parameters outside the model, as displayed with the model components in Supporting Information Table S1. A visual check for outliers was performed based on model residuals after which the model was tested with and without the potential outlier to see if conclusions changed; this was not the case and no outliers were deleted.

The significance value was set at $P < 0.05$ and a trend was declared at $0.05 \leq P < 0.10$.

RESULTS

Thirty cows finished the experiment. Two cows were excluded, one cow due to a hip injury after an accident not related to the experimental procedures (group CON), and one cow aborted just before the start of the experiment (group STEP).

Dry period exercise

During the dry period BW increased on average by 27 kg, but there was no difference between treatment groups. The average BW during the dry period was 679 vs. 705 kg for STEP and CON respectively (SED = 19.7 and P = 0.118). As expected, cows in STEP group had a lower NE_L balance (-33 vs. +23 $kJ/kg^{0.75} \cdot d^{-1}$ for STEP and CON respectively, SED = 18.0 and P < 0.01). Energy balance was below zero from 5 weeks before calving while CON cows had an energy balance below zero from 1 week before calving (Figure 1). Exercise affected feed intake: prepartum DM intake (**DMI**) was 0.8 kg DM/d lower for STEP relative to CON cows (10.6 vs. 11.4 for STEP and CON respectively, SED = 0.37 and P < 0.05).

During the prepartum training period, cows in BCS class HIGH had lower plasma cholesterol concentration (3.5 vs. 4.1 mmol/L, SED = 0.25 and P < 0.05) compared to LOW without a difference in NEFA or BHB; cows in group STEP showed significantly higher plasma NEFA (0.67 vs. 0.25 mmol/L, SED = 0.101 and P < 0.001) and BHB concentration (0.69 vs. 0.46 mmol/L, SED = 0.076 and P < 0.01) relative to cows in group CON.

Postpartum performance

Treatment or BCS class did not affect average gestation length (280 d) or birth weight of calves (44 kg). After calving, feed intake increased over six weeks from 15 to 24 kg DM per day, milk yield from 26 to 40 kg per day and the NEB slowly increased towards zero (Figure 1); average body weight and BCS are shown in Table 3. Neither DMI, milk yield nor NE_L balance were affected by exercise, BCS class or their interaction (Table 4). There was however a tendency for a reduced DMI in BCS class HIGH in the first two weeks after calving (interaction week × BCS, SED = 0.66 and P = 0.071) as well as a tendency for the interaction of week × exercise × BCS in FPCM production (SED = 2.40 and P = 0.053), due to the high FPCM yield in week 1 for non-exercised cows in BCS class HIGH (Table 4, Figure 1).

Table 3 Descriptive statistics for body weight in kg (BW) and body condition score on a scale from 1 to 5 (BCS) at start of the trial (6 weeks before calving), directly after calving and at the end of the experimental period (week 6 postpartum). For each treatment group, the average and standard deviation (between brackets) are shown (n = 8 per group).

	Start	**After calving**	**End**
Body weight			
LOW CON	662 (66)	631 (53)	647 (56)
LOW STEP	641 (68)	603 (58)	616 (55)
HIGH CON	713 (52)	677 (62)	660 (59)
HIGH STEP	719 (63)	643 (43)	662 (40)
Body condition score			
LOW CON	2.9 (0.4)	3.1 (0.5)	2.7 (0.9)
LOW STEP	2.8 (0.6)	2.5 (0.3)	2.3 (0.5)
HIGH CON	3.7 (0.5)	3.1 (0.4)	2.8 (0.6)
HIGH STEP	3.7 (0.3)	3.0 (0.3)	2.8 (0.4)

Cows with BCS class HIGH had initially greater milk fat content compared to LOW (interaction week × BCS, SED = 1.49 and $P < 0.001$; Table 4 or Supporting Information Figure S1) while the average milk fat content postpartum was reduced for treatment STEP compared to CON (44.2 vs. 46.3 g/kg, SED = 1.37 and $P < 0.05$; Table 4). Fat yield in kg per day was not affected by exercise, BCS class or their interaction and neither were protein content, lactose content or lactose yield. Protein yield showed a significant interaction of week × exercise × BCS (SED = 0.09 and $P < 0.05$; Table 4).

Postpartum NEFA concentration was affected by prepartum BCS: cows in BCS class HIGH had higher NEFA concentration compared to LOW (0.49 vs. 0.34 mmol/L, SED = 0.061 and $P < 0.05$, Table 5, Figure 2a). Concentration of NEFA was not affected by treatment, but plasma BHB was on average lower for treatment STEP compared to CON (0.65 vs. 0.76 mmol/L, SED = 0.068 and $P < 0.01$, Table 5, Figure 2b).

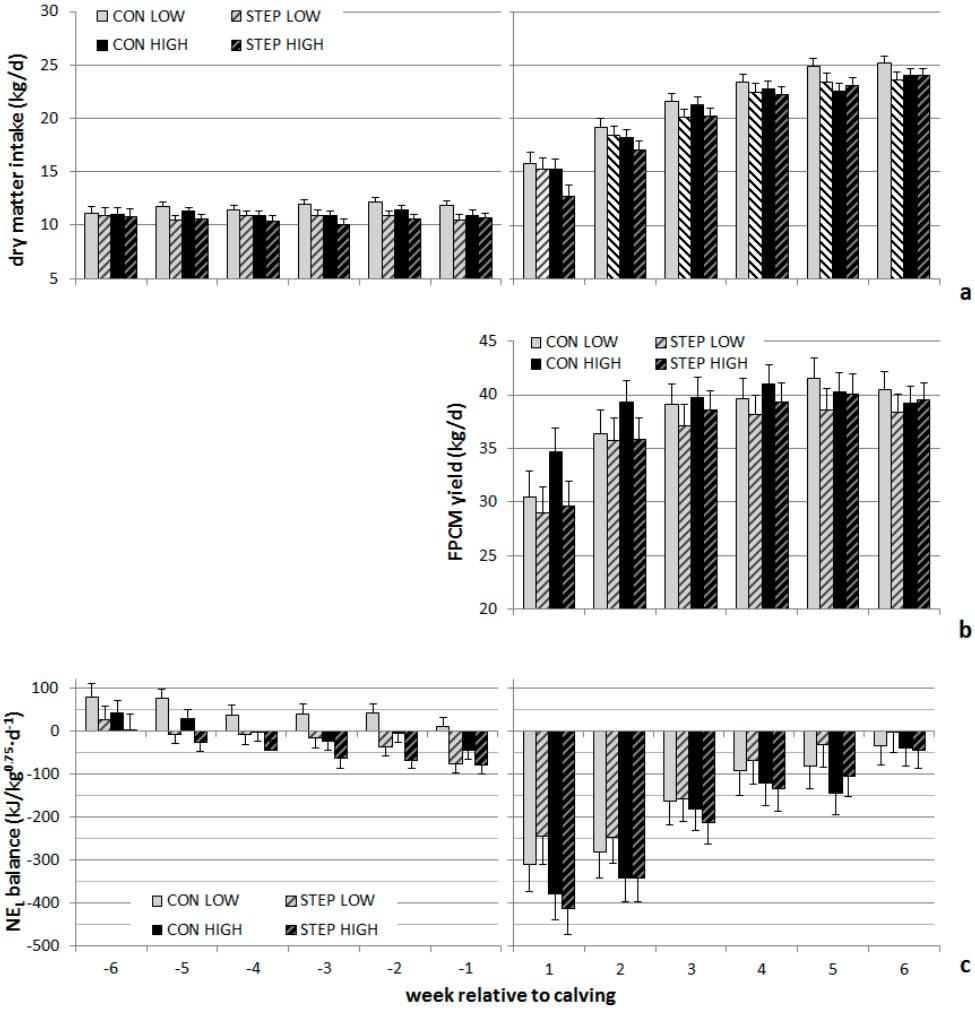

Figure 1 Cow performance regarding a) average dry matter intake (kg/d); b) fat- and protein corrected milk yield (FPCM yield, kg/d); and c) calculated net energy for lactation (NE_L) balance ($kJ/kg^{0.75} \cdot d^{-1}$) during the experiment from 6 weeks before until 6 weeks after calving. Figures showing predicted means by REML analysis with SEM.

Table 4 The effect of prepartum BCS class (HIGH vs. LOW) and exercise (CON vs. STEP) on dry matter intake (DMI), milk yield, fat- and protein corrected milk (FPCM), milk components (fat, protein, lactose), component yield and calculated net energy for lactation balance (NE_L bal) in week 1 to 6 postpartum.

	Treatment group					P-value[†]					
Item	**LOW CON (n = 7)**	**LOW STEP (n = 7)**	**HIGH CON (n = 8)**	**HIGH STEP (n = 8)**	**SED**	**exerc[‡]**	**BCS[§]**	**exerc × BCS**	**exerc × week**	**BCS × week**	**exerc × BCS × week**
DMI (kg/d)	21.7	20.5	20.7	19.9	0.97	0.184	0.556	0.485	0.654	0.071	0.383
Milk yield (kg/d)	35.7	35.2	36.3	35.2	3.03	0.905	0.905	0.831	0.195	0.773	0.150
FPCM (kg/d)	37.9	36.2	39.0	37.2	2.59	0.990	0.990	0.532	0.765	0.363	0.053
Milk fat (g/kg)	45.3	42.8	47.3	45.6	1.94	0.045	0.548	0.745	0.175	<0.001	0.735
Milk protein (g/kg)	36.5	34.1	34.2	34.9	1.87	0.840	0.498	0.086	0.873	0.936	0.963
Milk lactose (g/kg)	46.0	46.4	45.5	46.1	0.56	0.135	0.523	0.582	0.107	0.083	0.134
Fat yield (kg/d)	1.58	1.49	1.68	1.56	0.130	0.289	0.974	0.799	0.892	0.106	0.218
Protein yield (kg/d)	1.27	1.17	1.22	1.20	0.101	0.954	0.892	0.252	0.282	0.451	0.033
Lactose yield (kg/d)	1.64	1.63	1.66	1.64	0.128	0.775	0.850	0.721	0.154	0.744	0.238
NE_L bal ($kJ/kg^{0.75} \cdot d^{-1}$)	-161	-126	-202	-209	68.2	0.885	0.672	0.656	0.380	0.154	0.925

[†] the main effect "week" was significant for all parameters reported ($P < 0.001$).
[‡] exerc = exercise group (CON vs. STEP).
[§] BCS = body condition score class (LOW vs. HIGH).

Table 5 The effect of prepartum BCS class (HIGH vs. LOW) and exercise (CON vs. STEP) on average plasma concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyric acid (BHB), total cholesterol, high density lipoprotein cholesterol (HDL-chol), low density lipoprotein cholesterol (LDL-chol) and very low density lipoprotein (VLDL-chol) postpartum (determined on 3, 7, 10, 14, 21 and 42 days after calving), all in mmol/L; and liver TG determined in 16 cows in week -2, week 1 and week 2 relative to calving (expressed in g/kg wet weight (ww) and as a relative increase compared to week -2).

	Treatment group					**P-value**[†]					
Item	**LOW CON**	**LOW STEP**	**HIGH CON**	**HIGH STEP**	**SED**	**exerc**[‡]	**BCS**[§]	**exerc × BCS**	**exerc × time**	**BCS × time**	**exerc × BCS × time**
	(n = 7)	(n = 7)	(n = 8)	(n = 8)							
NEFA (mmol/L)	0.33	0.34	0.53	0.45	0.086	0.535	0.018	0.474	0.909	0.206	0.888
BHB (mmol/L)	0.69	0.59	0.84	0.72	0.096	0.009	0.127	0.493	0.891	0.477	0.500
Cholesterol (mmol/L)	2.94	2.94	3.01	2.70	0.280	0.416	0.440	0.460	0.039	0.272	0.498
HDL-chol (mmol/L)	2.08	2.07	2.12	1.93	0.181	0.586	0.650	0.912	0.059	0.196	0.713
LDL-chol (mmol/L)	0.84	0.85	0.86	0.72	0.104	0.504	0.142	0.372	0.397	0.813	0.548
VLDL-chol (mmol/L)	0.02	0.02	0.04	0.04	0.007	0.957	0.001	0.802	0.808	0.744	0.339
	(n = 4)	(n = 4)	(n = 4)	(n = 4)							
Liver TG (g/kg ww)	17	21	53	36	10.3	0.499	0.005	0.160	0.137	0.007	0.331
Liver TG (relative)	1.8	1.7	3.9	2.3	0.71	0.099	0.058	0.229	0.202	0.043	0.359

[†] the main effect "time of sampling" was significant for all parameters reported (P < 0.001), except for BHB (tendency, P=0.089) and VLDL-chol (P=0.235).
[‡] exerc = exercise group (CON vs. STEP).
[§] BCS = body condition score class (LOW vs. HIGH).

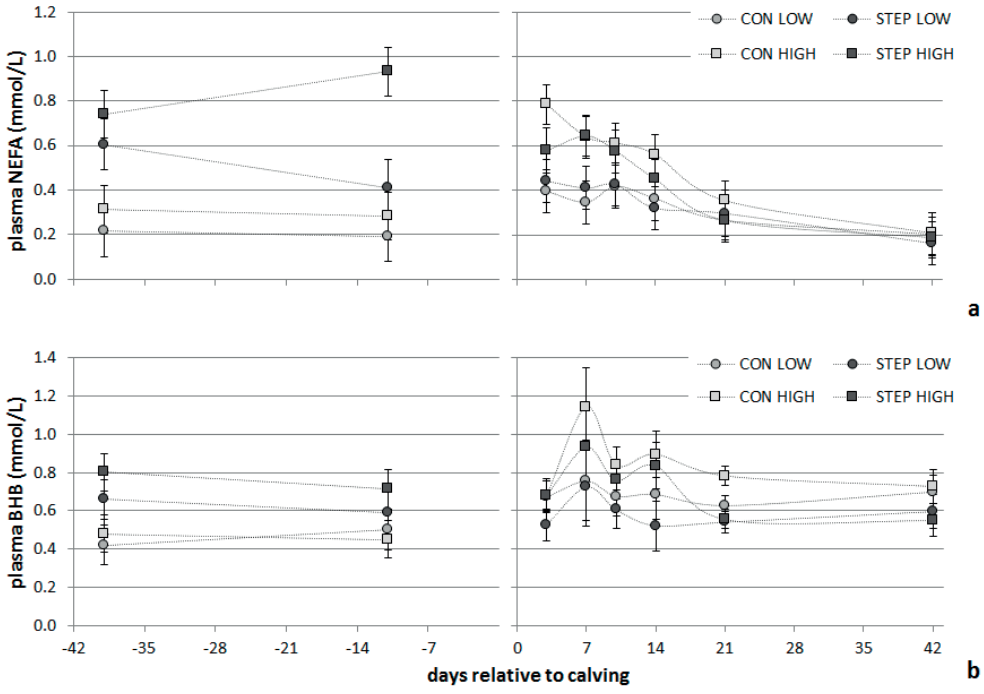

Figure 2 Fatty acid metabolism as measured by concentration of a) plasma non-esterified fatty acids (NEFA, mmol/L) and b) plasma β-hydroxybutyric acid (BHB, mmol/L). Figures showing predicted means by REML analysis with SEM.

Liver metabolism and lipoprotein transport

Liver TG concentration increased after calving and was affected by prepartum BCS. Cows in BCS class HIGH showed a greater increase in liver TG compared to LOW (BCS × time, SED = 9.3 and P < 0.01, Table 5, Figure 3a). Liver TG was not affected by prepartum exercise. The relative increase of liver TG postpartum compared to liver TG in week -2 tended to be lower in cows in group STEP compared to CON (2.1 vs. 2.8, SED = 0.50 P = 0.099, Table 5, Figure 3b).

Prepartum cholesterol was not affected by exercise. During prepartum, concentrations of plasma cholesterol were lower for cows in BCS class HIGH compared with LOW (3.5 vs. 4.1 mmol/L, SED = 0.25 and P < 0.05). At 3 d postpartum plasma cholesterol was relatively low and not affected by BCS class, but the increase over time was reduced for cows in group STEP compared with CON (interaction week × treatment, SED = 0.18 and P < 0.05, Table 5, Supporting Information Figure S2).

The subgroups of HDL and LDL were not affected by BCS class or exercise postpartum, with a tendency for the interaction of week × exercise for HDL cholesterol (SED = 0.118 and P = 0.059). Concentration of VLDL cholesterol was extremely low, but higher for BCS class HIGH compared to LOW (SED = 0.005 and P < 0.01) as shown in Table 5 and Supporting Information Figure S2.

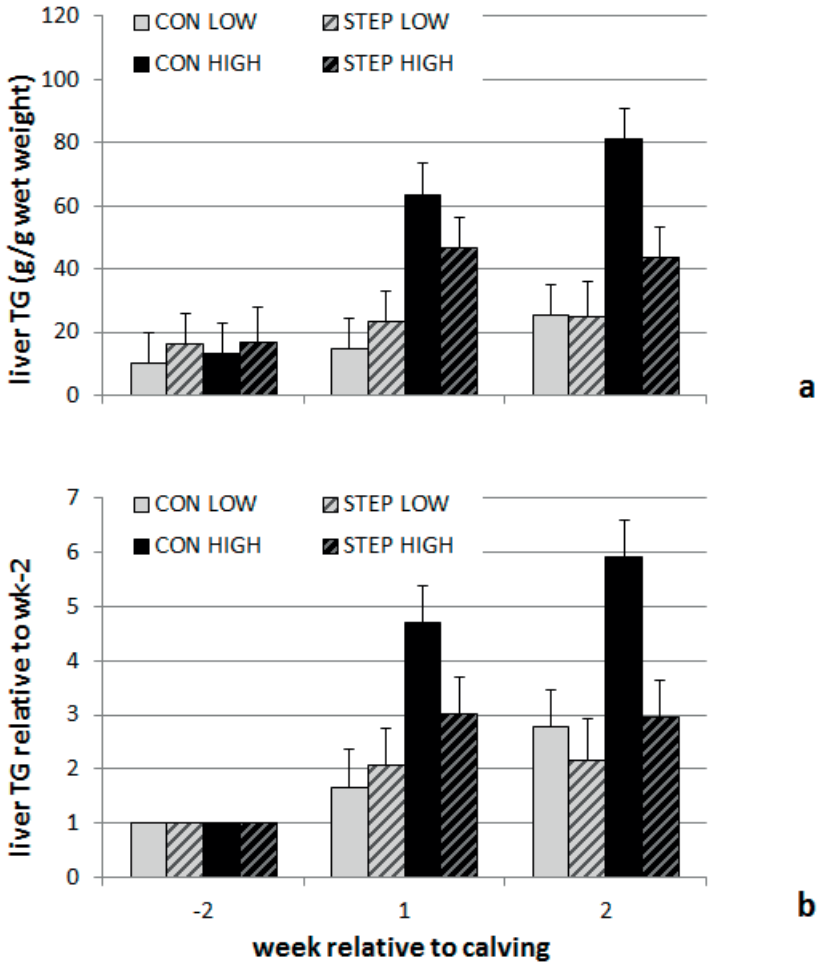

Figure 3 Liver triglyceride (TG) concentration for the subset of 16 cows with liver biopsies taken at week -2, week 1 and week 2 relative to calving with a) absolute TG concentration, in g/kg wet weight; and b) TG concentration expressed relative to week -2. Figures showing predicted means by REML analysis with SEM.

DISCUSSION

Our hypothesis that increasing physical activity will start fat mobilization prepartum is supported by the results of this proof of concept trial, which may reduce the risk for hepatic lipidosis in cows with a high BCS at dry-off. As expected, the increase in hepatic TG content postpartum was highest for cows in BCS class HIGH. The relative increase postpartum compared to week -2 prepartum tended to be reduced by exercise. This may be explained by a difference in plasma lipoprotein transport, as VLDL is presumed to be the main export route of TG from the liver, returning to the liver as LDL (Van Den Top *et al.*, 1995; Zom *et al.*, 2011; Newman, Mann, Nydam, Overton, & Behling-Kelly, 2016). Exercise did however not affect lipoprotein transport in the current trial. Lipid transportation by VLDL/LDL lipoproteins was low in plasma for all treatment groups after calving compared to their concentrations precalving. A reduction in VLDL and LDL cholesterol concentration immediately after calving when plasma NEFA concentrations are greater has also been reported by others (Gross, Kessler, Albrecht, & Bruckmaier, 2015; Newman *et al.*, 2016). Low lipid transport seems to be conflicting with the increased lipolysis early postpartum. This may be attributed to the homeorhetic changes in metabolism peripartum, such as the high priority for milk synthesis resulting in an increased utilization of lipids by the mammary gland. In a later stage of lactation, the metabolic adaptation changes in that feed restriction will directly result in increased VLDL and LDL cholesterol concentrations, in contrast to the situation in the periparturient period in the same cows (Gross *et al.*, 2015).

To our knowledge, the effects of exercise on lipoprotein metabolism or hepatic fat accumulation have not been studied before in dairy cattle. Initiation of lipolysis antepartum was expected to induce fat transport prepartum, earlier for exercised cows compared to non-exercised. This did however not affect plasma lipoprotein concentration as expected. The postpartum relative accumulation of hepatic fat of exercised cows tended to be lower, while the magnitude of lipolysis was comparable to non-exercised cows (no difference in plasma NEFA concentration postpartum). It may be hypothesized that the processing of lipids must have been improved in liver or in peripheral tissue. The actual rate of lipid transport from the liver may be underestimated by measuring plasma lipoprotein cholesterol, as this concentration represents the balance between liver excretion and peripheral uptake or utilization. Exercising cows prepartum likely induced lipolysis, increased lipid uptake and lipoprotein turnover, priming energy metabolism as suggested by Friggens *et al.* (2004) and could thereby reduce the risk for extreme hepatic fat accumulation in the first

weeks postpartum. In humans, exercise reduces intrahepatic liver accumulation through improved NEFA uptake in skeletal muscle and reduced NEFA uptake in liver without a change in VLDL secretion (Brouwers, Hesselink, Schrauwen, & Schrauwen-Hinderling, 2016).

Feed intake of STEP cows was reduced prepartum, which was not attributable to a difference in access time to the feed mixture in that feeding bins were inaccessible to all cows (STEP and CON) during training sessions. In a trial with heifers, forced exercise for 1.6 km/d until calving did not result in differences in feed intake; energy intake did reduce when forced exercise was continued for the first 10 d postcalving (Lamb, Barker, Anderson, & Walters, 1979). In contrast, forced exercise in multiparous animals for 3.2 or even 9.7 km/d did not affect prepartum DMI significantly (Anderson *et al.*, 1979). In a study with tied dairy cows, daily exercise of 2-3 km/d did not affect voluntary feed intake (Gustafson, 1993). The amount of exercise in the present trial was however greater and also involuntary, which may have reduced feed intake through stress. Exercise also increases heat production and rectal temperature in ruminants (Piguet, Bruckmaier, & Blum, 1994), which might be hypothesized to result in some degree of heat stress, thereby reducing feed intake (Koch, Lamp, Eslamizad, Weitzel, & Kuhla, 2016). Unfortunately, water intake was not measured in this trial but seemed to be increased directly after the training session upon return in the barn, especially on warm and sunny days (personal observation). This is suggestive for some degree of heat stress and could also have affected feed intake.

The forced physical activity in combination with lower feed intake prepartum resulted in a negative net energy balance from 5 weeks before calving in cows in the STEP group. Prolonged exercise by itself can also increase lipolysis and NEFA utilization in muscle as studied in sheep, where a walking speed of 4.5 km/h increased plasma NEFA from 0.1 to 0.9 after 45 min (Pethick, Harman, & Chong, 1987). These effects match with the increased concentration of plasma NEFA in cows in the STEP group before calving and further increasing towards calving for the cows with body condtion HIGH in the STEP group, indicating increased lipolysis. This concept may seem contradictory to results from field studies on dairy farms, where an increased NEFA concentration prepartum (>0.3 mmol/L) was found to be a risk factor for postpartum disease (Chapinal *et al.*, 2011). However, in our study NEFA concentration (and BHB concentration) was increased in healthy cows by physical activity, while cows in field studies with high NEFA prepartum may 1) already suffer from health problems before calving; and 2) have less metabolic capacity to start adaptation of fat metabolism sufficiently.

Postpartum, the calculated energy balance was negative for all cows, also indicated by a decreasing BCS, while DMI and milk yield level were comparable between groups. Cows in BCS class HIGH produced milk with a greater fat content, especially in the first weeks postpartum. High BCS is directly related to increased mobilization of adipose tissue and high milk fat excretion (Rukkwamsuk, Wensing, & Kruip, 1999), which was also confirmed in our study. HIGH cows had increased plasma NEFA concentrations during early postpartum compared with LOW cows. We found no effect of exercise on NEFA concentration postpartum. However, exercised cows (group STEP) had lower plasma BHB and lower milk fat content after calving, which suggests a carry-over effect of prepartum exercise on lipid mobilization and processing in early lactation. This reduction in milk fat postpartum is similar to results reported with multiparous animals subjected to prepartum exercise (Anderson *et al.*, 1979), but not with heifers (Lamb *et al.*, 1979).

The application of forced exercise for dry cows as used in this study is not immediately feasible on dairy farms, due to time- and labor commitments. Simply providing access to pasture does not directly stimulate cows to increase their activity sufficiently compared to forced exercise (Black, Van Amstel, & Krawczel, 2017). Cows will still need to be stimulated to exercise, for example by a daily return walk between barn and paddock, or by providing water and feed at different places in the exercise area.

CONCLUSIONS

A substantial amount of physical exercise in the dry period in this study affected fat metabolism of dairy cows in the prepartum period and these effects carried over into the postpartum period. Exercise can induce lipid mobilization and utilization prepartum and may thereby start metabolic adaptation of dairy cows before the onset of milk production. Other adaptive mechanisms related to the carbohydrate metabolism or endocrine regulation may also be involved, which were not measured in this study. The hypothesis that physical activity prepartum will reduce the risk on excessive fat mobilization postpartum is most promising for cows with a greater BCS at dry-off. Further research is needed to confirm our results in a larger group of animals and to improve our understanding of the underlying physiological pathways.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

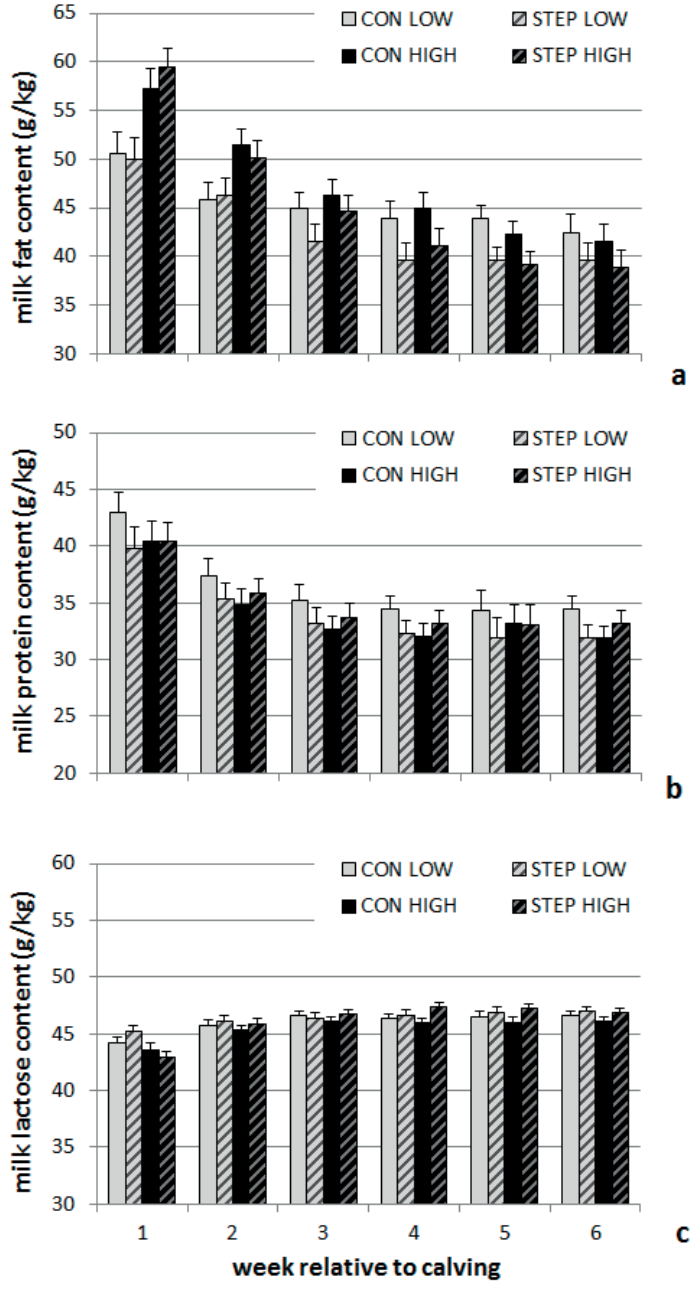

Figure S1. Average milk content (g/kg) of a) fat; b) protein; and c) lactose for each treatment group during the experiment. Figures showing predicted means by REML analysis with SEM.

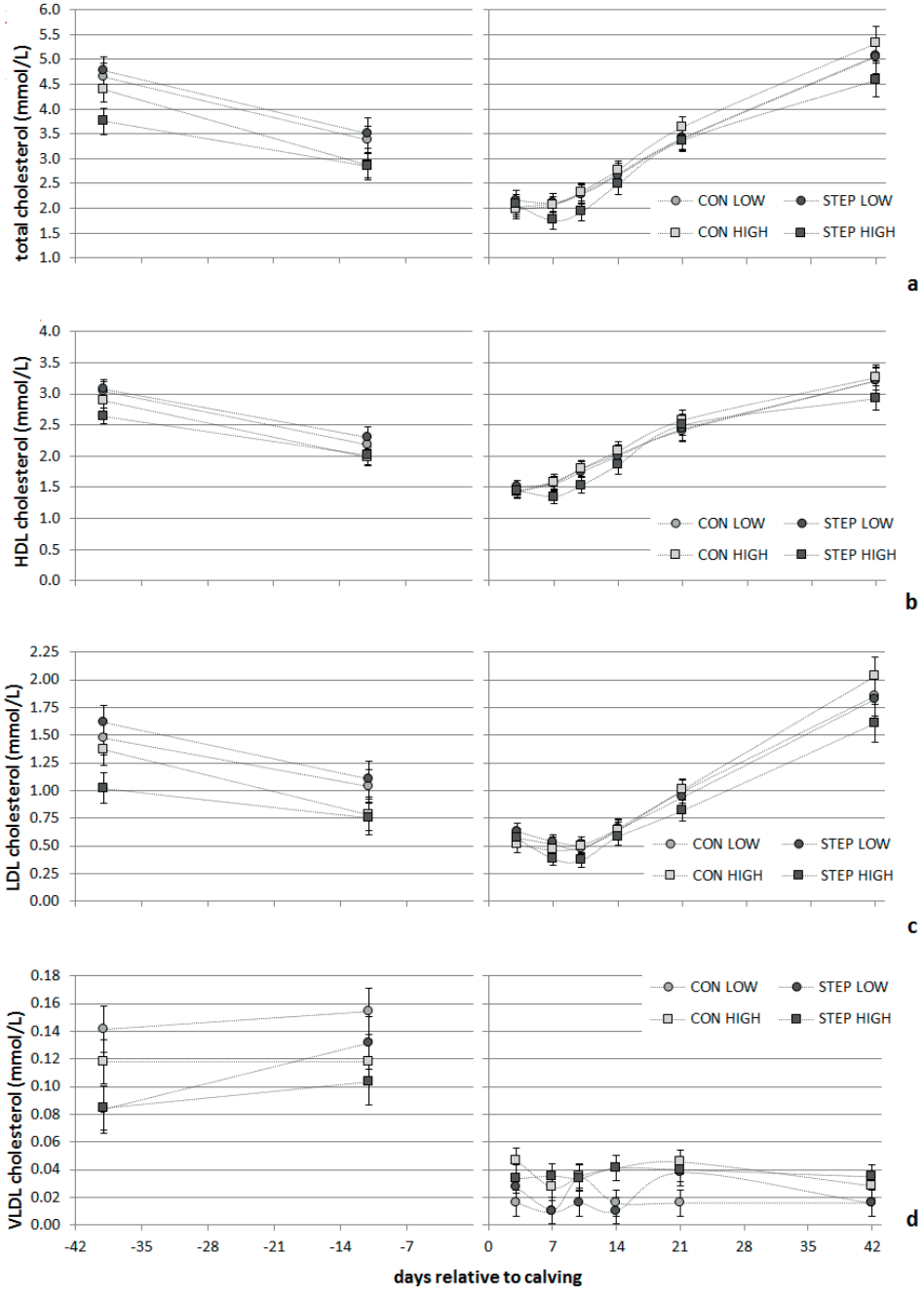

Figure S2. Average plasma concentration (in mmol/L) of a) total cholesterol; b) HDL cholesterol; c) LDL cholesterol; and d) VLDL cholesterol. Figures showing predicted means by REML analysis with SEM.


4

Chapter 4

Restricting energy intake of dairy cows in the dry period with grass-based rations

R. M. A. Goselink, R. E. Weurding, G. van Duinkerken, and J. T. Schonewille

ABSTRACT

In dairy cattle the prevention of excessive energy intake in the dry period has shown to be important for optimal metabolic health and performance postpartum. The majority of studies investigating energy intake in the dry period have used experimental rations based on corn silage and alfalfa hay or silage. The aim of the present study was to validate the effect of reducing prepartum energy intake under grass-rich, low-starch dietary conditions. We compared an intake level at 150% of energy requirements with 100% of energy requirements realized by either restricting intake or feeding a low energy ration ad libitum.

Overfeeding a grass-based ration in terms of energy intake resulted in an increased BCS and bodyweight prepartum. Unexpectedly, postpartum feed intake was not reduced compared to the other groups. Postpartum liver TG accumulation was greatest after overfeeding in the dry period, showing an imbalance in lipid processing and a high risk for metabolic disorders. Restricting feed intake and rumen fill prepartum while maintaining energy intake at requirement did not limit DMI nor induce health disorders in the first weeks postpartum compared to ad libitum feeding. Even though postpartum intake or performance may not seem different, dairy cows are ideally fed at or just below their energy requirements during the dry period for a healthy start of the lactation.

INTRODUCTION

Various studies have demonstrated the benefit of restricting energy intake prepartum relative to overfeeding energy above requirements to support metabolic health postpartum (Douglas et al., 2006; Janovick, & Drackley, 2010; Mann et al., 2015). Preventing excessive energy intake prepartum can improve DMI in early lactation (Holcomb et al., 2001; Douglas et al., 2006; Janovick, & Drackley, 2010). It can also reduce milk fat yield, thereby improving the energy balance (Janovick, & Drackley, 2010; Bjerre-Herpoth et al., 2014; Richards et al. 2020) and reducing the incidence of ketosis postpartum (Mann et al., 2015). Within the dry period, prevention of excessive energy intake is specifically important in the first weeks (the far-off period) relative to the final 2-3 weeks before calving (the close-up period). High energy intake in the close-up period does not further impair nor improve postpartum performance (Dann et al., 2006; Richards et al., 2020, Vasquez et al., 2021).

In most studies investigating energy levels in the dry period, the reduction in feed intake was often realized by restricting total intake compared to a control group fed ad libitum. In practice, dairy farmers and nutritionists are hesitating to feed a prepartum ration restricted. Next to some practical difficulties regarding feeding individual animals restricted under group-housing conditions, a widely shared idea is that rumen fill of dry cows should be maximized – both for health reasons as well as to maximize rumen feed intake immediately after calving. The scientific support to this theory is however unavailable. Low rumen fill has been suggested to increase the risk for a left displaced abomasum (Shaver, 1997) but the causal relationship between DMI, rumen fill and abomasal displacements is still unclear. Two studies investigated rumen fill in the transition period, where no correlation was found between rumen volume and feed intake postpartum (Stanley et al., 1993; Park et al., 2011).

To our current knowledge, only few studies compared the effect of reducing energy intake prepartum by either feeding a high energy ration restricted, or feeding a low energy ration ad libitum. In the study of Dann et al. (2006), during the far-off period a control ration was fed to meet but not exceed requirements at ad libitum intake, relative to a high nutrient density ration fed either ad libitum or restricted to realize 150% or 80% of NRC recommendations for NEL, respectively. Cows were then randomly allocated to two different rations in the close-up period, being the 150% (ad libitum) or 80% (restricted) ration, thereby missing a 100% treatment group in the final three weeks before calving (Dann et al., 2006). In the study of Janovick and Drackley (2010), comparable rations

were fed throughout the entire dry period. A high energy ration was fed either ad libitum or restricted to realize 150% or 80% of NRC recommendations for NEL, compared to a ration at reduced energy density fed ad libitum to meet NE requirements (Janovick and Drackley, 2010). Both studies showed overfeeding energy in the dry period resulted in reduced feed intake in the first two weeks postpartum compared to reducing energy intake either by restricting intake or reducing the energy density of the dry cow ration (Dann et al., 2006; Janovick and Drackley, 2010).

The majority of studies investigating energy intake in the dry period, including the studies of Dann et al. (2006) and Janovick and Drackley (2010), use experimental rations mainly based on corn silage and alfalfa hay or silage. In grass-based dairy systems as present in Northwest Europe and New Zealand, the most important roughage fed to dairy cows is grass - either fresh or ensiled - due to the favorable climate and legislative circumstances stimulating local grass production (Klootwijk et al., 2016). This will affect the nutrient composition of the dry cow rations, resulting in lower starch levels compared to the rations fed in earlier studies. Two trials with 150 transition cows each under grazing circumstances showed that a small restriction of energy requirements (to ~90%) from 3 weeks before calving was beneficial in terms of metabolic health (Roche et al., 2015; Roche et al., 2017). Individual energy balances were however unavailable in these studies as feed intake had to be estimated per treatment group, based on the difference between pre- and postgrazing mass. In a pilot study with only 16 animals, overfeeding of grass silage at 141% of NE recommendations resulted in beneficial effects regarding glucose metabolism compared to a mixture of grass silage and wheat straw realizing 108% of NE recommendations (Salin et al., 2018).

The aim of the present study is to validate the effect of reducing prepartum energy intake under grass-rich dietary conditions with dry cow rations low in starch. We compared an intake level at 150% of energy requirements (high energy ration fed ad libitum) with an intake level at 100% of energy requirements which was realized by either restricting intake of the high energy ration or feeding a low energy ration ad libitum.

We hypothesize that overfeeding a grass-based ration in terms of energy intake will result in an increase in BCS prepartum and a strong lipolysis postpartum, reaching higher plasma NEFA, milk fat content and liver TG concentration as well as a reduced DMI in early lactation. Additionally we expect that restricting feed intake and thereby rumen fill in the dry period will not limit DMI in the first weeks postpartum compared to high energy or low energy rations fed ad libitum.

MATERIALS AND METHODS

Animals and treatments

All experimental protocols and interventions were approved by the Ethical Committee on Animal Experiments of the Animal Sciences Group of Wageningen University & Research, Wageningen, The Netherlands. The experiment comprised 48 pregnant Holstein-Friesian cows entering the second or greater lactation selected from our 450-cow research dairy farm in Lelystad, The Netherlands. The animals were selected based on parity and expected calving date. The selected cows were blocked in groups of three by similarities in parity, previous 305 day milk production, BCS and expected calving date resulting in 16 blocks. Within each block, cows were randomly allocated to one of three treatments relating to the dietary management of the dry period: a high energy ration fed ad libitum (**HE-AL**), the same ration fed restricted (**HE-RES**) to match calculated energy requirements of maximally 60 MJ of NEL/cow (CVB, 2016), or a low energy ration fed ad libitum (**LE-AL**) calculated to match the energy intake of HE-RES. After calving, all treatment groups were fed the same lactation ration.

Cows were dried-off weekly according to the farm protocol at approximately 55 d (± 3 d) before expected calving date and were enrolled a week later in the experiment. Until calving, all cows were fed their respective treatment diets. After calving, all cows received the same lactation ration and were monitored for 8 wk after calving.

Cows were housed in a cubicle shed with 1 cubicle available per cow. During the dry period cows were kept in separate treatment groups; post-calving animals were housed together in a lactation group.

Rations and feeding management

Fresh feed mixtures were provided daily between 0800 and 0900 h. From 7 wk before the expected calving date until calving, cows in groups HE-AL and HE-RES received the High energy ration and cows in group LE-AL the Low energy ration (Table 1).

Table 1 Ingredients, chemical composition and feeding value of the roughage feed mixtures fed during the dry period (High energy for groups HE-AL and HE-RES, Low energy for group LE-RES) and during the lactation period.

	Dry period		**Lactation**
	High energy	**Low energy**	
Ingredient (% of total DM)			
Wilted grass silage[1]	60.3	38.4	53.4
Corn silage[2]	27.4	17.1	24.4
Chopped wheat straw	3.0	30.0	-
Chopped grass seed straw	-	-	7.7
Premix dry_H	9.2		
Premix dry_L		14.4	
Premix lactation			14.3
Chemical composition (g/kg DM)			
Crude protein	118	117	138
Crude fat	33	27	32
Crude ash	107	100	99
Sugars	29	27	31
Starch	111	76	104
NDF	413	501	418
ADF	257	321	261
Feeding value			
NE_L (MJ/kg DM)	6.33	5.38	6.26
DVE[3] (g/kg DM)	63	55	75
OEB[4] (g/kg DM)	7	8	17
FOM[5] (g/kg DM)	549	495	536

[1] Wilted grass silage with a DM content of 332 g/kg, chemical composition (in % of DM): Crude protein 14.0, Crude fibre 24.9, Crude fat 3.7, Crude ash 12.1, Sugars 3.2, NDF 43.9 and ADF 28.0, delivering FOM 578 g/kg DM and NE_L of 6.48 MJ/kg DM.
[2] Corn silage with a DM content of 352 g/kg, chemical composition (in % of DM): Crude protein 6.4, Crude fibre 19.4, Crude fat 2.8, Crude ash 4.8, Starch 34.4, NDF 39.5 and ADF 22.3, and delivering FOM 498 g/kg DM and NE_L of 6.45 MJ/kg DM.
[3] Intestinal digestible protein (Tamminga et al., 1994).
[4] Rumen-degradable protein balance (Tamminga et al., 1994).
[5] Fermentable organic matter.

After calving, all cows were fed the lactation feed mixture ad libitum (Table 1). Throughout the entire study, the cows had access to troughs with individual transponder-controlled access gates (Roughage Intake Control system, Hokofarm Group, Marknesse, The Netherlands). The troughs were equipped with an electronic balance and allowed automatic registration of individual feed intakes. Except during feeding time (from 0800 to 0900 h), cows had continuous access to the feed troughs. Overall, the amount of feed mixture that was supplied to

the cows was 110% of actual intake to ensure maximum (restricted) feed intake per cow. The cows were fed two compound concentrates individually, using transponder-controlled concentrate dispensers which made the individual allowance available in equal portions over six 4-h periods. One of those concentrates was also supplied at milking (0.5 kg per milking, at 0545 and 1700 h). The amount of concentrates supplied was increased after calving up to 9.0 kg per day at d 28 and maintained at that level until the end of the experiment (Table 2). All cows had unrestricted access to fresh water.

Table 2 Chemical composition, feeding value and feeding scheme of concentrates postpartum (all treatment groups).

	Concentrate 1	**Concentrate 2**
Chemical composition (g/kg DM)		
Dry matter (g/kg)	891	888
Crude protein	204	191
Crude fat	58	44
Ashes	76	68
Sugars	84	92
Starch	216	285
NDF	256	242
ADF	128	133
Feeding value		
NEL (MJ/kg DM)	7.82	7.94
DVE[1] (g/kg DM)	128	140
OEB[2] (g/kg DM)	32	3
FOM[3] (g/kg DM)	613	512
Feeding scheme (kg/d)		
d 0 to d 7	1.0	0.5
d 7 to d 12	1.5 to 2.5	0.5 to 1.5
d 12 to d 28	2.5 to 5.0	1.5 to 4.0
d 28 to d 56	5.0	4.0

[1] Intestinal digestible protein (Tamminga et al., 1994).
[2] Rumen-degradable protein balance (Tamminga et al., 1994).
[3] Fermentable organic matter.

Feed sampling and feed analysis

Feed mixtures were sampled daily for DM analysis. The DM content was determined after oven drying at 104°C during 36 h. Grass and corn silage were sampled three times per week and other feed mixture ingredients as well as the compound concentrates were sampled weekly; all samples were stored at –20°C. For grass and corn silage, samples of 4-5 consecutive weeks were pooled into 1 composite sample;

for concentrates and wheat straw, all samples were pooled in two samples per feedstuff. The pooled samples were used for chemical analysis and determination of the feeding values at the Agricultural Laboratory Northern Netherlands (ALNN, Ferwert, the Netherlands). Briefly, the crude fat content was determined gravimetrically as the ether extract (ISO 6492; ISO, 1999) and crude ash content after incineration at 550°C (ISO 5984; ISO, 2002). Content of NDF and ADF were determined according to Van Soest, Robertson, & Lewis (1991) and expressed without residual ash. Crude protein was calculated as 6.25 × N-Kjeldahl (ISO 5983; ISO, 2005) and sugar concentrations were determined as described by Van Vuuren, Van der Koelen, Valk & De Visser (1993). Starch was released by heating in a boiling water bath in the presence of 2 N HCl (Cone, 1991) after which starch concentration was determined using the amyloglucosidase method (ISO 15914; ISO, 2004). The NEL, intestinal digestible protein (DVE), rumen-degradable protein balance (OEB) and fermentable organic matter (FOM) were calculated according to the guidelines from the Central Bureau for Livestock Feeding (CVB, 2016).

Milk yield and milk composition

Cows were milked twice daily around 0545 and 1700 h with milk weights recorded automatically at each milking. Weekly, milk samples of each cow were taken at 4 consecutive milkings (2 morning milkings and 2 afternoon milkings). Both morning milk samples were pooled to 1 composite sample; afternoon milk samples were processed likewise. The composite morning and afternoon milk samples were analyzed for fat, protein, and lactose concentrations by Qlip (Zutphen, the Netherlands) using a Foss MilkoScan infrared automatic analyzer (Foss Electric, Hillerød, Denmark). Weighted means were calculated from the recorded morning and afternoon milk weights and the analyses of the composite morning and afternoon milk samples.

Body weight, body condition score and energy balance

The precalving BW was recorded weekly at the same time and postcalving BW was automatically recorded at the same weighing scale, twice per day at entry into the milking parlor. Cows were scored in wk -8, -3, 1, 4 and 8 for body condition from 1 (thin) to 5 (fat) with 0.25 point increments according to Ferguson, Galligan, & Thomsen (1994). Energy balance was calculated as described by Van Knegsel et al. (2007) as the difference between net energy for lactation (NEL) supplied with feed and the NEL required for gestation, maintenance and milk production. Calculations were based on the stage of gestation, average feed intake, milk yield, milk composition and body weight results of that week.

Blood collection and analysis

The first 8 blocks of cows (based on expected calving dates) were selected for additional blood and liver sampling. Blood samples were taken three times prepartum, at 3, 2 and 1 wk before calving; postpartum blood was sampled within 24h after calving (d 0), and weekly for the first 8 weeks of lactation. Blood samples were collected from the tail vein (vena cauda) using lithium-heparin-coated tubes (Vacuette, type 455084, Greiner Bio-One, Frickenhausen, Germany) for analyses of beta-hydroxybutyrate (**BHB**) and NEFA. Immediately after collection, blood samples were placed in ice water and centrifuged at 1,500 × g for 10 min within 1 h after collection. Subsequently, 1 mL of blood plasma was transferred to a vial and stored at −20°C until analysis. NEFA were determined using a colorimetric assay and BHB by an enzymatic method (both kits from Randox Laboratories Ltd, Crumlin, United Kingdom).

Liver biopsies

Liver biopsies were taken 8 and 2 wk before calving and in wk 1, 3 and 5 after calving according to the method described by Zom et al. (2011). Briefly, a skin incision was made at the 11th intercostal space after local anesthesia and a biopsy needle was inserted to collect ~2 g of liver tissue for analysis. Samples were immediately frozen in liquid N and stored at −80°C until analysis. Before analyses, liver samples were thawed and adhered water was removed using paper tissues. The concentrations of liver TG were determined using enzymatic hydrolysis of triglycerides with lipase into glycerol and fatty acids using the Triglycerides LiquiColor Mono test kit (Instruchemie BV) by photometric analysis at 550 nm (HumaLyzer 3000, Human Diagnostics). Categories of fatty liver were defined as described by Bobe, Young, & Beitz (2004) based on liver TG concentrations: normal < 10, mild 10 to 50, moderate 50 to 100 and severe > 100 mg/g wet weight.

Calculations and statistical analysis

FPCM was calculated according to CVB (2016):

FPCM (kg) = milk (kg) × (0.337 + 0.116 × fat (%) + 0.06 × protein (%))

with the weekly determined contents of fat and protein, and the mean daily milk production of each week.

Net energy requirements (NE_{req}) for maintenance and milk production were also calculated according to CVB (2016) in Dutch VEM energy units per day:

NE_{req} for maintenance (VEM/d) = 42.4 × BW0.75

NE_{req} for milk production (VEM/d) = (442 × FPCM (kg)) × (1 + FPCM (kg) – 15) × 0.00165)

The NE_{req} for gestation was calculated according to CVB (2000) in Dutch VEM energy units per day as:

NE_{req} for gestation = 0.025 × EBW (kg) × ((0.0201 × e-0.0000576*DIP) × (10151.665-151.64 × e (-0.0000576 × DIP)) × 0.6/0.15 × 1000/6.9

Where EBW = expected birth weight in kg, defined at 44 kg in the present study and DIP = days in pregnancy.

Finally, NE in MJ was calculated by transforming NE in VEM:

NE_L (MJ) = NE_L (VEM) / 1000 × 6.9

Energy balance was calculated per week as the difference between NE intake and total NE requirements for maintenance, milk production, and pregnancy and expressed in kJ/kg $BW^{0.75}$ per day.

Daily feed intake, milk yield, BW and energy balance were averaged per cow per week relative to calving. Blood and liver results were available for a subset of 21 cows (from 7 blocks).

A mixed model analysis with repeated measures was performed using the REML procedure in Genstat 19th edition (2018). Each parameter was modelled over time for each individual cow using a random coefficient regression model, i.e.

$$Y_{ijk} = \mu + \varepsilon_i + T_j + W_k + T_j \times W_k + \varepsilon_{i\cdot k}$$

where Y_{ijk} = dependent variable; μ = overall mean; ε_i = random effect of cow (where $\varepsilon_i \sim N(0,\sigma^2_i)$; T_j = effect of treatment (HE-AL, LE-AL or HE-RES); W_k = effect of time (prepartum -8 to -1; postpartum 1 to 8); $T_j \times W_k$ = interaction between treatment and time; and $\varepsilon_{i\cdot k}$ = residual error (or cow × time). The necessity of an auto-regressive function as well as heterogeneity were tested and judged by the difference in deviance of each model with the change in degrees of freedom in a chi squared distribution. Auto-regressive function improved model deviance of all parameters; heterogeneity was not always relevant but for most parameters outside the model, as displayed with the model components in Supporting Information Table S1. A visual check for outliers was performed after which the model was tested with and without the potential outlier to see if conclusions changed; this was not the case and no outliers were deleted.

RESULTS

Forty seven cows finished the experiment. One cow was excluded after calving due to an accident in week 3 resulting in a leg injury (group HE-RES). Prepartum blood samples were missing for 2 cows in 1 block; this block was excluded from further analysis.

Prepartum period

As planned, the average DMI of the cows fed restricted was much lower in the prepartum period than DMI of cows fed ad libitum (interaction of treatment x week, P=0.003). Restricted feeding resulted in a relatively constant DMI during the 8 weeks before calving, without a decline in intake as seen for HE-AL and LE-AL in the last three weeks (Figure 1). Total DMI of cows in group LE-AL was higher than expected, resulting in a higher NEL intake than planned. Average intake of NEL during the final 8 weeks prepartum compared to calculated requirements was 175%, 145% and 115% for HE-AL, LE-AL and HE-RES, respectively. For cows fed restricted, the average net energy balance declined to 0 at calving (Figure 1). Body weight of both HE-AL and LE-AL increased throughout the dry period, while it stayed relatively constant for cows in group HE-RES (Table 3, Figure 1). Body condition score increased for both ad libitum fed groups while restricted cows maintained a relatively stable BCS in the dry period (Figure 2).

Table 3 Average performance prepartum during the final 8 weeks before parturition.

Item	Treatments			SED	P-value[1]	
	HE-AL (n = 16)	LE-AL (n = 16)	HE-RES (n = 16)		TRT[2]	TRT × week
DMI (kg/d)	14.6[a]	14.0[a]	9.2[b]	0.44	<0.001	0.003
Grass silage	8.7	5.4	5.6			
Corn silage	4.0	2.4	2.5			
Wheat straw	0.5	4.2	0.3			
Premix dry_H	1.4	-	0.8			
Premix dry_L	-	2.0	-			
Body weight (kg)	731[a]	729[a]	689[b]	15.5	<0.001	0.005
NE_L balance (MJ/kg $BW^{0.75}$)	0.28[a]	0.16[b]	0.05[c]	0.018	<0.001	0.006
Plasma NEFA (mmol/L)	0.20[a]	0.21[a]	0.47[b]	0.066	<0.001	0.090
Plasma BHB (mmol/L)	0.56[a]	0.43[b]	0.51[a]	0.037	0.010	0.369

[1]week effects were significant for all parameters (P<0.001)
[2]TRT = treatment effect

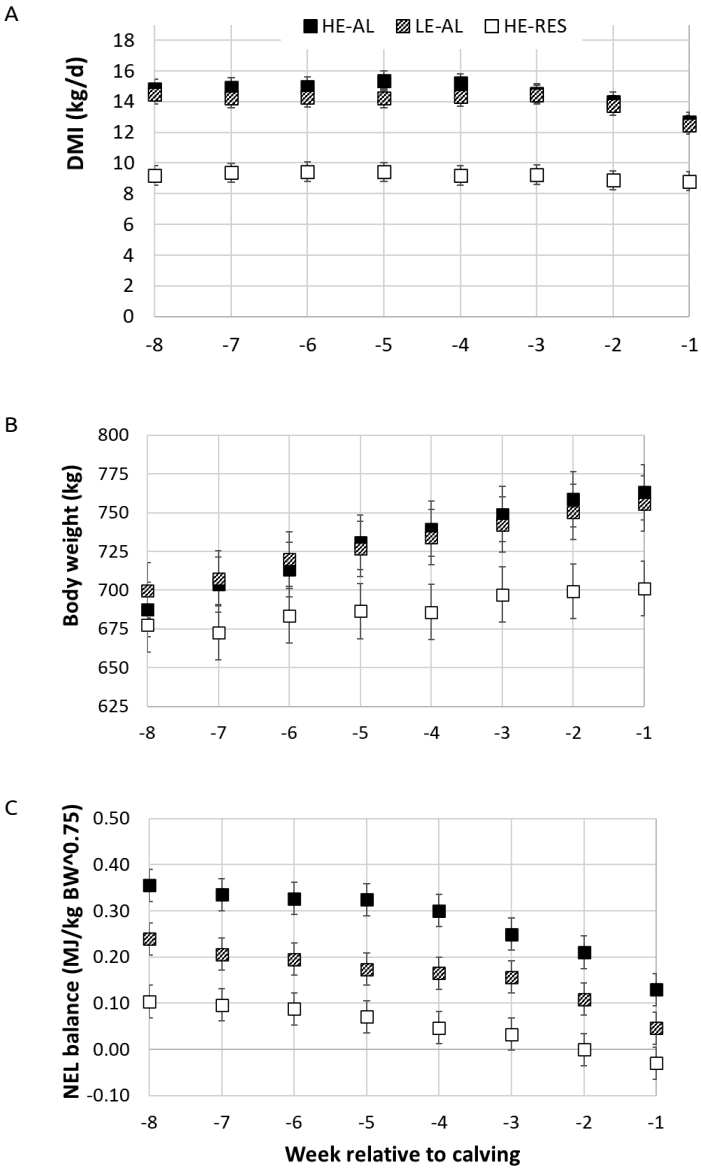

Figure 1 Average dry matter intake (A), body weight (B) and net energy balance (C) for cows fed a high energy ration ad libitum (HE-AL), a low energy ration ad libitum (LE-AL) or a high energy ration with restricted intake to match requirements (HE-RES) in the dry period. Figures showing predicted means by REML analysis with SED during the last 8 weeks before calving.

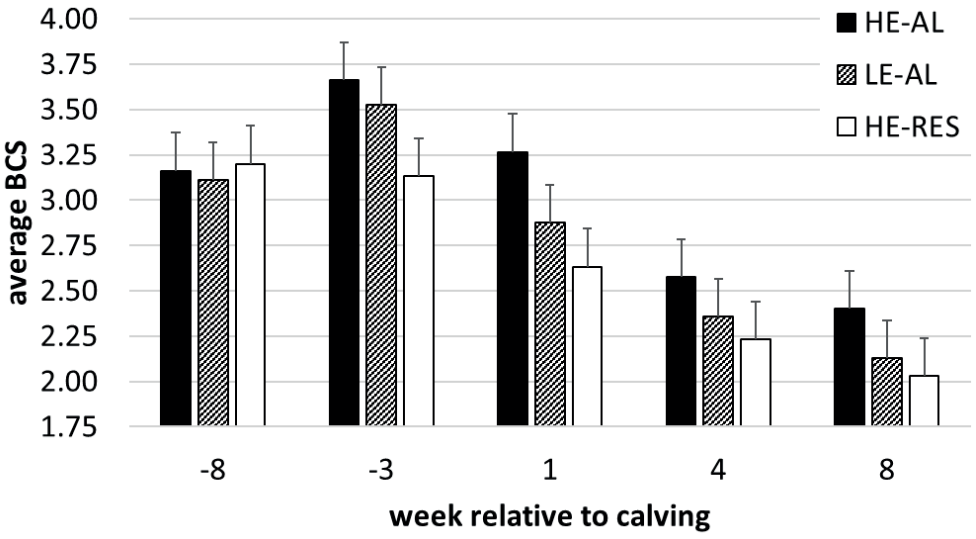

Figure 2 Average BCS for cows fed a high energy ration ad libitum (HE-AL), a low energy ration ad libitum (LE-AL) or a high energy ration with restricted intake to match requirements (HE-RES) in the dry period. Figures showing predicted means in the respective weeks relative to calving by REML analysis with SED. Different letters show significant differences between treatment groups in that relative week.

Postpartum period

After calving there was no significant effect of the precalving treatment on average dry matter intake, milk yield or milk composition. (Table 4). The net energy balance was negative throughout the first 8 weeks for all groups without a significant effect of the prepartum dietary treatment (Table 4). Body condition score decreased after calving for all groups as expected based on the negative energy balance of early lactation (Figure 2). At the end of the trial in week 8 postcalving, BCS was still higher for HE-AL cows compared to HE-RES cows (Figure 2).

Blood and liver parameters

Prepartum, plasma NEFA was increased for cows in group HE-RES compared to cows fed ad libitum while plasma BHB was lowest for cows fed a low energy ration ad libitum (Table 3). Postpartum, there were no significant effects of treatments on plasma NEFA or BHB concentrations (Table 4).

Table 4 Average performance postpartum.

Item	Treatments			SED	P-value[1]	
	HE-AL (n = 16)	LE-AL (n = 16)	HE-RES (n = 15)		TRT[2]	TRT × week
DMI (kg/d)	20.3	20.1	19.5	0.65	0.395	0.807
Milk yield (kg/d)	41.5	40.8	39.5	1.48	0.295	0.712
FPCM (kg/d)	45.6^{a}	43.6^{ab}	42.0^{b}	1.50	0.102	0.521
Milk fat (g/100g)	4.96	4.72	4.66	0.146	0.412	0.400
Milk protein (g/100g)	3.41	3.37	3.45	0.085	0.631	0.530
Milk lactose (g/100g)	4.46	4.50	4.52	0.035	0.105	0.190
Body weight (kg)	647	639	614	18.0	0.497	0.083
NE_L balance (MJ/kg $BW^{0.75}$)	-0.39	-0.35	-0.33	0.044	0.450	0.642
Plasma NEFA (mmol/L)	0.57	0.49	0.53	0.139	0.625	0.900
Plasma BHB (mmol/L)	0.90	0.80	0.83	0.142	0.657	0.581

[1]week effects were significant for all parameters (P<0.001)
[2]TRT = treatment effect

Liver TG concentration increased for all cows after calving but was significantly higher for group HE-AL compared to LE-AL and HE-RES (treatment x time effect, P=0.030). Moreover, the incidence of severe hepatic lipidosis (liver TG content > 100 mg/g wet weight) was 5/7 for HE-AL, 2/7 for LE-AL and 1/7 for HE-RES.

The results of the average net energy balance, plasma NEFA and plasma BHB concentrations are combined in Table 5 with the results of the liver biopsies taken in the same week for TG analysis. During the prepartum period, the differences in net energy balance in wk -2 might be linked to the increased NEFA concentration but without a difference in liver TG concentration (Table 5). In the postpartum period however, cows in group HE-AL seem to be metabolically challenged as shown by the plasma NEFA, BHB and liver TG concentrations in the first week after calving without any differences in net energy balance between the three groups (Table 5).

Table 5 Energy balance, plasma non-esterified fatty acids (NEFA), plasma beta-hydroxybutyric acid (BHB) and liver triacylglycerol (TG) in week -2, 1, 3 and 5 relative to calving.

Item	Treatment	Week relative to calving				SED	P – value[1]	
		-2	1	3	5		TRT[2]	TRT x wk
Energy balance (kJ/kg $BW^{0.75}$)	HE-AL	0.23^{ax}	-0.53^{b}	-0.45^{b}	-0.29^{c}	0.081	<0.001	0.007
	LE-AL	0.10^{ay}	-0.47^{b}	-0.42^{b}	-0.30^{c}			
	HE-RES	-0.01^{az}	-0.50^{b}	-0.34^{c}	-0.30^{c}			
NEFA (mmol/L)	HE-AL	0.37^{ax}	1.06^{b}	0.67^{ab}	0.52^{a}	0.194	0.001	0.536
	LE-AL	0.26^{ax}	0.86^{b}	0.62^{bc}	0.43^{ac}			
	HE-RES	0.63^{y}	0.78	0.75	0.58			
BHB (mmol/L)	HE-AL	0.41^{a}	1.44^{b}	0.97^{c}	0.85^{c}	0.198	0.370	0.152
	LE-AL	0.38^{a}	0.87^{b}	0.91^{b}	0.83^{b}			
	HE-RES	0.42^{a}	0.94^{bc}	1.05^{b}	0.77^{c}			
Liver TG (mg/g wet weight)	HE-AL	14^{a}	102^{b}	117^{bx}	77^{cx}	19.2	0.009	0.054
	LE-AL	18^{a}	56^{b}	62^{by}	51^{bxy}			
	HE-RES	15^{a}	59^{b}	45^{by}	31^{acy}			

[1] week effects were significant for all parameters (P<0.001)
[2] TRT = treatment effect

DISCUSSION

Our hypothesis that overfeeding a grass-based ration in terms of energy intake results in an increase in BCS prepartum, a strong lipolysis and increased concentration of liver TG postpartum was confirmed by the results of the present trial. This is in line with previous studies with corn-based, starch-rich rations (Dann et al., 2006; Janovick, & Drackley, 2010; Mann et al., 2015).

Unexpectedly, cows overfed at a net energy intake ~175% of requirements in the dry period did not show a decreased DMI in the first wks postpartum compared to the other two treatment groups. Around 70% of the cows in group HE-AL had severe hepatic lipidosis (liver TG content >100 mg/g wet weight) in wk 3 or 5 postpartum which is known to be associated with reduced feed intake and milk production (Bobe, Young, & Beitz, 2004). The effect of energy intake prepartum on DMI postpartum varies among studies however. Some studies demonstrate a reduced DMI after overfeeding (Dann et al., 2006; Douglas et al., 2006; Janovick, & Drackley, 2010) while others do not find differences in DMI (Mann et al., 2015, Richards et al. 2020, Akhtar et al., 2021). The variability in effects on DMI postpartum may be related to differences in the experimental design of each study. Restricting energy intake to a level below requirements relative to overfeeding seems te be more effective in improving DMI (Dann et al., 2006; Douglas et al., 2006; Janovick, & Drackley, 2010), compared to studies where all

energy intake levels are at or above requirements (Mann et al., 2015, Richards et al. 2020, Akhtar et al., 2021). Moreover, studies with multiparous cows show a stronger negative effect of overfeeding than studies with primiparous cows included (Janovick & Drackley, 2010). The type of nutrients in grass-based diets may be relevant for these contrasting results. Overfeeding a ration of grass silage realizing 141% compared to 108% of ME recommendations even seemed to benefit dry cows regarding their early postpartum metabolic state (Salin et al., 2018). With TMR diets, overfeeding in the far-off period was a risk for metabolic disorders postpartum while the plane of energy in the close-up period was less relevant (Dann et al., 2006); in studies with grazing cows an energy restriction specifically in the close-up period improved metabolic health postpartum (Roche et al., 2015; Roche et al., 2017).

Our dietary strategy after calving with a partially mixed ration may also have influenced the variability in DMI between groups. All cows received a high quality roughage mixture containing ~53% well-digestible grass silage ad libitum (Table 1), and additionally an increasing amount of concentrates fed in concentrate dispensers (Table 2). Concentrate allowance was kept stable in wk 1 at 1.5 kg/d, slowly increasing afterwards to a total of 9 kg/d by the end of wk 4. It can be hypothesized that the differences in DMI may have been larger if the ration was fed as a totally mixed ration ad libitum to all animals.

We also hypothesized that restricting feed intake in the dry period will not limit DMI in the first weeks postpartum compared to feeding a high or low energy ration ad libitum, which was confirmed by the results of HE-RES compared to HE-AL and LE-AL. Dairy farmers and nutritionists in practice strive for maximal rumen fill in dry cows, to prevent an extreme reduction of rumen volume during the dry period. It could be hypothesized that this reduced rumen capacity prepartum results in a lower DMI in the first weeks postcalving. To our knowledge, the only two studies investigating the role of rumen fill in the transition period did not find a correlation between rumen volume and feed intake postpartum. Stanley et al. (1993) measured rumen capacity and showed that at 22 days postpartum, ruminal capacity was only 5% greater than at 61 d before calving and postpartum DMI was 69% greater than DMI measured 61 d before calving. Park et al. (2011) measured rumen capacity and showed that with ad libitum feeding, capacity is not limiting feed intake in the dry period or lactation. Next to studies measuring rumen capacity, Bertics et al. (1992) tried force-feeding dry cows precalving to maximize rumen fill, but force-fed cows still showed the same DMI depression in the first days postpartum as control cows. Postpartum DMI was also comparable in two studies comparing restricted feed intake of a high energy diet with ad

libitum feeding of a high or a low energy diet (Dann et al., 2006; Janovick and Drackley, 2010). Factors other than ruminal capacity and distension must play a (more) critical role in causing intake changes during the periparturient period.

A low rumen fill was previously related to an increased risk for a left displaced abomasum (Shaver, 1997), but in the present trial there were no abomasal displacements in any of the treatment groups.

Restricting energy intake in the dry period in practice will have disadvantages in terms of welfare. A shorter eating time than necessary will reduce welfare, simply because cows need oral manipulation time (Lindstrom and Redbo, 2000). Feed restriction also imposes a general (social) stress factor on cows related to competition and agonistic behaviour among cows - that can increase the risk for disease (Proudfoot et al., 2018). Feeding a low energy ration is therefore a better alternative under practical situations, preventing overfeeding while reducing the social stress of restricted feeding.

CONCLUSIONS

As shown in previous studies with alfalfa and corn-based rations, overfeeding cows in the dry period with a grass-based ration will result in metabolic disturbances postpartum. Differences may not be very overt in daily practice, as DMI and milk yield postpartum may not seem to be affected; underlying metabolic processes will be hampered resulting in liver fat accumulation. Ideally, dry cows are fed at or just below their energy requirements during the dry period for an optimal start of the lactation.

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5

Chapter 5

Dry period length affects rumen adaptation in dairy cattle precalving and during the first weeks after calving

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ABSTRACT

Omitting or shortening the dry period may result in a fairly constant ration throughout the transition period of dairy cows, reducing the need for adaptation of cow metabolism and rumen function to a new lactation. The objective of this study was to determine the effect of dry period length on rumen adaptation and cow metabolic state during the transition period. Twelve pregnant, rumen-cannulated Holstein Friesian dairy cows at the end of their first lactation were assigned to one of three treatments: a conventional (**60 d**), short (**30 d**) or no dry period (**0 d**). At dry-off, cows received a dry cow ration until calving. Lactating cows received a lactation ration. Cows were monitored from 8 wk before calving until 8 wk after calving for milk yield and dry matter intake (**DMI**). Rumen biopsies were taken from 3 locations in the rumen at 60, 40 and 10 d before calving and 3, 7, 14, 28 and 56 d after calving to assess papillae dimensions. Blood was sampled weekly from 3 wk before until 8 wk after calving, and liver biopsies were taken at wk -2, wk 2 and wk 4 relative to calving. Prepartum, DMI and milk yield were greater for cows with a short or no dry period, compared with cows with a conventional dry period. Postpartum, DMI was greater for cows with a short dry period compared with cows with a conventional dry period. Plasma glucose concentration was greater for cows without a dry period, compared with the other dry period lengths postpartum. Plasma concentrations of non-esterified fatty acids and β-hydroxybutyrate, and liver triglyceride content, did not differ among dry period lengths. Rumen papillae differed in size based on biopsy location, but there was no interaction between biopsy location and the effect of dry period length. Rumen papillae surface area for cows managed for a 30 d or 60 d dry period decreased towards calving. At 40 d prepartum, papillae surface area was greater for short and no dry period treatment compared with a conventional dry period. At 10 d prepartum, papillae surface area was greater for the no dry period treatment compared with both other treatments, and this difference was still present 3 d postpartum. Cows managed for a short dry period showed faster increase in papillae dimensions after calving compared to cows managed for a conventional dry period. From d 28 onwards, no differences in papillae surface area were observed. The faster rumen adaptation postpartum may be related to the increased DMI during the first weeks postpartum for cows managed for a short dry period. However, this did not result in improved metabolic status or milk yield. The results from the present study demonstrate that the dietary changes related to a conventional dry period length affected rumen papillae development, not only prepartum but also early postpartum. Further optimization of dry period length as well as dietary composition throughout the transition period may support cows in their adaptation to a new lactation.

INTRODUCTION

In the transition period around calving, the cow's physiological status changes dramatically which coincides with, amongst others, housing and dietary changes (Ingvartsen, 2006; Van Knegsel et al., 2014). High-yielding dairy cows require a large adaptive capacity to cope with the transition from late gestation to early lactation. In case this adaptation fails and feed intake decreases, the energy balance (**EB**) postpartum may become more negative, which is associated with a greater incidence of diseases such as mastitis, displaced abomasum, ketosis and hepatic lipidosis (Collard et al., 2000). Nutrition plays a key role in a successful transition from gestation to lactation in dairy cattle. This notion is illustrated by the well-known negative effect of excessive energy intake during the dry period on the cow's health after calving (Janovick et al., 2011). To prevent excessive energy intake during the dry period, dry cows are commonly fed diets with a low energy density. In contrast, a high energy density of the diet is required after calving to meet the cow's energy requirements related with the onset of milk production. Thus, dairy cows are subjected to dietary, housing and physiological changes around calving and the adaptation process around these changes is crucial for cow health and fertility in early lactation (Zebeli et al., 2015).

The dietary changes during the transition period require a large adaptive capacity of the rumen wall including rumen papillae and its epithelial layer (Steele et al., 2015). A main factor driving the proliferation of rumen papillae is rumen fermentable organic matter (**fOM**) intake (Dirksen et al., 1985; Dieho et al., 2016a). When fOM intake is low during the dry period, rumen papillae regress (Dieho et al., 2016a), whereas greater fOM intake by supplemental concentrate during the dry period increased papillae surface area (Dieho et al., 2017a). Total rumen papillae surface area is considered an important factor in rumen performance as it may directly be related to the capacity for VFA absorption. The evidence for a positive relationship between papillae surface area and VFA absorption capacity is ambiguous though (Bannink et al., 2008; Martens et al., 2012; Dieho et al., 2016a; Dieho et al., 2016b). In order to avoid significant regression of rumen papillae, total fOM intake should remain relatively high during the last weeks prior to calving. Continuation of milk production before calving may maintain such a fairly constant papillae surface area, due to limited dietary changes and a relatively high fOM intake to support milk production. In an earlier study, a short dry period (i.e., 35 d) tended to result in a higher ruminal pH and lower concentration of total VFA before calving and higher concentration of VFA after calving, compared with a conventional dry period (Jolicoeur et al., 2014).

Limited knowledge is available concerning the dynamics of rumen papillae size during the transition period for cows with and without a dry period. Therefore, the objective of the current study was to monitor the regression and subsequent proliferation of the rumen papillae of dairy cows from 60 d before calving until 56 d after calving with either a conventional 60 d dry period, a short dry period of 30 d, or a complete omission of the dry period. We hypothesized that omitting or shortening the dry period compared with a conventional dry period, induces a smaller decline in rumen papillae surface area prepartum, and results in greater rumen papillae surface area in the first weeks postpartum.

MATERIALS AND METHODS

Animals and Treatments

All experimental protocols and interventions were approved by the Ethical Committee on Animal Experiments of the Animal Sciences Group of Wageningen University & Research, Wageningen, The Netherlands (experimental protocol 2010026). Twelve rumen-cannulated Holstein dairy cows were selected at the end of their first lactation from the Dairy Campus Research herd (Wageningen Livestock Research, Lelystad, the Netherlands). Cows were blocked in 4 groups of 3 animals, based on similarity in the 305 d milk production during the first lactation. Cows from each block were randomly assigned to one of 3 treatment groups varying in dry period length: a conventional dry period (**CON**) of 60 d, a short dry period (**SHORT**) of 30 d or no dry period (**NODRY**) with continuous milking. All cows were monitored from 60 d before their expected calving date until 8 wk postpartum. Our study was part of a larger trial with 168 cows subjected to the same three treatments, monitored over two full lactations (Van Knegsel et al., 2014). All animals were housed in a cubicle shed in separate dry cow and post-calving groups. On the day of calving, cows were separated from the dry cow group and housed in a straw bedded calving pen. After calving, the cows were moved to the post-calving group. Cows were milked twice daily (around 0500 and 1630 h). The drying-off protocol for cows allocated to the groups CON or SHORT started 7 d before actual drying-off with a transition to the dry-cow ration (rations are described in Tables 1 and 2). Four days before drying-off, the milking frequency was reduced to once daily.

Rations and Feeding Management

All cows had unrestricted access to fresh drinking water. Rations and feeding management are described by Van Knegsel et al. (2014). In short, three forage mixtures were fed: two prepartum rations, viz. one for lactating cows and one

for dry cows, and a postpartum ration for all lactating animals. Forage mixtures were supplied ad libitum in feed weighing troughs with individual transponder-controlled access gates (Roughage Intake Control system, Hokofarm Group, Marknesse, The Netherlands). The stocking density was 2 cows per trough. For each visit of a cow to a feed trough, the start and end time of the visit, as well as the start and end weight of the trough, were recorded. Daily, between 1030 and 1100 h, feed refusals were removed from the troughs and fresh forage mixture was supplied. All cows in the current study were supplemented with a glucogenic concentrate (concentrate A) (Table 1). Concentrate A was fed individually using transponder-controlled concentrate dispensers starting with 1.0 kg/d from 10 d before the expected calving date. The amount supplied was gradually increased from the day of calving up to 8.5 kg/d at d 17 after calving. The maximum level of concentrate A was maintained from d 17 until the end of the experimental period at d 56. Additionally, cows received 0.5 kg of standard concentrate B at each milking in the milking parlor (1 kg per day during lactation). The chemical composition of the different rations pre- and postcalvingbased on the realized total feed intake is presented in Table 2. To calculate DMI, all forage mixtures were sampled daily. The DM concentration was determined by oven drying at 104 °C during 36 h. The daily DMI of each cow was calculated by multiplying the daily fresh feed intake with the DM concentration.

Table 1. Average intake of compound concentrate as well as ingredients, chemical composition and feeding value of concentrates

Item	**Concentrate A** *fed in the barn*	**Concentrate B** *fed in the milking parlor*
Average intake (kg/d)		
Precalving, all cows[1]	1.0	-
Precalving, lactating cows[1]	-	1.0
Postcalving[2]	8.5	1.0
Ingredient (%)		
Corn	53.5	30.3
Palm kernel, expeller	-	22.4
Rapeseed meal	10.9	18.3
Soybean meal	9.1	2.5
Citrus pulp	-	10.0
Sugar beet pulp	7.4	-
Molasses	5.8	5.0
Wheat middlings	3.8	-
Rapeseed meal, formaldehyde treated	3.1	1.2

Table 1. Continued

Item	**Concentrate A** *fed in the barn*	**Concentrate B** *fed in the milking parlor*
Soybean meal, formaldehyde treated	2.5	4.3
Vinasses	-	2.3
Wheat	-	0.9
Palm oil	0.1	0.2
Calcium carbonate	1.9	0.7
Sodium chloride	0.9	0.5
Magnesium oxide	0.8	0.5
Urea	-	0.6
Mineral-vitamin mixture[3]	0.2	0.3
Chemical composition (g/kg)		
DM	873	881
Crude protein	158	186
Crude fat	29	43
NDF	155	246
ADF	68	143
Starch	365	222
Sugars	68	82
Ash	70	59
Feeding value (g/kg)		
DVE[4]	106	110
OEB[5]	11	27
fOM[6]	457	480
NE_L (MJ/kg)[7]	6.78	6.77

[1]Prepartum, all cows (dry and lactating) received 1.0 kg/d of concentrate A in individual concentrate feeder in the barn, from 10 days before expected calving date. All lactating cows received 1.0 kg/d of concentrate B during milking in the milking parlor.
[2]Postpartum, concentrate A allowance in individual concentrate feeder was gradually increased for all cows from 1.0 kg/d on the day of calving, to 8.5 kg/d on d 17 postpartum.
[3]Premix 2016 (Pre-Mervo UA Cooperatie, Utrecht, the Netherlands).
[4]Intestinal digestible protein (Tamminga et al., 1994).
[5]Rumen degraded protein balance (Tamminga et al., 1994).
[6]Fermentable organic matter.
[7]Net energy for lactation calculated with the Dutch net energy evaluation (VEM) system (Van Es, 1975).

Table 2. Ingredient, chemical composition and feeding value (g/kg of DM, unless otherwise stated) of prepartum and postpartum rations based on realized average feed intake

	Prepartum		**Postpartum**
	Dry cow diet	**Lactation diet**	**Lactation diet**
Ingredient			
Wilted grass silage	358	464	335
Corn silage	172	301	218
Soybean meal	-	60	44
Rapeseed meal	106	77	55
Wheat straw	344	22	16
Rapeseed straw	-	11	8
Mineral and vitamin premix	10	11	8
Concentrate A	10	5	277
Concentrate B	-	49	39
Chemical composition			
DM (g/kg of product)	587	487	556
Crude protein	115	157	163
Crude fat	25	33	33
NDF	531	392	333
ADF	320	227	186
Starch	68	126	206
Sugars	73	92	88
Ash	86	86	84
Feeding value			
DVE[1]	45	77	89
OEB[2]	10	25	21
fOM[3]	463	552	544
NE_L[4] (MJ/kg of DM)	5.24	6.45	6.82

[1]Intestinal digestible protein (Tamminga et al., 1994).
[2]Rumen degraded protein balance (Tamminga et al., 1994).
[3]Fermentable organic matter.
[4]Net energy for lactation calculated with the Dutch net energy evaluation (VEM) system (Van Es, 1975).

Each separate ingredient of the forage mixtures as well as compound concentrates were sampled weekly and stored at −20 °C until chemical analyses. Before analyses (BLGG AgroXpertus BV, Oosterbeek, the Netherlands), forage samples were pooled per batch. Briefly, crude fat concentration was determined gravimetrically as the ether extract (ISO, 1999) and crude ash content after incin-

eration at 550 °C (ISO, 2002). Concentrations of NDF and ADF were determined according to Van Soest et al. (1991) and expressed without residual ash, crude protein was calculated as 6.25 × N-Kjeldahl (ISO, 2005) and sugar concentrations were determined as described by Van Vuuren et al. (1993). Starch was released by heating in a boiling water bath in the presence of 2 N HCl (Cone, 1991) after which starch concentration was determined using the amyloglucosidase method (ISO, 2004). The values of NE_L, intestinal digestible protein (DVE), rumen degradable protein balance (OEB) and fOM were calculated from the chemical composition and according to the guidelines of the Central Bureau for Livestock Feeding (CVB, 2021).

Milk Yield and Milk Composition

Milk weights were recorded automatically at each milking. Weekly, milk samples of each cow were taken at 4 consecutive milkings (2 morning and 2 afternoon milkings). Both morning milk samples were pooled to 1 composite sample; afternoon milk samples were processed likewise. The composite morning and afternoon milk samples were analyzed for fat, protein, and lactose concentration (ISO, 2013; Qlip Zutphen, the Netherlands), using a Foss MilkoScan infrared automatic analyzer (Foss Electric, Hillerød, Denmark). Weighed means were calculated from the recorded morning and afternoon milk weights and the analyses of the composite morning and afternoon milk samples.

BW, BCS and EB

The precalving BW was recorded weekly at the same time of day. For lactating cows, BW was automatically recorded twice per day when cows left the milking parlor and averaged per week. Every 4 weeks, cows were scored for body condition from 1 (thin) to 5 (fat) according to Ferguson et al. (1994). Energy balance was calculated according to the VEM system (Van Es, 1975; CVB, 2021) as the difference between NE_L intake with feed and the NE_L required for gestation, maintenance and milk production and 1,000 VEM = 6.9 MJ of net energy. Regarding the NE_L for gestation, 1500 VEM/d is required in the 8^{th} month and 2700 VEM/d for the 9^{th} month of gestation. Animal maintenance requirements are 42.4 VEM/ $kg^{0.75}$·d and requirements for milk production are 442 VEM/kg of fat and protein-corrected milk (**FPCM**), which is calculated as milk yield × (0.337 + 0.116 × milk fat % + 0.06 × milk protein %). A correction factor was applied to scale maintenance and production requirements to an average cow, as described by Van Es (1975).

Rumen Sampling

Rumen morphology was further investigated on d-60, d-40 and d-10 relative to the expected calving date, and on d3, d7, d14, d28 and d56 after calving. Each time, the rumen content was evacuated completely. Biopsies were taken at 3 locations: the right dorsal sac, directly opposite of the rumen cannula, cranially of the dorsal coronary groove; the right wall of the caudodorsal blind sac; and the ventral wall of the caudoventral blind sac. At each location, 5-15 papillae were harvested from the rumen wall using forceps (No. 631319, Stuemer, Würzburg, Germany) and gently rinsed in 0.9% NaCl solution before placing them on a clean paper tissue to measure physical dimensions (height and width). Papillae were photographed using a digital camera (Canon IXUS 130, Canon Inc., Tokyo, Japan), including a ruler in each photograph. Papilla length was measured from the tip to the base of the papilla along its axis, papilla width was measured halfway of and perpendicular to the papilla length. Papillae damaged by the biopsy procedure were easily recognized as damaged papillae, as they showed distinctive circular cut marks.

Blood Collection and Liver Biopsies

To determine the metabolic status during the transition period, blood samples and liver biopsies were taken and analyzed as described by Chen et al. (2015). In short, blood was taken weekly from wk -3 relative to expected calving date until wk 8 postpartum, decanted, aliquoted and frozen at -20 °C until analysis. Plasma was analyzed for glucose content using commercial kit no. 61269 (BioMérieux, Marcy l'Etoile, France), NEFA and BHB concentrations were measured enzymatically using kits no. 994-75409 (Wako Chemicals, Neuss, Germany) and RB1007 (Randox, Ibach, Switzerland) and insulin was measured using a radioimmunoassay (RIA). Liver biopsies were performed in wk –2, wk 2, and wk 4 relative to calving under local anesthesia and stored at -20 °C until analysis. Liver TG was extracted and concentration was determined through enzymatic colorimetric analysis (Triacylglycerols LiquiColor Mono kit, Human Gesellschaft for Biochemica und Diagnostica mbH, Wiesbaden, Germany).

Statistical Analysis

Daily feed intake, milk yield, BW and EB were averaged per cow per week relative to calving. Precalving data were available for all cows from d-59 relative to actual calving date. Rumen papilla surface area was calculated as 2 × papillae height × papillae width.

Data was analyzed separately for the pre- and postpartum period (except for liver TG content) by mixed model analysis using the REML procedure in Genstat

19th edition (2018). The model structure for all measures, except papillae characteristics, was:

$$Y_{ijk} = \mu + B_i + DP_j + T_k + DP_j \times T_k + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = effect of block (1 to 4); DP_j = effect of dry period management (CON, SHORT or NODRY); T_k = effect of time (prepartum wk -8 to -1; postpartum wk 1 to 8); $DP_j \times T_k$ = interaction between treatment and time; and ε_{ijk} = residual error. Cow was considered as the repeated effect.

For papillae characteristics, the model structure was:

$$Y_{ijkl} = \mu + B_i + S_j + DP_k + T_l + DP_k \times T_l + \varepsilon_{ijkl}$$

where Y_{ijkl} = dependent variable; μ = overall mean; B_i = effect of blocks (1 to 4); S_j = effect of rumen site (right dorsal sac, caudodorsal blind sac, caudoventral blind sac); DP_j = effect of dry period management (CON, SHORT or NODRY); T_k = effect of time (prepartum -60, -40 or -10 d; postpartum 3, 7, 14, 21, 28 or 56 d); $DP_j \times T_k$ = interaction between treatment and time; and ε_{ijk} = residual error. Cow was considered as the repeated effect. The necessity of an auto-regressive function as well as heterogeneity were tested and judged by the difference in deviance of each model with the change in degrees of freedom in a chi squared distribution. Auto-regressive function improved model deviance of all parameters; heterogeneity was not relevant for most parameters, but for some parameters outside the model. Significance of effect was declared at $P < 0.05$ and trends at $0.05 \leq P < 0.10$.

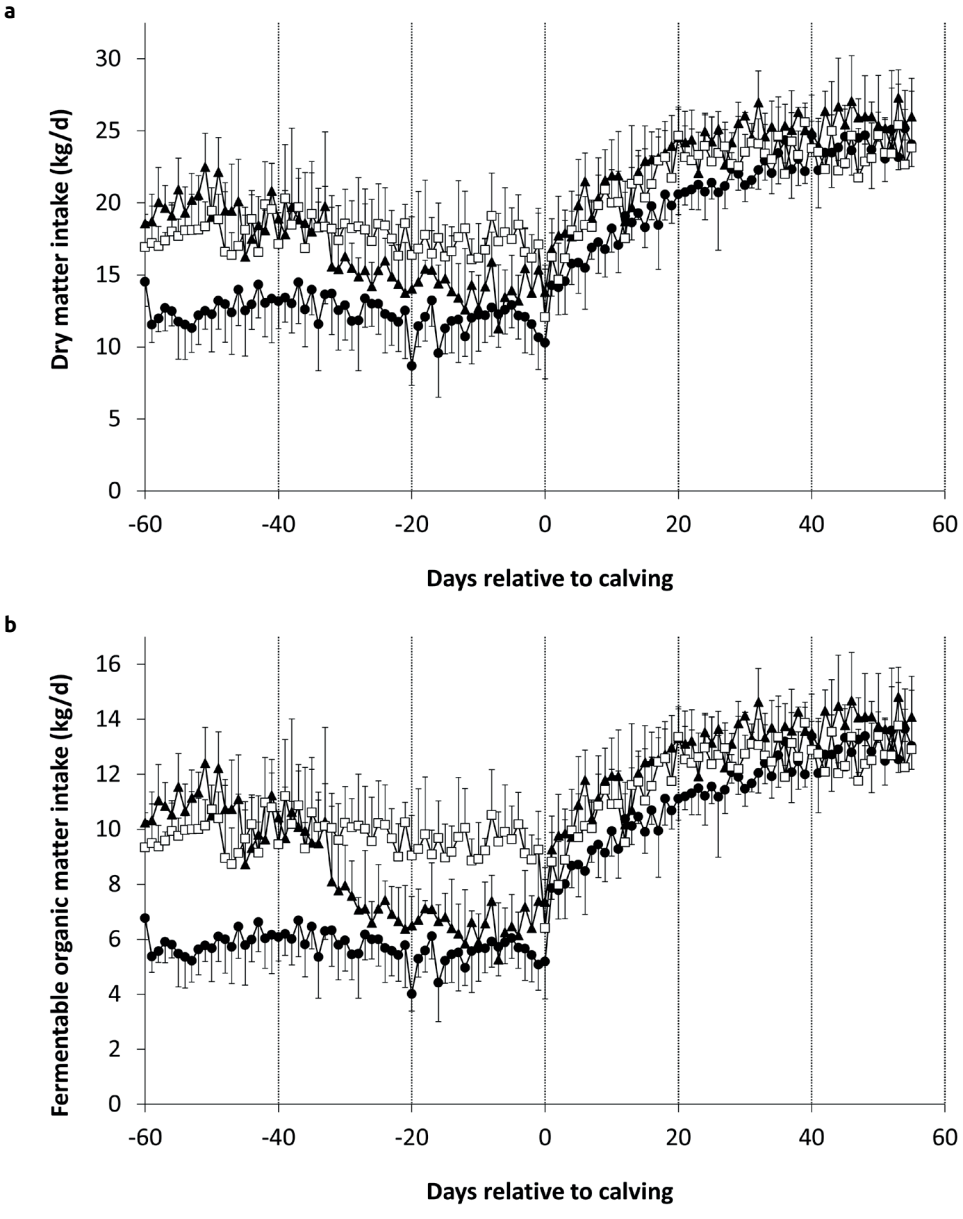
RESULTS

Actual dry period length was 59.3 d (± 4.6 d) for group CON and 26.5 d (± 4.9 d) for group SHORT. In group NODRY, one cow dried off naturally 4 days before calving; others were milked until calving as planned. Two cows in group NODRY encountered mastitis postcalving, and milk yield was affected for one cow during wk 7 and wk 8; data of this cow were therefore taken into account until wk 6.

Feed Intake

Before calving, the experimental treatments resulted in different DM and fOM intake, with the level of these differences depending on week before calving

as indicated by a significant interaction between dry period length and week before calving (Figure 1; Table 3). After calving, daily DMI and fOM were on average 2.5 and 1.4 kg/d higher, respectively, for cows with a short dry period length compared with cows with a conventional dry period, but without significant differences compared with no dry period.



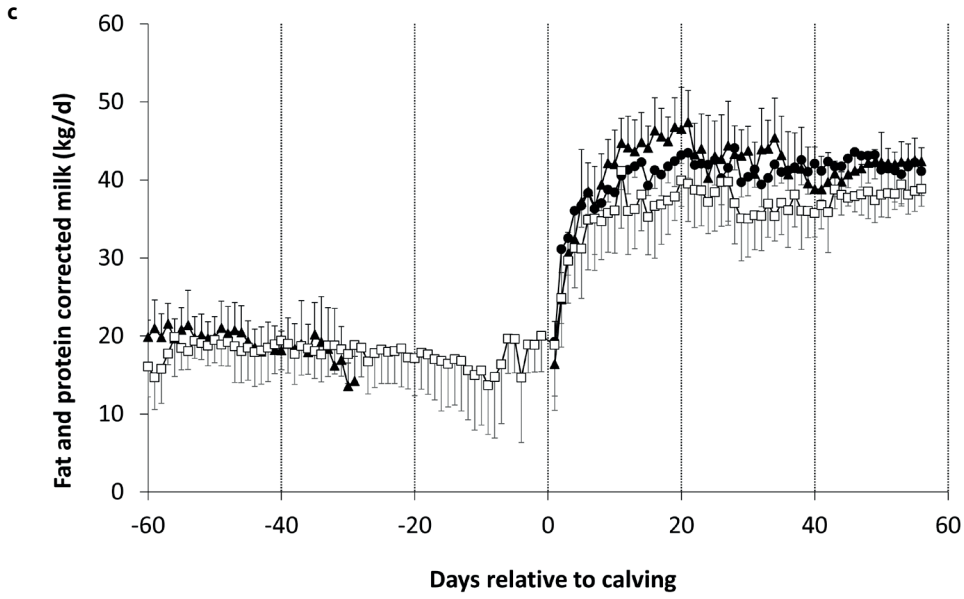


Figure 1. Average daily dry matter intake (a), fermentable organic matter intake (b) and fat- and protein correct milk (c) in kg/d for cows on a 60 d dry period (CON, black circles), 30 d dry period (SHORT, black triangles) or no dry period (NODRY, open squares) relative to the day of calving.

Table 3. Dry matter intake (DMI), fermentable organic matter (fOM) intake, milk yield, fat- and protein corrected milk (FPCM), milk components, body weight, body condition score (BCS) and calculated net energy balance (EB) in the prepartum and postpartum period of cows with a dry period length of 60 (CON), 30 (SHORT) or 0 days (NODRY).

	Dry period length[1]				P-value[3]		
	CON	SHORT	NODRY	SED[2]	DP	wk	DP × wk
Prepartum wk -8 to -1							
DMI (kg/d)	12.4[a]	16.7[b]	17.9[b]	1.04	0.005	< 0.001	< 0.001
fOM intake (kg/d)	5.7[a]	8.5[b]	9.9[b]	0.62	0.001	< 0.001	< 0.001
Milk yield (kg/d)	0.0[a]	8.0[b]	15.7[c]	2.27	< 0.001	< 0.001	< 0.001
FPCM (kg/d)	0.0[a]	9.6[b]	17.7[c]	2.44	< 0.001	< 0.001	< 0.001
Body weight (kg)	663	693	669	31.6	0.618	< 0.001	0.015
BCS	2.9	3.0	2.9	0.19	0.658	0.022	0.333
EB (kJ/kg$^{0.75}$·d)	66	89	21	57.2	0.204	0.097	0.219
Postpartum wk 1 to 8							
DMI (kg/d)	21.1[a]	23.6[b]	22.4[ab]	0.76	0.040	< 0.001	0.184
fOM intake (kg/d)	11.5	12.9	12.2	0.45	0.055	< 0.001	0.152
Milk yield (kg/d)	41.0	40.3	34.6	3.05	0.218	< 0.001	0.610
FPCM (kg/d)	40.8[ab]	41.7[a]	36.1[b]	2.48	0.008	0.035	0.314
Milk fat (g/kg)	39.6	41.0	42.3	2.08	0.082	< 0.001	0.054
Milk protein (g/kg)	34.2[a]	37.0[ab]	38.3[b]	1.48	< 0.001	< 0.001	0.825
Milk lactose (g/kg)	46.4	46.1	46.6	0.60	0.467	0.008	0.153
Fat yield (kg/d)	1.60[ab]	1.63[a]	1.43[b]	0.128	0.023	0.010	0.101
Protein yield (kg/d)	1.39	1.48	1.30	0.081	0.067	0.282	0.613
Lactose yield (kg/d)	1.92	1.87	1.62	0.145	0.218	< 0.001	0.299
Body weight (kg)	613	661	636	36.5	0.474	0.053	0.236
BCS	2.1	2.4	2.6	0.40	0.586	< 0.001	0.687
EB (kJ/kg$^{0.75}$·d)	-209[a]	-121[ab]	-12[b]	65.4	0.011	< 0.001	0.410

[a-c]Means within a row with different superscripts differ ($P < 0.05$).
[1]Dry period length of 60 days (CON, conventional), 30 days (SHORT) or 0 days (NODRY).
[2]SED = standard error of differences.
[3]*P*-value for the effects of dry period length (DP), week (wk) or their interaction

Milk Production and EB

As intended by the imposed treatments, before calving, the greatest milk and FPCM yield was observed for cows without dry period and lowest for cows with a conventional dry period, with cows on a short dry period in between (Table 3); the level of this effect was dependent on week relative to calving as indicated by significant interaction between both factors. After calving, dry period length did not affect average milk yield, but FPCM was 5.6 kg/d lower for group NODRY

compared with group SHORT. Milk protein content was greater, and milk fat content tended to be greater, in cows without dry period compared with cows with a conventional dry period.

Prepartum, BW was affected by an interaction between dry period length and week relative to calving (P = 0.015) based on a relatively lower BW of the cows with a conventional dry period at the start of the experiment that became more similar to BW of the other groups nearer to calving (Figure S1). Prepartum, BCS was not affected by dry period length or interaction between dry period length and week relative to calving. After calving, neither BW nor BCS were affected by dry period length (Table 3). The calculated EB did not differ between the three treatments prepartum. In the postpartum period, the calculated EB was more negative for cows in group CON compared with group NODRY.

Plasma Metabolites and Liver TG

In the final 3 weeks before calving, plasma NEFA concentrations were low and similar between groups (Table 4). Plasma BHB concentrations were higher for group NODRY compared with group CON or SHORT. Plasma glucose concentrations were greater for cows in group SHORT or NODRY compared with cows in group CON. Plasma insulin concentrations showed an interaction between dry period length and time, with low levels for the cows in CON, high levels for cows in NODRY and initially high but decreasing levels for cows in SHORT during the final 3 weeks before calving (Figure S2). During the postpartum period plasma NEFA and BHB concentrations were comparable between the three treatment groups (Table 4). Average plasma glucose concentrations postpartum were higher for cows in NODRY, compared with cows in SHORT or CON. The effect of dry period length on plasma insulin concentrations depended on week postpartum, with low levels for the cows in CON, increasing levels for cows in SHORT from wk 4 to 8, and an even stronger increase during the same period for cows in NODRY (Figure S2). Liver TG content analyzed in wk -2, 2 and 4 relative to calving was not affected by dry period length.

Table 4. Plasma non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose and insulin and liver triacyl glycerides (TG) concentrations of cows with a dry period length of 60 (CON), 30 (SHORT) or 0 days (NODRY). Values represent means ±SED.

	Dry period length[1]				*P*-value		
	CON	**SHORT**	**NODRY**	**SED[2]**	**DP**	**wk**	**DP × wk**
Plasma prepartum wk -3 to -1							
NEFA (mmol/L)	0.13	0.10	0.12	0.025	0.448	0.153	0.863
BHB (mmol/L)	0.39[a]	0.41[a]	0.55[b]	0.057	0.044	0.326	0.220
Glucose (mmol/L)	3.42[a]	3.87[b]	3.82[b]	0.113	0.005	0.616	0.589
Insulin (µIU/mL)	15.2	21.1	20.1	4.88	0.617	< 0.001	0.023
Plasma postpartum wk 1 to 8							
NEFA (mmol/L)	0.26	0.27	0.19	0.070	0.387	< 0.001	0.184
BHB (mmol/L)	0.48	0.51	0.49	0.039	0.668	0.449	0.562
Glucose (mmol/L)	3.43[a]	3.49[a]	3.71[b]	0.065	< 0.001	< 0.001	0.765
Insulin (µIU/mL)	12.3	12.2	18.5	2.56	0.075	< 0.001	0.002
Liver biopsies wk -2, 2, 4							
TG (mg/g of wet weight)	27.3	25.7	17.8	8.28	0.238	0.017	0.868

[a-b]Means within a row with different superscripts differ ($P < 0.05$).
[1]Dry period length of 60 days (CON, conventional), 30 days (SHORT) or 0 days (NODRY).
[2]SED = standard error of differences.

Rumen papillae

An average of 8.0 (SD= 2.4) papillae per site per cow per sampling day were collected. In general, rumen papillae length and surface area differed between the three biopsy locations ($P < 0.001$, Figure S3). Papillae harvested from the right dorsal sac were shortest with on average 5.6 mm prepartum and 6.7 mm postpartum. Papillae from the caudoventral blind sac were longest (11.6 mm prepartum and 12.6 mm postpartum) and papillae from the caudodorsal blind sac were intermediate (7.7 mm prepartum and 9.3 mm postpartum). There was no interaction between biopsy location and the effect of dry period length in prepartum and postpartum period.

Before calving, papillae length was not affected by dry period length whereas papillae width and papillae surface area showed an interaction of dry period × day (Table 5). Cows with a dry period showed narrower papillae and a reduced papillae surface area after switching to the dry cow ration, where cows without a dry period had a relatively constant papillae width and surface area. At 40 d before the expected calving, cows in group CON had a lower papillae width and

surface area than the groups with other dry period lengths. At 10 d before the expected calving date, cows in both groups CON and SHORT had a lower papillae width and surface area compared with cows in NODRY (Table 5).

Table 5. Main effects in rumen papillae size averaged across sampling sites during the dry period as affected by dry period length (DP) of 60 (CON), 30 (SHORT) or 0 days (NODRY), by day relative to calving (day) and by the interaction between DP and day relative to calving

	Dry period length[1]				*P*-value		
	CON	**SHORT**	**NODRY**	**SED[2]**	**DP**	**day**	**DP × day**
Papillae length (mm)							
d -60	8.3	8.5	8.0				
d -40	8.0	8.7	9.0	0.60	0.859	0.093	0.164
d -10	7.9	7.8	8.4				
Papillae width (mm)							
d -60	2.4^{x}	2.8^{x}	2.7				
d -40	1.9^{ay}	3.0^{bx}	2.8^{b}	0.24	<0.001	0.521	0.001
d -10	2.0^{axy}	2.3^{ay}	3.1^{b}				
Papillae surface area (mm^2)							
d -60	40.0	48.3^{xy}	46.2				
d -40	31.3^{a}	54.5^{bx}	53.2^{b}	6.07	0.006	0.408	0.013
d -10	33.8^{a}	39.0^{ay}	53.4^{b}				

[a-b]Means within a row with different superscripts show differences between treatments at the specific time point ($P < 0.05$).
[x-z]Means within a column with different superscripts show a time effect for a specific treatment ($P < 0.05$).
[1]Dry period length of 60 days (CON, conventional), 30 days (SHORT) or 0 days (NODRY).
[2]SED = standard error of differences.

Table 6. Main effects in rumen papillae size averaged across sampling sites during early lactation as affected by dry period length (DP) of 60 (CON), 30 (SHORT) or 0 days (NODRY), by day relative to calving (day) and by the interaction between DP and day relative to calving

	Dry period length[1]				*P*-value		
	CON	SHORT	NODRY	SED[2]	DP	day	DP × day
Papillae length (mm)							
d 3	8.8^{x}	8.2^{x}	9.2				
d 7	8.7^{x}	9.7^{yz}	9.4				
d 14	9.6^{xy}	9.6^{y}	9.0	0.61	0.804	<0.001	0.031
d 28	10.4^{yz}	10.2^{yz}	9.4				
d 56	10.7^{z}	10.6^{z}	9.7				
Papillae width (mm)							
d 3	2.3^{ax}	2.9^{bx}	3.4^{cx}				
d 7	2.3^{ax}	3.0^{bx}	3.4^{cx}				
d 14	2.4^{ax}	3.3^{by}	3.1^{bx}	0.20	<0.001	<0.001	<0.001
d 28	3.1^{ay}	3.7^{bz}	3.4^{abx}				
d 56	3.5^{z}	4.0^{z}	3.9^{y}				
Papillae surface area (mm^2)							
d 3	40.7^{ax}	49.6^{ax}	61.8^{bxy}				
d 7	40.4^{ax}	60.2^{by}	64.3^{bxz}				
d 14	47.5^{ax}	64.2^{by}	56.4^{aby}	6.38	<0.001	<0.001	0.017
d 28	66.1^{y}	76.4^{z}	64.8^{xyz}				
d 56	75.1^{y}	83.1^{z}	76.5^{z}				

[a-c]Means within a row with different superscripts show differences between treatments at the specific time point ($P < 0.05$).
[x-z]Means within a column with different superscripts show a time effect for a specific treatment ($P < 0.05$).
[1]Dry period length of 60 days (CON, conventional), 30 days (SHORT) or 0 days (NODRY).
[2]SED = standard error of differences.

After calving, papillae length, width and surface area were affected by the interaction between dry period length and day after calving (Table 6). The difference in papillae surface area before calving was still present at 3 days postpartum: cows in NODRY had about 25 to 50% larger papillae surface area than cows in SHORT or CON (Table 6). Papillae dimensions in the NODRY group remained relatively constant, whereas papillae in the SHORT and CON group increased with day after calving. The increase in papillae surface area between d 3 and 7 was larger for cows in SHORT than in CON, reaching a papillae surface area

comparable with cows in NODRY at 7 days postpartum. At 4 weeks postpartum, the effect of dry period length on average papillae surface area had disappeared.

DISCUSSION

The current data indicate that shortening or omitting the dry period affects rumen papillae development not only prepartum, but also during the first 2 weeks postpartum. By omitting the dry period, cows received the same lactation ration throughout the transition period, not interrupted by the low-energy dry cow ration fed to cows with a shortened or conventional dry period. Prepartum papillae surface area followed the changes in fOM intake related with the differences between the dry cow and lactation ration (Figure 2). These differences in fOM intake resulted in a reduction in rumen papillae surface area for the two groups consuming a dry cow ration (30 d and 60 d dry period) during the final weeks before calving. Such a decline in rumen papillae surface area upon decreased fOM intake is in line with observations during the dry period by Dieho et al. (2016a). It is also in line with the decline in papillae surface area upon a reduction in NE_L intake (Dirksen et al., 1984) and upon a reduction in fOM intake during first days on fresh pasture diet compared with a total mixed ration (Schären et al., 2016). For group CON fed the dry cow ration during the entire 60 d before calving, this reduction in surface area was already apparent 40 d before calving, whereas for group SHORT cows still received the lactation ration 40 d before calving and papillae surface area at this day was not reduced compared with 60 d before calving. This absence of a decline in papillae surface area between 60 and 40 d before calving in SHORT agrees with Dieho et al. (2017a), where supplemental concentrate from 28 d before calving prevented a decline in papillae surface area between 28 and 8 days before calving. The difference in papillae surface area after a short or conventional dry period length compared with the continuously milked group was still present in the first days after calving but quickly resumed to similar values from 28 days after calving onwards.

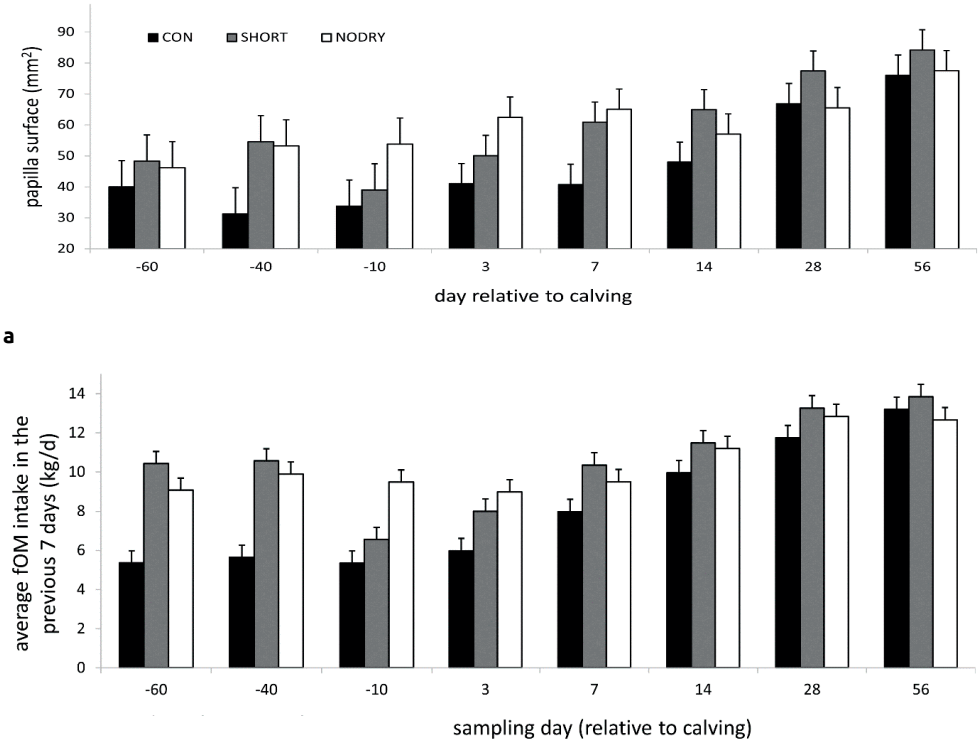


Figure 2. Average papillae surface area in mm^2 at rumen sampling days pre- and postcalving, averaged across sampling sites (a) with the daily fermentable organic matter intake of the 7 days preceding the rumen sampling day (b) for cows on a 60 d dry period (CON, black bars), 30 d dry period (SHORT, grey bars) or no dry period (NODRY, open bars).

Shortening the dry period relative to a conventional dry period instead of omitting the dry period subjects cows to the same rations in the final weeks before calving but with a shorter interval between the two dietary changes. In our study, cows without a dry period had larger papillae surface area directly after calving (at d 3) than cows in the other treatments, but cows managed for a short dry period of 30 d already reached a papillae surface area comparable to the NODRY cows at d 7 after calving. Cows managed for a conventional 60 d dry period had lowest papillae surface area after calving until 28 d after calving when all groups had comparable papillae surface area. Papillae growth can be evoked by supplemental concentrate prepartum (Dieho et al., 2017a) as well as with a faster increment of concentrate allowance postpartum (Dieho et al., 2016a). The cows participating in our study as well as in the study of Dieho et al. (2016a) were relatively young animals, transitioning from first to second parity. The precise effect of age on adaptation capacity of the rumen, however, is still unclear. Our

study adds new data on papillae growth during the transition period in response to changes in dry period length and associated changes in diet composition and feed intake, showing that the duration of feeding a low-energy dry cow ration prepartum affects the size of papillae at the onset of lactation and the time required to let papillae regain their full size postpartum.

The higher DMI and fOM intake at the onset of lactation for the short or no dry period treatment relative to the conventional dry period will have stimulated papillae growth. As described earlier, the 12 primiparous cows enrolled in the current study were part of a larger trial with 60 primiparous and 108 multiparous cows subjected to the same three treatments (Van Knegsel et al., 2014). In the overall study, no main effect of dry period length on DMI postpartum was found during the first 14 wks after calving (Van Knegsel et al., 2014). The rumen study had fewer animals per treatment (n = 4) and monitored only primiparous cows, which may have a specific response to dry period length. In any case, the higher DMI in cows with a shortened dry period needs to be considered when interpreting the results on rumen performance. In general, the effect of dry period length on DMI is not consistent among studies. Some studies show a positive effect of omitting the dry period on DMI postpartum (Rastani et al., 2005), where others report no differences (Andersen et al., 2005; De Feu et al., 2009). The variation in outcome may be related to the experimental design of the rations with different dry period lengths. Rastani et al. (2005) showed an improved DMI during the first 3 wks of lactation for cows without a dry period compared to a short dry period. Interestingly, cows without a dry period as well as cows on a short dry period received the same high energy ration while cows on a conventional dry period received a low to moderate energy diet prepartum (Rastani et al., 2005). The energy balance of the cows on a short dry period was therefore highest of all treatments prepartum which increased the risk for metabolic disturbances postpartum, as shown by increased plasma NEFA and liver TG concentrations, resulting in a reduced feed intake in early lactation (Rastani et al., 2005). In other studies, continuously milked cows and cows with a conventional dry period did not have different DMI postpartum (Andersen et al., 2005; De Feu et al., 2009). In the study of Andersen et al. (2005), cows received exactly the same ration prepartum, having dry cows fed restricted to a maximum of 9 kg DM/d from dry-off to wk 3 prepartum to prevent overfeeding. Feeding the same dietary ingredients to cows with or without a dry period while preventing overfeeding at the same time, may have supported rumen adaptation differently compared with our study based on ad libitum intake of a low energy diet during the dry period. In the study of De Feu et al. (2009) no effect was found on DMI during the first 12 weeks postpartum for cows with a conventional or no dry period,

but unfortunately no information was given on milk yield or EB prepartum to understand the consequences of the dietary management precalving. Our results are in line with the results of Jolicoeur et al. (2014), comparing a short dry period with a conventional dry period. In the first 3 weeks postpartum, DMI was highest for the cows managed for a short dry period precalving. In that study, however, cows with a conventional dry period were subjected to three dietary changes: 1) from lactation to far-off ration; 2) from far-off to close-up ration at 28 d before the expected calving date; and 3) from close-up to lactation ration at calving. Cows with a short dry period did not receive the far-off ration at all, resulting in only two dietary changes (Jolicoeur et al., 2014), and such differences with our study hamper direct comparison.

Assuming a relationship between papillae development and nutrient absorption capacity (Schären et al., 2016), the improved papillae surface area for continuously milked cows may have improved rumen VFA absorption and thus rumen fermentation the first weeks of lactation. Papillae surface area is however not the only factor involved in nutrient absorption rate. Other rumen factors like papillary blood flow and VFA transporter expression in papillae may also be relevant (Dieho et al. 2016b, Dieho et al. 2017b), but the morphological proliferation of papillae seemed to be most associated with observed nutrient absorption rate from the rumen (Laarman et al., 2015, Schären et al., 2016; Dieho et al. 2017b). If DMI remains higher around parturition and nutrient absorption at onset of lactation is improved, this may not only support fermentation and lead to increased DMI, but may also reduce the risk for metabolic disease. In our study with second parity cows, however, no clinical cases of metabolic disease were reported; the concentrations of plasma NEFA, BHB and liver TG were generally low and comparable among treatment groups. The absence of such differences may be related to the small number of animals (n = 12) in the present study. The numerically lower NEFA concentrations of group NODRY in the subset of animals in our study is in line with the significantly lower NEFA concentrations of group NODRY compared with SHORT and CON of the main trial with a total of 168 cows as reported by Chen et al. (2015). Similarly, the numerically lower liver TG content of NODRY compared with SHORT and CON in the present study is in in line with significant differences between these groups reported by Chen et al. (2015). An effect of improved nutrient absorption with increased papillae surface area may become more relevant in a situation where cows experience more challenging conditions prone to stronger metabolic disturbances than in our study.

CONCLUSIONS

The results from the present study demonstrate that shortening or omitting the dry period, with associated dietary changes, led to a less pronounced or no decline in rumen papillae surface area precalving and to a greater papillae surface area during the first weeks after calving compared with a conventional dry period of 60 d. This may facilitate rumen adaptation and improve energy balance in early lactation. Further optimization of the combination of dry period length as well as dietary composition and associated dietary changes throughout the transition period in support of adaptation to a new lactation remains to be studied.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

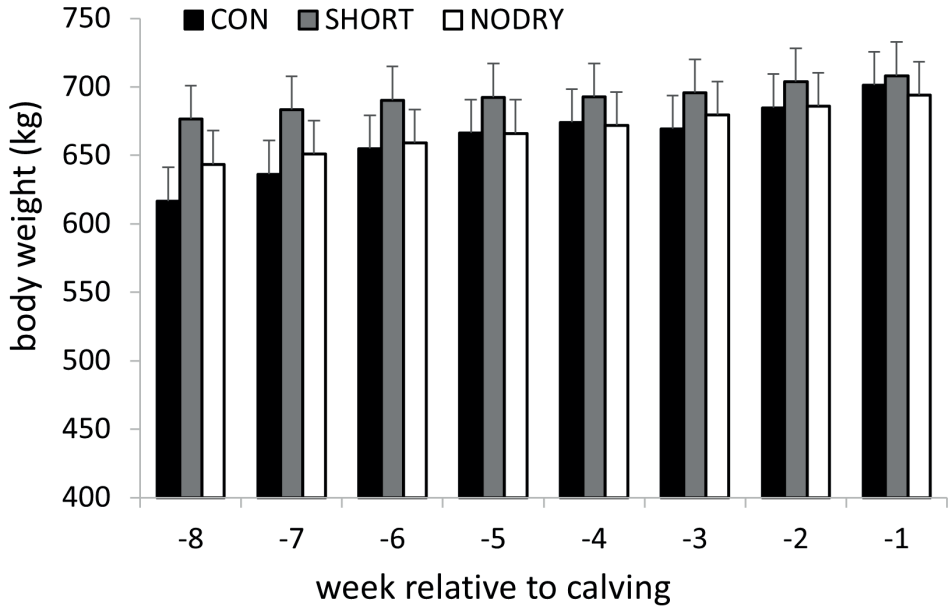

Figure S1 Average body weight precalving for cows on a 60 d dry period (CON, black bars), 30 d dry period (SHORT, grey bars) or no dry period (NODRY, open bars).

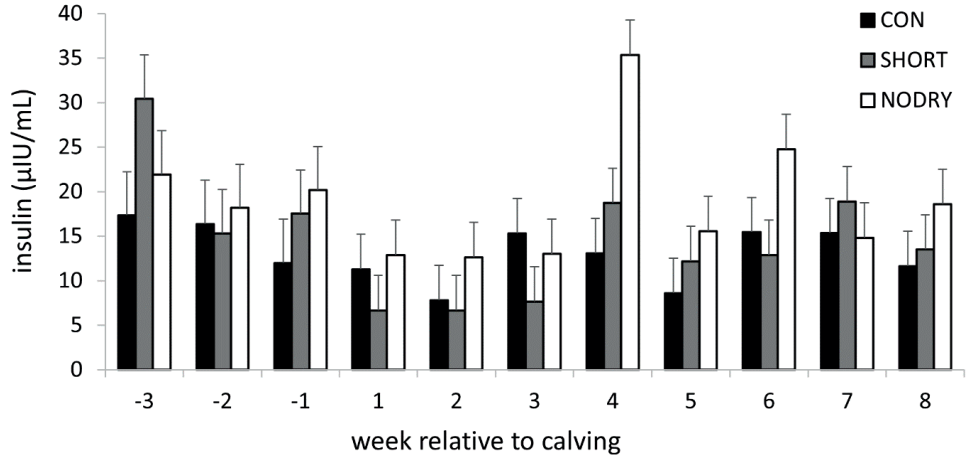

Figure S2 Average plasma insulin concentrations in µIU/mL pre- and postpartum for cows on a 60 d dry period (CON, black bars), 30 d dry period (SHORT, grey bars) or no dry period (NODRY, open bars).

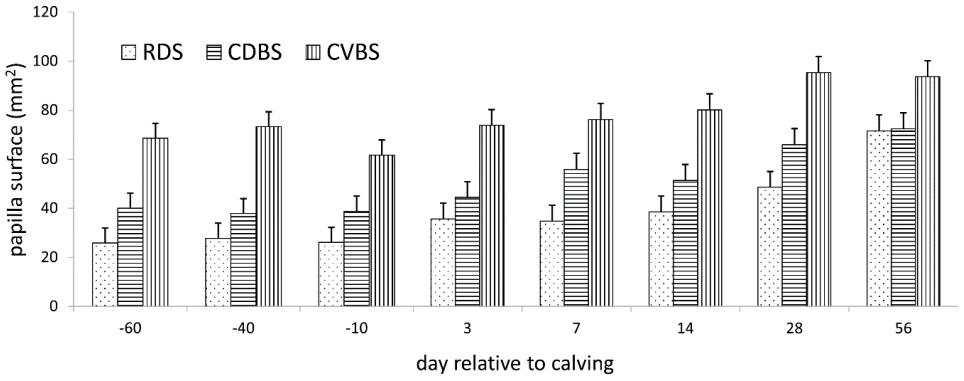

Figure S3 Average papillae surface for each of the three locations (RDS, dotted bars), 30 d dry period (CDBS, bars with horizontal striping) or no dry period (CVBS, bars with vertical striping).
P-values
Prepartum: location < 0.001, location × day 0.511, location × DP 0.382
Postpartum: location < 0.001, location × day 0.063, location × DP 0.585


6

Chapter 6

General discussion

R.M.A. Goselink

1 Adaptation to lactation

As described in **Chapter 1**, the transition from the final phase of gestation to early lactation is very turbulent requiring effective adaptive mechanisms involving both homeostatic and homeorhetic processes. Supporting dairy cows in this peripartum period is considered essential to improve cow health and welfare.

This thesis deals with nutrition and management strategies to start metabolic adaptation prepartum, with a focus on the liver as the central organ in energy metabolism. The hypothesis was that preparing the liver prepartum for the increased metabolic requirements of lactation would improve metabolic capacity and reduce the incidence of clinical metabolic disorders like ketosis and hepatic lipidosis. Four different strategies were tested in this thesis:

- *Hepatic support*
  Improving the fatty acid processing capacity by increasing the availability of choline, one of the essential metabolites required to export triacylglycerides (**TG**) from the liver (Chapter 2)
- *Increase dry cow energy output*
  Starting adaptation processes to increased non-esterified fatty acid (**NEFA**) release in the dry period by starting lipolysis through physical activity prepartum (Chapter 3)
- *Reduce dry cow energy input*
  Starting adaptation processes to a negative energy balance prepartum by restricting energy intake in the dry period to a level at requirements (Chapter 4)
- *Maintain lactating state*
  Reducing the need for adaptation to lactation through continuous milking or a shorter dry period, maintaining digestive and metabolic pathways active aiming for an improved energy input postpartum (Chapter 5)

1.1 Hepatic lipidosis

The main parameter to monitor the success of each strategy in this thesis was liver TG content, supported by plasma NEFA and beta-hydroxybutyric acid (**BHB**) values. A small increase in liver TG concentration in the transition around parturition can be considered physiological and part of the homeostatic and homeorhetic processes explained in Chapter 1. As described by Bobe et al. (2004), a liver TG content > 50 mg/g wet weight is considered a moderately fat liver, associated with a moderately increased risk for disease and reduced reproductive performance. At a liver TG content > 100 mg/g wet weight, liver

function is often severely reduced resulting in symptoms like reduced appetite, reduced milk production, ketosis, reduced reproductive performance, a reduced immune response and strong risk for disease (Table 1).

Table 1. Categories of fatty liver in dairy cows (adapted from Bobe et al., 2004).

Liver category	Liver TG (mg/g wet weight)	Urinary ketones	DMI, milk yield	Health, reproduction	Liver histology
Normal	<10	No change	No change	No change	Normal
Mild fatty liver	10-50	May be slightly increased	No change	May be slightly decreased	Centrilobular TG infiltration
Moderate fatty liver	50-100	Moderately increased	No change	Moderately decreased	TG infiltration throughout liver
Severe fatty liver	>100	Strongly increased	Strongly decreased	Strongly decreased	Enlarged, necrotic

The incidence of moderate or severe fatty liver in dairy practice is not regularly monitored. To assess the status of the liver of a dairy cow, liver biopsies are required. The minor surgical procedure of taking a liver biopsy can be performed relatively easy under practical circumstances, but is not quite common among farmers and their veterinarians (Ouweltjes et al., 2007). Imaging techniques like quantitative analysis of ultrasound images may provide non-invasive solutions to determine liver TG content, with quite good accuracy (92-94%) in discriminating mild from moderate or severe fatty liver (Bobe et al., 2008; Starke et al., 2010). These techniques, however, still require further validation under practical circumstances. The prevalence of hepatic lipidosis in the field can, therefore, not be easily quantified, due to a low availability of data in dairy practice. The most recent evaluation under Dutch production standards was performed more than 25 years ago (between 1995-1997) by Jorritsma et al. (2001) who investigated nine dairy herds with in total 219 cows between 6 and 17 days postpartum. At that time, 40% of the cows had “moderate fatty livers” with TG concentrations between 50 and 100 mg/g wet weight and 14% experienced “severe fatty livers” with concentrations > 100 mg/g wet weight (Jorritsma et al., 2001).

In the four trials presented in this thesis, liver TG content was quite variable (Figure 1, Table 2). The maximum level of liver TG concentration detected in each individual cow in the four experiments in this thesis can also be classified according to the severity of lipidosis described in Table 1. A total of 27 cows was managed according to common dairy practice in the control groups of the different trials - excluding the positive control of Chapter 4 which was actively overfed relative to

requirements. Among these 27 control cows, seven cows (26%) were qualified as having moderate fatty livers and seven (26%) having severe fatty livers. Of the 30 cows treated with nutritional or management strategies to improve adaptation to lactation, still nine cows (30%) experienced moderate fatty livers and three (10%) were qualified as having severe fatty livers. The severeness of liver TG accumulation in the four trials could not always be related to clinical symptoms of (metabolic) disease, which may partially be due to the low number of animals (65 individuals in four experiments). The risk of disease may have increased with increasing liver TG concentration, but the cut-off levels defined in the classification system will not provide a sharp distinction between no metabolic disease vs. clear symptoms. Arshad and Santos (2022) analyzed a dataset of four experiments including 329 cows with liver TG concentration analyzed at 6-11d after calving. Strikingly, a liver TG concentration up to 70 mg/g wet weight was associated with increasing milk yield in the first 100 days after calving; higher liver TG concentrations were associated with lower milk yields and an increased risk for culling. The high milk yield with a moderate fatty liver was realized at the expense of tissue catabolism, as the increasing TG concentration also correlated with reduced DMI, increased BW loss and an increased risk for hyperketonemia, hypocalcemia, metritis and multiple postpartum disease (Arshad and Santos, 2022).

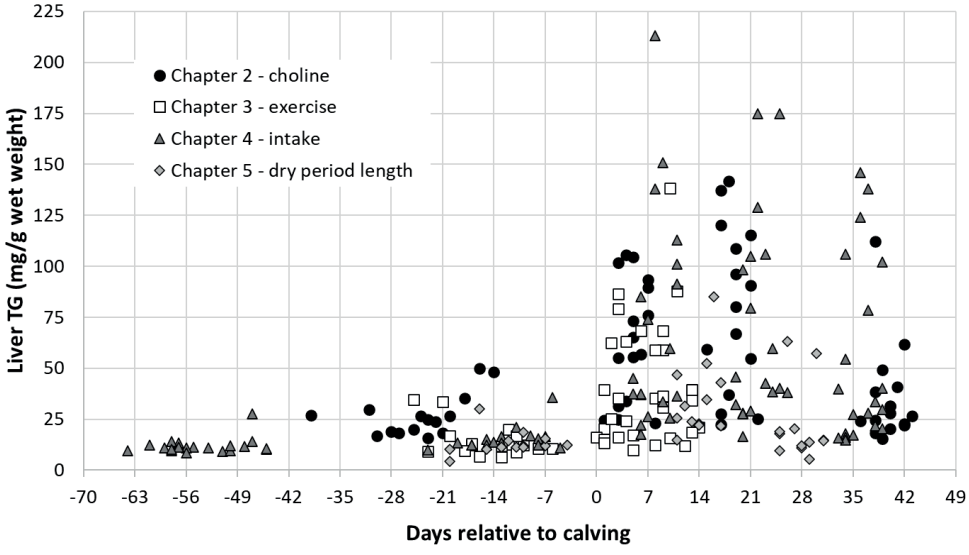

Figure 1. Individual liver triacylglycerol (TG) content of dairy cows participating in each of the thesis trials, including both control groups as well as cows treated with preventive measures (Chapter 2: hepatic support with choline supplementation, Chapter 3: prepartum physical exercise, Chapter 4: reducing prepartum energy intake and Chapter 5: maintaining lactating state by shortening or omitting the dry period).

Table 2. Summary of trials described in this thesis.

Ch[1]	Strategy	Prepartum parity	Trial period (wk)[2]	Biopsy wk[2]	Trt grps[3]	Cows (with liver biopsies)	Individually max liver TG (mg/g wetweight)[4]		
							Min	Med	Max
2	Choline supplement	2 to 6	-4 to 6	-3, 1, 3, 6	Control Choline	8 (8) 8 (8)	32 27	98 63	142 120
3	Dry period exercise	1 to 3	-6 to 6	-2, 1, 2	Control Exercise	16 (8) 16 (8)	16 17	37 37	138 68
4	Dry period energy intake	1 to 3	-8 to 8	-8, -2, 1, 3, 5	HE-AL HE-RES LE-AL	16 (7) 16 (6) 16 (7)	29 18 28	106 44 38	213 113 138
5	Dry period length	1	-8 to 8	-2, 2, 4	Control Short dry No dry	4 (4) 4 (4) 4 (4)	34 22 15	45 23 24	63 85 52

[1]Ch = chapter; [2]wk = wk relative to calving; [3]Trt grps = description of treatment groups, where for Chapter 4: HE-AL = high energy density, ad libitum intake; HE-RES = high energy density, restricted intake; and LE-AL = low energy density, ad libitum intake; [4]Individually max liver TG = the highest level of liver triacylglycerol content for each individual cow in a treatment group, which was always in one of the postpartum biopsy weeks

1.2 The role of lipoprotein transport

As in all other vertebrate species, fat transport in dairy cattle is handled by plasma lipoprotein transport. The different types of lipoproteins are classified based on their density and contain different types of apoproteins (Table 3). Chylomicrons have the lowest density and are involved in the transport of dietary lipids. Intestinally absorbed TG and cholesterol are engulfed in chylomicrons and subsequently transported through the lymphatic system. At the thoracic duct, chylomicrons are released in the venous system, for distribution to peripheral tissue. In ruminants, dietary fat content is generally low (3-5% of DM); plasma chylomicron concentration is, therefore, lower than in monogastric species.

Table 3. Classification of plasma lipoproteins for fat transport in dairy cows (based on Palmquist, 1974).

Parameter	Density (g/mL)	Main apolipoproteins	Role
Chylomicron	< 0.93	C, E	Dietary lipid transport of absorbed triacylglycerol and cholesterol
Very-low-density lipoprotein (VLDL)	0.93 – < 1.006	B100, C	Triacylglycerol export from liver to peripheral tissue
Low-density lipoprotein (LDL)	1.006 – < 1.063	B100	Cholesterol-rich remnant of VLDL, returning to liver
High-density lipoprotein (HDL)	1.063 – < 1.121	A1, C, E	Exchange of apolipoprotein C with VLDL, cholesterol transport

As described in the introduction, very-low-density lipoprotein (**VLDL**) particles are required to excrete the TG formed and stored in the liver. Synthesis of VLDL in the liver requires phospholipids, cholesterol esters, microsomal triglyceride transfer protein and apolipoproteins such as apolipoprotein B100 (Bernabucci et al., 2004). When passing peripheral tissue, TG is released from VLDL increasing its density and transforming it into low-density lipoprotein (**LDL**; Figure 2). When returning to the liver, LDL can be 'reloaded' with TG to form VLDL. High-density lipoproteins (**HDL**) have a different role, coordinating lipid transport by the exchange of apolipoproteins and also returning cholesterol from peripheral tissue to the liver.

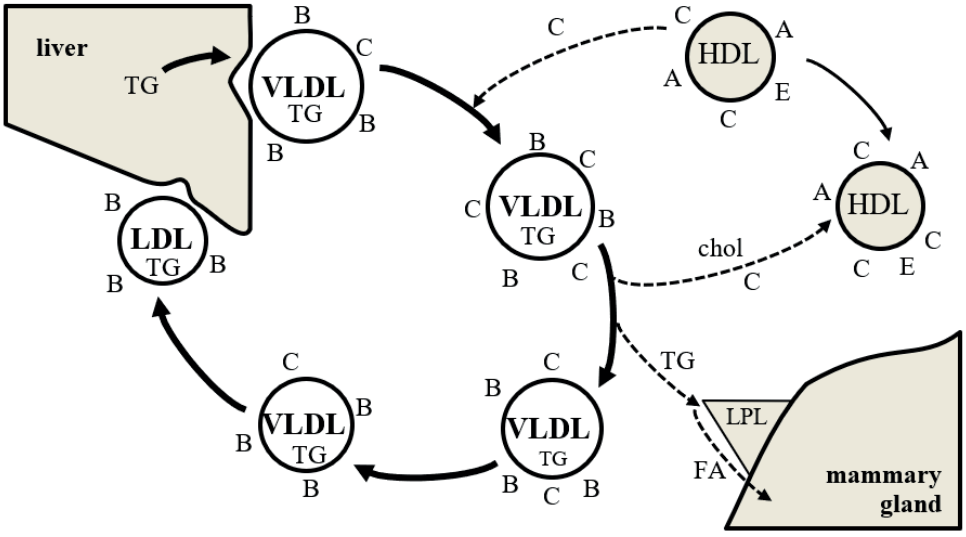

Figure 2. Schematic representation of lipoprotein conversions: exchange of lipoprotein components between VLDL, HDL and the mammary gland (dashed lines) and transformation of VLDL to LDL upon return to the liver (solid lines). Based on Palmquist (1974) and Puppione (1978).

VLDL = very low density lipoprotein
LDL = low density lipoprotein
HDL = high density lipoprotein
TG = triglycerides
chol = cholesterol
FA = fatty acids

A = apoprotein A1
B = apoprotein B100
C = apoprotein C
E = apolipoprotein E
LPL = lipoprotein lipase

In ruminants, VLDL secretion is considered to be low compared to other mammals based on its concentration in plasma, which could imply a low rate of TG export from the liver (Kleppe et al., 1988). The relevance of VLDL in liver TG regulation may, however, be larger than expected based on plasma concentrations. Supplementation with rumen-protected choline in the transition period, a precursor for phosphatidylcholine as a constituent of VLDL, resulted in

increased expression of genes relevant for VLDL synthesis (microsomal transfer protein, apolipoprotein B100) and effectively reduced liver TG accumulation in the first 4 weeks after calving (Chapter 2). These results indicate a potential to improve VLDL synthesis by supplying the components required for its assembly. In Chapter 3, VLDL was analyzed around parturition showing that the VLDL concentration decreased threefold at parturition, remaining rather low in the first weeks of lactation. The LDL and HDL concentrations, however, increased after calving. This may confirm the hypothesis that plasma concentration of VLDL in postpartum dairy cows does not reflect actual VLDL (and TG) export from the liver, as the conversion rate of VLDL to LDL may be strongly increased (Palmquist, 1974). The increased energy requirement of the mammary gland will increase turnover rate from VLDL to LDL during the transition period. The well-known increase in absolute milk fat excretion in the first weeks after parturition is largely realized by fatty acids mobilized from adipose tissue relative to fatty acids originating from de novo synthesis in the udder. In mid lactation, fatty acids require processing within the liver into VLDL, as there is no direct uptake of NEFA by the udder at normal plasma NEFA concentrations (Glascock et al., 1974). When plasma NEFA concentration is >0.2 mM like in early lactation, some direct uptake of NEFA by the mammary gland will also take place (Miller et al., 1991). The half-life of VLDL is generally short in all species and turnover from VLDL to LDL is more rapid than the extraction of LDL and HDL from the blood pool (Palmquist, 1974). In a lactating cow, the transfer of fatty acids from TG in VLDL by lipoprotein lipase to the mammary gland is extremely fast and the half-life of VLDL is short (Glascock et al., 1974) relatively increasing LDL to VLDL ratio in early lactation. More insight is required in the analytical methods and quantification of lipoprotein classes and the relation between lipoprotein concentrations and the actual status of lipid metabolism in transition cows.

1.3 Lipoprotein analysis

In studies with dairy cattle, lipoprotein concentrations are not commonly determined. This is likely due to both the limited understanding of its relevance for cow health as well as the relatively complicated analytical procedures to quantify bovine lipoprotein concentrations. Increasing the availability of reliable data on lipoprotein metabolism in periparturient dairy cattle is, however, required to better understand the variation and regulation of lipoproteins. With current modern analytical procedures, methodological limitations can be addressed to overcome the impasse in our understanding of bovine lipoprotein metabolism.

The gold standard to determine the relative abundance of the various lipoprotein fractions is (ultra-) centrifugation with salt solutions of specific densities,

separating the serum lipoproteins based on their differential density (Terpstra et al., 1981). Lipoprotein particles cannot be counted and their structure will be damaged by the ultracentrifugation procedure. Individual classes of lipoproteins are, therefore, quantified by measuring the volume of each fraction and determining cholesterol concentration within that fraction, to calculate the respective lipoprotein-cholesterol concentrations per mL of serum. Alternative measurements have been developed to reduce the costs and labour of the gold standard. Commercial kits are available to analyze the concentration of specific apolipoproteins that may provide an indication of the quantitative presence of VLDL, LDL or HDL. In addition, kits have been developed to determine HDL-cholesterol or LDL-cholesterol specifically after precipitation of the other lipoprotein classes.

A simplified method to determine the relative abundance of the three classes VLDL, LDL and HDL at low cost is developed and validated for use in human medicine by Friedewald et al. (1972). The procedure is based on two observations: 1) the mass of plasma triglyceride relative to that of VLDL-cholesterol is around 5 to 1 and relatively constant; and 2) when chylomicrons are not detectable, most plasma triglycerides are present in VLDL lipoproteins. The analytical procedures needed to calculate the different lipoprotein fractions include the measurement of plasma total cholesterol, plasma triglycerides and the determination of HDL-cholesterol. The concentration of VLDL-cholesterol is calculated by dividing total TG by 5, and subsequently the concentration of LDL-cholesterol is calculated by subtracting HDL-cholesterol and VLDL-cholesterol of total cholesterol concentration. In human, samples are collected in fasted subjects (12-14 hours after their last meal) to reduce the presence of chylomicrons to a minimum. Fasting would not be required for ruminants, where chylomicron concentration is generally low. The Friedewald method validated for human medicine is also used to determine lipoprotein fractions in dairy cows. Calculation of LDL-cholesterol in early lactating dairy cows correlated very well with enzymatically measured LDL-cholesterol with a commercial kit (correlation of 0.99 at $P < 0.001$, Kessler et al., 2014). The numerical abundance of VLDL-cholesterol is however very low in periparturient dairy cattle, requiring a high accuracy of the analytical procedure determining total triglycerides. Further validation of the Friedewald method with the gold standard ultracentrifuge method in (early lactating) dairy cattle would be required to assess the accuracy and understand the limitations of this fast method for lipoprotein transport analysis in ruminants.

1.4 Quantifying lipid processing in early lactation

Our understanding of the relevance of lipoprotein transport in ruminants, and specifically in dairy cows in early lactation, is still limited. As discussed in the previous sections, the half-life of VLDL is generally short in all species and specifically short in early lactating cows, where the mammary gland is an efficient docking station for VLDL. Table 4 shows the average plasma cholesterol concentrations of the various lipoprotein fractions in late gestation (week 2 before expected parturition) and early lactation (week 2 and week 6 postpartum), showing the relatively low VLDL-cholesterol concentration after calving.

Table 4. Average cholesterol concentration in mmol/L of plasma and lipoprotein fractions at 2 weeks before parturition, and 2 and 6 weeks after parturition in control cows of Chapter 3.

Parameter	Wk -2	Wk 2	Wk 6
Total cholesterol	3.12	2.73	5.19
HDL-cholesterol	2.08	2.05	3.23
LDL-cholesterol	0.91	0.65	1.94
VLDL-cholesterol	0.14	0.03	0.02
LDL- + VLDL-cholesterol	1.05	0.68	1.96

The VLDL-cholesterol concentration seems overall low compared with the HDL- or LDL-cholesterol in bovine plasma (Table 4). This is both related to a low number of VLDL particles present in plasma, as well as the relatively low cholesterol content of each VLDL particle compared to HDL. The VLDL particles exported from the liver are large and buoyant, due to their high TG and low cholesterol content. The TGs are hydrolyzed and taken up by the mammary gland, reducing the VLDL diameter, until it is fully transformed into an LDL particle (Figure 2). Considering potential differences in the release rate of VLDL and the uptake rate of LDL, both lipoprotein classes may be summed as the circulating pool of TG "transport vesicles" shown in Table 4 as "LDL- + VLDL-cholesterol". The decrease in week 2 after parturition can be considered part of the process of adaptation to lactation, reflecting the initial pull of the mammary gland to retrieve TG from the circulation; while at the same time the processing capacity to provide the mammary gland with TG from adipose tissue is increasing. In week 6, total TG transport capacity in LDL- + VLDL-cholesterol has almost tripled (Table 4).

Even though the plasma concentrations of VLDL-cholesterol are low in the periparturient period, the impact of VLDL export from the liver may be more relevant than expected when quantifying the mobilization of lipids from adipose tissue, their transport and processing in the liver. An estimation is displayed in

Table 5 based on the results of Chapter 3, where liver TG content of 16 cows was available in week 1 and 2 postpartum in combination with data on energy balance, blood and milk constituents. The calculations are based on actual data measured: the average of the 16 cows with liver biopsies, the cow with the highest liver TG content (cow 5876, with high BCS and no physical exercise in the dry period) and the cow with the lowest negative energy balance in week 1 and 2 postpartum (cow 5743, with high BCS and physical exercise in the dry period). To calculate the amount of preformed (long chain) fatty acids in milk, the relative milk lipid composition of week 1 and 2 postpartum reported in Chapter 2 where used.

Table 5. Average amount of lipids mobilized, transported in plasma, stored in the liver and secreted in milk during the first two weeks of lactation based on cow data of Chapter 3.

Parameter	**Average (n=16)**		**Cow 5876** with highest liver TG content		**Cow 5743** with deepest negative EB	
Week after parturition	**wk 1**	**wk 2**	**wk 1**	**wk 2**	**wk 1**	**wk 2**
Body weight (kg)	635	635	582	572	720	707
Liver TG content (g/kg)	37	44	86	138	62	68
Liver weight (kg)[1]	9.5	9.5	8.7	8.6	10.8	10.6
Liver TG storage (kg)	**0.35**	**0.42**	**0.75**	**1.19**	**0.67**	**0.73**
Liver TG change from wk 1 to 2 (kg)		**0.07**		**0.44**		**0.06**
Net energy balance (MJ/d)	-44.8	-35.8	-74.6	-60.3	-87.1	-75.3
Metabolizable energy balance (MJ/d)[2]	-56.0	-44.7	-93.2	-75.4	-108.9	-94.1
Lipid mobilization (kg/d)[3]	1.5	1.2	2.5	2.1	3.0	2.6
Ratio liver TG : daily lipid mobilization	23%	34%	30%	58%	23%	28%
Lipid mobilization (kg/wk)	**14.3**	**8.6**	**17.8**	**14.4**	**20.8**	**18.0**
Average lipid mobilization (kg/wk)		**9.6**		**16.1**		**19.4**
Milk fat production (kg/d)	1.24	1.42	1.48	1.55	1.78	1.89
Preformed milk fat (kg/d)[4]	0.73	0.82	0.87	0.90	1.05	1.10
Preformed milk fat (kg/wk)	**5.1**	**5.8**	**6.1**	**6.3**	**7.3**	**7.7**
Average preformed milk fat (kg/wk)		**5.4**		**6.2**		**7.5**

[1] average liver weight estimated at 1.5% of live body weight (Baldwin et al., 2004; Gibb et al., 1992).
[2] assuming the energy deficit being fully compensated by the release of lipids from adipose tissue, the ratio between NE and ME is 0.80 (NRC, 2001).
[3] assuming the energy deficit being fully compensated by the release of lipids from adipose tissue (36.6 MJ/kg, CVB 2021).
[4] amount of preformed fatty acids in milk (50% of C:16 and all ≥ C:17), based on milk fatty acid composition in week 1 and 2 as reported in Chapter 2, i.e. including both mobilized as well as dietary FA.

The total amount of TG stored in liver tissue is highest for cow 5876 with the highest liver TG content. For cow 5876 total TG storage accumulated up to 1.19 kg in week 2 postpartum. Focusing on the change in TG storage in the liver between week 1 and week 2 after parturition, total TG storage increased with 0.07, 0.44 and 0.06 kg for the average cow with liver biopsies taken (n=16), cow 5876 and cow 5743, respectively. The estimated amount of lipid mobilization in the same period of 7 days between week 1 and week 2 is much higher, estimated at 12.9, 21.5 and 25.9 kg. The actual TG "accumulation" in hepatic tissue in a week is very limited, compared to the total amount of lipids released from adipose tissue in the same period. A small disbalance in the processing of the vast amount of lipids mobilized in early lactation can however easily affect liver TG content. Interestingly, cow 5743 with the deepest negative energy balance in the trial (and highest rate of lipid mobilization) has relatively low TG accumulation in the liver. The amount of preformed milk fat is high in this cow, confirming the adequate processing of the mobilized lipids through the liver towards the mammary gland.

1.5 Identifying cows at risk

The efficacy of lipid processing and the risk for hepatic TG accumulation can be quite different in cows, even if lipid mobilization is high as shown for the two individual cows in Table 5. This example confirms that the variation in the success of transition management may not only depend on environmental factors of influence to a herd (heat stress, disease status), but also on individual factors. The metabolic response of individual dairy cows under the same management is variable: some cows are less susceptible to lipid related metabolic disorders. Differences in susceptibility are also shown by the resistance of individuals to a ketosis induction protocol, which might even be related to their immunometabolism, based on differentially expressed genes with a role in immune function (Pralle et al., 2021). To optimally support individual dairy cows in the periparturient period, the identification of cows at risk is key.

Body Condition Scoring (BCS)

A relatively basic and non-invasive measurement to consider potential individual differences in energy metabolism based on the risk of overfeeding in the prepartum period is the determination of BCS. In all trials presented in this study, BCS monitoring was included to investigate the relationship between BCS and a successful transition to a new lactation. However, there are some restrictions to the value of BCS with regards to methodology and relevance. First, BCS scoring by manual inspection may vary between observers and is, therefore, required to be performed by a single person to increase reliability of trial data. Second, only externally visible subcutaneous body reserves are scored (on ribs, lumbar

vertebrae and tailhead) and the relationship with internal fat accumulation around viscera in dairy cattle as well as the regulation of different types of adipose tissue are unclear. Thirdly, the relevance of BCS for a successful transition to a new lactation may again depend on other individual or environmental factors. A high BCS at calving is generally considered a risk factor for reduced DMI and performance postpartum, however, results are not consistent. A higher BCS at calving will result in a larger BCS decrease in early lactation, simply because the potential to lose BCS is larger compared with cows with a low BCS at calving. Cows with higher backfat thickness precalving mobilized a greater amount of backfat during the first weeks of lactation (Van der Drift et al., 2012; McCabe et al., 2021). For three of the 34 cows in this study, extreme mobilization resulted in high NEFA concentrations and severe hyperketonemia; for the other 31 cows, the degree of hyperketonemia did not correlate with the amount of fat mobilization (Van der Drift et al., 2012). This indicates that other factors determine whether the level of fat mobilization in early lactation can be handled by an individual cow, or results in metabolic disturbances. Likewise, there is no direct correlation between BCS at calving and liver TG accumulation in practice (Jorritsma et al., 2001).

The BCS at calving is influenced by 1) the BCS at the end of lactation, and 2) the plane of energy fed during the dry period, as described in the previous section. In a study by Bjerre-Harpoth et al. (2014), both aspects were included by 1) feeding a high or low energy diet during the final phase of lactation and 2) feeding a high or low energy diet during the dry period, in a two by two trial design with 51 cows. There was no interaction between the two phases, but overfeeding in either phase resulted in increased plasma NEFA, BHB and liver TG concentration (Bjerre-Harpoth et al., 2014). Cows fed a high energy diet during late lactation were dried off at a higher BCS (3.40 vs. 3.18), but feeding a low energy diet in the dry period reduced the risk for metabolic disturbances after calving independent of BCS at dry-off (Bjerre-Harpoth et al., 2014). On the other side of the spectrum, overfeeding thin cows in the dry period to “improve” their BCS towards calving was considered counterproductive: it does not support metabolic adaptation to the new lactation but increases the risk for metabolic disturbances (Bjerre-Harpoth et al., 2014).

In Chapter 3 the effect of prepartum exercise was described, comparing cows with low (< 3.25) and high BCS (≥ 3.25) at dry-off to investigate the interaction of management and BCS (Chapter 3). During the dry period, all animals were fed at their requirements preventing an increase in BCS towards calving. Postpartum, cows with a high BCS had higher plasma NEFA, milk fat content and liver TG

concentrations but without a reduced DMI. The strategy of physical exercise in the dry period in combination with the restricted intake during the dry period showed to be most effective for cows with a high BCS at dry off. It is, however, uncertain what the individual effects of both measures - either physical exercise or an intake restriction - were in determining the success of transition to a new lactation. Chapter 4 focused on an intake restriction without an additional strategy to increase energy requirements by physical exercise. Unlike control cows in Chapter 3 that were also fed at their energy requirements, cows in the unrestricted feeding groups of Chapter 4 had increasing BCS in the dry period while restricted cows showed a constant BCS until calving. Cow performance in early lactation did not seem to be affected by increasing BCS during the dry period but metabolic disturbances (plasma NEFA, liver TG) were reduced by feed restriction, keeping BCS stable in the dry period. This is in line with earlier results showing that high dry matter intake prepartum, at best, has no advantage over restricted energy intake (Holcomb et al., 2001).

Sensors and imaging

Technical solutions monitoring behavior are widely used at dairy farms for automated estrus detection. With fast developments in sensor technology and imaging, the automated detection of other aspects of cow behavior or general cow characteristics has regained attention for animal health and welfare purposes. The management of large herds may benefit from automated and standardized detection, when supported by algorithms to translate the data collected into specific actions when needed.

The BCS can also be detected by imaging techniques, with three-dimensional sensors or camera systems detecting anatomical points of interest (e.g. ribs, tailhead) calibrated to a 5-, 8 or 10-point BCS scale. These systems can deliver repeatable and standardized scores, without inter- or intra-observer differences with manual observations (Spoliansky et al., 2016; Albornoz et al., 2022). The accuracy of the more extreme scores is low (BCS ≤ 2 (thin) or BCS ≥ 4 (fat) on a 5-point scale), as calibration and validation are focused on the most frequently observed scores between 3 and 3.5 (Albornoz et al., 2022). Using the camera system to select cows 'at risk' – with high BCS – may, therefore, be difficult at present. Time series can still be helpful to monitor the energy balance of cows by detecting the increase and decrease of BCS in individual cows over longer periods.

The use of wearable sensors or video techniques to monitor cow behavior may also be valuable in tracing cows 'at risk' in the transition period. In a study using

neck and leg sensors in the last two weeks precalving, inactive time (negatively correlated with average eating time), irregular rumination periods and irregular bouts of standing up were good predictors for disease, where disease was defined by a "total deficit score" in week -2 to week 6 relative to calving (Van Dixhoorn et al., 2023).

Biomarkers in blood and milk

As new and wide analytical methods have become more available, also for high throughput usage, blood and milk of dairy cows prepartum can be screened for various known and unknown metabolites to discover their potential relation with the risk for transition disorders. For each technique, the interpretation of results can be challenging - but proper interpretation is of high importance. Understanding the physiological background for any relationship between the thousands of parameters that can potentially be analyzed and the subsequent success of a transition is highly relevant for the extrapolation of results to dairy cows world-wide, but also for the development of preventive measurements supporting the cows defined to be 'at risk'.

The continuous development of data processing and data analysis techniques in the field of bioinformatics further supports the fast screening of large sets of metabolites in blood ("metabotypes") for their relationship with the risk for transition disorders, or even dairy cow lifespan (Huber et al., 2016). Using proteomics to find differences between over-conditioned cows (n = 5) with a disturbed fat metabolism postpartum compared with control cows show that already 49 days before calving, cows have an increased activity of the acute inflammatory response (Ghaffari et al., 2019). A larger field study related a set of metabolic, immunologic and oxidative parameters at dry-off (51-60 days before calving) with disease incidence until day 30 postpartum shows the opportunity to use precalving biomarkers to identify cows at risk (Wisnieski et al., 2019). These predictive models, however, still require external validation studies to confirm their value in practice.

Relevant metabolites may also be reflected in milk, providing a non-invasive method to screen for potential indicators of an increased risk for transition disorders. During early lactation, the glycine content in milk, for example, was found to be related with the energy balance of dairy cattle (as shown by Xu et al., 2020). To be able to anticipate which cows require additional support in the transition period, biomarkers need to be detectable before calving. Further studies need to be performed to see if indicators in milk could be relevant

identifiers for the risk of transition disorders when analyzed in milk samples obtained at the end of the preceding lactation.

Data integration

Todays' world relies more and more on data collection and analysis. Big data is also used in the dairy sector, for example, to develop and renew breeding programs for dairy herds. The amount of and variety in data collected on individual cows is increasing fast with the help of technical on farm solutions: milk yields, milk components, physical activity, rumination or eating time, and many other parameters to come such as video imaging. Artificial intelligence will support a breakthrough here in the coming years by translating raw data from sensors and imaging techniques into relevant information such as feeding behavior or even feed intake (De Vries et al., 2023). The integration of the various individual cow parameters into early warning systems as well as prediction models for decision support will facilitate precision farming in general, and can specifically contribute to optimize management of transition cows.

2 Synergies of transition management strategies

The effect size of the different nutritional and management strategies evaluated in each of the four trials presented in this thesis are difficult to compare, due to differences in trial design (cow characteristics, duration) as well as other factors varying among the trials such as the dietary composition of the control diet or the incidence of (subclinical) hypocalcemia that may affect DMI in early lactation. This can also be illustrated with the "Utrecht fatty liver model of dairy cows" (Geelen and Wensing, 2006). In this model, high yielding dairy cows are severely overfed ante partum, resulting in a high BCS at calving. Postcalving cows were fed ad libitum or shortly fasted to further stimulate lipolysis. All overfed groups will show significantly higher liver TG concentrations than their controls. Nevertheless, even small differences in the experimental set-up (such as the duration of overfeeding or the plane of energy) will result in variation in the severity of liver TG accumulation (Table 6).

Table 6. Summary of trials in the development of the "Utrecht fatty liver model of dairy cows".

Groups (cows)	Strategy		Liver TG in mg/g wet weight		Ref
	Prepartum	**Postpartum**	**Max**	**SEM**	
	Duration 2-3 months	ad libitum feeding			1
control (8)	control: fed restricted at 48 MJ/d		68	9	
test (10)	test: fed ad libitum (~130 MJ/d)		170	23	
	Duration 8 wks	ad libitum feeding			2
control (16)	control: restricted at 48 MJ/d		31	4	
test (16)	test: ad libitum (~116 MJ/d)		86	8	
	Duration 8 wks	6 h fasting			3
control (4)	control: TMR restricted + hay ad lib		50	10	
test (6)	test: TMR restricted + grass ad lib		117	28	
	Duration 2-3 months	7 h fasting after calving	175	40	4
test (8)	fed ad libitum				
	Duration 10 wks	8 h fasting			5
control (6)	control: TMR restricted + wheat		49	9	
test (7)	straw ad lib (~44 MJ/d)		125	24	
	test: TMR restricted + grass silage ad lib (~96 MJ/d)				
	Duration 2-3 months	8 h fasting and 5 d			6
control (7)	control: fed restricted at 47 MJ/d	restricted feeding (at 70	59	12	
test (10)	test: fed ad libitum (~91 MJ/d)	MJ/d)	174	25	

References: 1 Van den Top 1996; 2 Rukkwamsuk 1999b; 3 Rukkwamsuk 1998; 4 Van den Top 1995; 5 Rukkwamsuk 1999a; 6 Murondoti 2004.

Independent of the effect size of each separate intervention tested in the present thesis, there may be synergies in a combination of different measures. Feeding rumen-protected choline (Chapter 2) in the transition period to support the adaptation to lactation can be easily combined with any of the other measures. Additional choline will ensure sufficient input of building blocks (phosphatidylcholine) for VLDL synthesis, supporting the homeostatic and homeorhetic processes. A recent meta-analysis showed that the efficacy of choline supplementation does not depend on the prepartum dietary net energy for lactation (varying between 5.9 to 7.2 MJ/kg DM in underlying studies); low metabolizable methionine intake postpartum will result in a more pronounced effect of choline (Arshad et al., 2020). Choline and methionine are both methyl donors and a deficiency in methionine can be at least partially mitigated by choline supplementation (and vice versa).

The theoretical background of increasing energy requirements through exercise (Chapter 3) and restricting energy intake below requirements (Chapter 4) are comparable: preventing a positive energy balance prepartum, to start adaptation to lactation prepartum. A combination can be practiced if the result is a prepartal energy balance at or just below requirements towards calving as in the trials in Chapter 3 and 4; pushing towards a more negative energy balance will likely reach a point at which there are no additional benefits for metabolic adaptation processes, while functional metabolic processes or even calf development may be compromised. The energy requirements for physical exercise are relatively low, compared to the difference in energy input of 1 kg DM. Walking 5 km per day can be quite a challenge to realize under practical circumstances and that would result in an additional energy requirement of 4.6 MJ of NE (Chapter 3); while 1 kg of a low energy density dry cow diet would realize ~5.4 MJ of NE (Chapter 4).

At the same time, it can also be difficult to feed individual cows at their energy requirements precalving under practical conditions, as there may be differences among group-housed cows in factors determining the energy requirements: parity, gestation length and body weight. This can be overcome by providing a low-energy basal ration, with additional concentrate on an individual level to cows that require supplementation based on their energy requirements towards the end of gestation and their feed intake. Another possibility is to separate cows at dry-off in two groups: cows with a high BCS (> 3.25) and low BCS. Feeding the same (low energy density) diet with additional physical exercise for the high BCS cows could combine the benefits of both measures at best, as the effects of exercise were particularly interesting for over-conditioned cows (Chapter 3).

Shortening or omitting the dry period reduced the effect of the dry cow diet on rumen adaption processes (Chapter 5). A combination with either physical exercise (Chapter 3) or restricting prepartum energy intake (Chapter 4) may help to prevent overfeeding in cows with a short or no dry period. The intention of omitting the dry period may sometimes not be actually reached in cases where the daily milk yield of an individual cow is decreasing already weeks before calving to a level where frequent milking is not efficient and may even be detrimental for udder health. These animals are at risk of overfeeding, as energy requirement is relatively low while energy intake is calculated based on a certain level of milk yield. Restricting energy intake to match requirements or providing additional physical exercise to increase energy requirements can be helpful. If the overfeeding due to low milk output would only take place in the final 2-3 weeks before calving, the relevance of additional measures to reduce the energy balance is limited. In experiments with overfeeding energy only in the

final 2-3 weeks before calving, no negative effects on metabolic performance where found. In a study comparing 6.9 vs. 5.4 MJ NEL/kg DM for the final 21 days until calving, cows fed the high energy diet had improved DMI and EB in the first 6 weeks of lactation with reduced plasma NEFA and liver TG concentrations (Doepel et al., 2002). Overfeeding during the far-off period starting 9 weeks before calving had a greater negative impact on metabolic adaptation after calving than overfeeding in the final 3 weeks antepartum (Dann et al., 2006).

Shortening or omitting the dry period also has an additional effect outside the rumen: the postpartum milk yield is decreased, resulting in a less negative energy balance (Van Knegsel et al., 2014). The improvement of the postpartum energy balance is in support of adaptive capacity, as the metabolic differences to overcome by homeostasis and homeorhesis are not that large. Other methods described to reduce energy output in the first weeks postpartum are a reduced milking frequency (two times instead of three times per day, Moallem et al., 2019) or a low protein deficient diet (Whelan et al., 2014).

2.1 Implementing transition management in practice

Even though the amount of knowledge on optimal transition management that has become available in the past twenty years is enormous, the incidence of metabolic disturbances in early lactation remains high. Farmers are well aware of the relevance of transition management for animal welfare and production, but attitude and behavior may not always match (Redfern et al., 2021). Based on the multifactorial background of transition disorders, there is no silver bullet to be enrolled world-wide in dairy practice. A network of support like veterinarians and nutritionists around the dairy farmer is needed to be able to analyze the current situation of the transition cow management and the potential for improvement, in line with the dairy farmers' goals. Obviously, these advisors should not provide conflicting advice based on their personal perspective of the problem, leaving the farmer in confusion (Kristensen and Jakobsen, 2011). As an example, veterinarians showed to be reluctant to ask farmers for their goals and make assumptions about farmers' motivation even resulting in a directive or paternalistic role of the advisor without success (Derks et al., 2013; Bard et al., 2017). Without understanding personal aspects and motives of the farmer, an advisor cannot succeed in creating an environment for behavioral change. A close collaboration between farmers (i.e. farm owners, staff members) and farm advisors (nutritionists, veterinarians) with effective and open communication is needed to improve transition management (Mills et al., 2020; Redfern et al., 2021).

2.2 Effect on cow health and lifespan

Improving adaptation to lactation will support the integral sustainability of dairy production (Van Knegsel et al., 2014). Worldwide the main reasons for culling are mastitis, infertility and lameness – frequently in combination with low productivity. Culling decisions are made by farmers based on animal welfare as well as economic reasons that may be related to a production system such as a seasonal grazing herd (De Vries and Marcondes, 2020). Most of the culling-related disorders find their origin in the transition period. Reducing the risk for metabolic imbalances in the transition period will, therefore, be instrumental to increase the productive lifespan of dairy cattle (Seifi et al., 2011; Huber et al., 2016). The impact of productive lifespan on the sustainability of dairy production is large, considering the environmental and economic losses of the rearing period. In the first two years of life, a dairy cow is growing towards her first lactation - without producing milk. The nutrient losses (nitrogen, phosphorus, methane) and economical investments of the first two years of life should be diluted over many productive years, as long as the individual cow can efficiently produce milk during each new lactation (De Vries and Marcondes, 2020; Van Knegsel et al., 2014).

The natural lifespan of dairy cows would be around 20 years, while the average lifespan of dairy cows in most countries with a developed dairy industry is around 4.5 to 6 years (De Vries and Marcondes, 2020). To reduce or prevent societal concern about this relatively young age at culling, an increase in lifespan is actively promoted in The Netherlands by the "Sustainable Dairy Chain" (Duurzame Zuivelketen). Lifespan is, however, a complex trait, not only affected by production and health but also by herd management, the availability of replacement heifers forcing cows out, national policies such as limiting phosphate production, and system or even personal factors of the farmer (De Vries and Marcondes, 2020; Schuster et al., 2020). Maximizing lifespan for each individual animal is no guarantee for optimal production efficiency (regarding nutrient losses or carbon footprint). Keeping each animal as long as possible will have a negative impact on herd profitability and can even negatively affect animal welfare.

The influence of nutrition or management measures potentially increasing lifespan is hard to investigate in experimental research, as it requires either a large group size with long trial duration (reaching actual culling age), or new parameters that can be used to accurately predict the risk of culling in a shorter trial. Alternatively, outcome parameters with an indirect effect on the risk for culling are used in experiments, such as a reduction of metabolic disorders in early lactation by choline supplementation in the transition period. Another

option is to reduce the relative number of transition periods per annum and thereby the risk for transition disorders and early culling, by extending lactations. Extending lactations will also reduce herd milk production and potentially increase greenhouse gas emissions per kg of milk. This may be compensated by specifically selecting heifers for an extended lactation (as heifers are generally more persistent), or individual cows that are known for a higher production persistency. The reduced milk production may be compensated by overall reduced feed costs, reduced costs for animal healthcare and by considering the improvement of lifespan a reduction of the replacement rate and costs for youngstock rearing (Burgers et al., 2022; Kok et al., 2019).

Instead of experimental research with indirect outcome parameters suggestive for lifespan, data analysis can be performed on historical data of dairy herds in practice with actual lifespan information. This type of analysis can help to define potential risk factors (e.g. age at first calving) or breeding influences (Pritchard et al., 2013). Liver TG accumulation beyond 70 mg/g wet weight is shown to be such a risk factor, associated with impaired performance, health and survival (Arshad and Santos, 2022). The influence of production level and breeding programmes towards high yielding dairy cows in the past decades has also been a topic of investigation. A low milk yield may increase the risk to be culled early, if production efficiency and, thereby, the economic efficiency of the individual cow is reduced. On the other hand, a clear antagonistic relationship exists between lifespan and milk (or fat) yield, potentially related to the higher risk for transition disorders in these animals when nutrition and management are not adequate (Pritchard et al., 2013; Schuster et al., 2020). It has been widely recognized that breeding goals should not only focus on maximal production, but also on animal health and resilience. Still, dairy farmers show difficulties in focusing on more health related traits, as production traits are more directly related to farm economics and emission reduction (Rilanto et al., 2022). Further support and education is required here to help dairy farms find an optimal production level integrating farm economics, animal health and environmental sustainability.

3 Integration in a sustainable dairy sector

At present the dairy chain faces several challenges towards increasing sustainability of dairy production. Dairy farmers need to comply with environmental sustainability goals such the reduction of methane emission, phosphate production limits and reduction of nitrogen losses. Other topics of concern regarding our environment are the carbon footprint of livestock diets,

the worldwide loss of biodiversity and the pressure on arable land use (feed, food and fuel competition) to provide the growing world population with sufficient food. Reducing methane and nutrient losses should therefore be aligned with local feed sourcing and a reduction of the amount of human-edible ingredients in the dairy cow ration. For the Dutch dairy sector, depending on the development of agricultural policies and alternative revenue models, the combination of these environmental challenges may reduce the number of farms towards 2030 with 33 to 50%, while the remaining farms will increase their herd size (Beldman et al., 2020). The effects of the various environmental goals on other sustainability goals, such as the effect on animal health and welfare or the economic viability of the dairy industry, is uncertain. The overall sustainability of individual dairy farms as well as the dairy production chain needs to be considered from an integral perspective.

The anticipated changes in nutrition and management in dairy practice in the coming decades will influence the outcome of the proposed strategies to improve transition cow health in the present thesis. Specifically, the reduction of nitrogen and phosphorus losses and the change in diet composition based on local sourcing can be directly related to the transition period strategies.

3.1 Nitrogen

The reduction of nitrogen losses may have the largest impact on transition management. To improve nitrogen efficiency of dairy production, dietary protein concentration will be reduced. The effect of reducing prepartum protein level towards calculated requirements on postpartum performance is limited. A meta-analysis showed that for primiparous cows a reduction of prepartum protein intake below 14-15% crude protein (1,100 g/d metabolizable protein) may reduce pre- and postpartum DMI, milk yield and milk protein yield. For multiparous cows, there was no effect on DMI or milk yield, but milk protein production was somewhat limited when metabolizable protein supply decreased (Husnain and Santos, 2019). No interactions were found between the metabolizable protein level fed prepartum and the protein level in the first weeks postpartum (Underwood et al., 2022).

Other than the effect of feeding protein above calculated requirements, a protein deficiency prepartum may have negative effects, by reducing the capacity to adapt to the new lactation. This could increase the risk for both metabolic and infectious disorders. Studies with a protein deficiency prepartum while feeding energy at requirements are however very scarce. Two studies in beef cattle show that there may not be an effect on birth weight, milk production (López

Valiente et al., 2018) or colostrum quality, but IgG absorption in calves may be reduced (Blecha et al., 1981). Data on dairy cow or calf health and performance are, however, unavailable. On the other hand, an intentional protein deficiency in early lactation could also have a positive outcome, if this dietary protein restriction will limit total milk production and, therefore, energy output in early lactation, improving overall energy balance (Whelan et al., 2014).

Although the impact of dietary protein levels on performance is relevant, the effect of reducing protein content was out of the scope of the present thesis. To prevent interactions of energy and protein metabolism in the four experiments, protein intake was controlled by feeding at calculated protein requirements in each trial (crude protein 12% prepartum and 16-17% postpartum; intestinal digestible protein ~55 g/kg DM prepartum and ~97 g/ kg DM postpartum). Postpartum, however, protein intake cannot be matched with the high requirements of early lactation, resulting in a negative intestinal digestible protein balance. Sustainability strategies to further reduce protein intake in dairy cows throughout a full lactation should, therefore, always consider the potential effects of decreasing protein intake in the transition period. A deficiency of the amino acid methionine will increase the need for other methyl donors such as choline, for example, resulting in an even more pronounced effect of choline supplementation around parturition (Arshad et al., 2020). Choline itself ($C_5H_{14}NO$) also contains nitrogen but supplementation with 14.4 g choline per day would relate to only ~0.4% of total crude protein intake.

3.2 Phosphorus

The strong focus on nitrogen reduction also renewed the interest in phosphorus intake of dairy cattle. The maximal allowance of animal manure to be applied per hectare reduced over the past decade in The Netherlands, reducing phosphorus application on grassland. Since 2017, the average phosphorus concentration of compound feed was also restricted, reducing the phosphorus input on farms. Farmers, feed advisors and veterinarians worried about a decreasing phosphorus content of grass and eventually dairy diets, especially for transition cow health, based on four points: 1) the calcium and phosphorus metabolism in dairy cows are linked through their shared storage in bone as calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); 2) directly after calving, phosphorus requirements increase steeply with the initiation of lactation; 3) around parturition, dairy cows often have (very) low plasma phosphorus levels, and 4) some cases of “Downer cows” – cases of periparturient paresis that do not recover after calcium balance therapy – have been anecdotally linked to hypophosphatemia. Some veterinarians and farmers advocate oral supplementation of phosphorus in cases of hypocalcemia,

next to the restoration of calcium balance with intravenous calcium infusions and oral calcium supplements. The scientific evidence for a relation between low phosphorus intake and parturient paresis is, however, lacking. The decrease in plasma phosphorus around parturition is a common phenomenon in dairy cattle, not necessarily related to clinical disease. It is not associated with the loss of phosphorus through milk production, as the same drop in plasma phosphorus concentration also occurs in mastectomized cows around parturition (Goff et al., 2002). The plasma phosphorus decrease may be related to a combination of reduced feed intake around calving, an increase in plasma corticosteroids, and more generally a redistribution of intracellular and extracellular phosphorus (Grünberg, 2014). Testing different phosphorus levels in the transition period showed that dairy cows can handle relatively low levels of phosphorus intake (~70% of requirements) well in the first 8 weeks of lactation (Keanthao et al., 2021). Strikingly, overfeeding phosphorus relative to requirements in the dry period even increases the risk for hypocalcemia in the first week postpartum (Keanthao et al., 2021; Cohrs et al., 2018). Still, caution is warranted regarding strongly deficient diets in the first weeks of lactation (~55% of requirements) as feed intake may be strongly reduced (Grünberg et al., 2019) or long term feeding of phosphorus deficient diets (~67% of requirements for almost two lactations (Valk et al., 1999)).

3.3 Glucogenic nutrients

The inclusion of more locally grown feeds and human-inedible feed sources will under Western European circumstances result in a grass-based diet with by-products from the food industry like rapeseed meal, dried distillers grains, sugar beet pulp and potato pulp, instead of human-edible cereal grains or soybean meal. These ingredients will reduce the dietary starch content and increase the amount of crude fibre in the ration. For mid-lactating dairy cows this can be realized without differences in production performance, but it may decrease nitrogen efficiency (Karlsson et al., 2018; Pang et al., 2018). For early lactating cows, this change may have a stronger impact because the amount of lipogenic nutrients will increase, relative to the glucogenic nutrients. A shift towards a more lipogenic diet may impose a risk on transition cows, as glucogenic diets help to improve the calculated energy balance by reducing milk energy output and support gluconeogenesis, which is important in the homeostatic and homeorhetic processes of early lactation reducing the risk for metabolic disturbances (Van Knegsel et al., 2007; Chen et al., 2016). A lack of glucogenic nutrients is particularly relevant in the first 8 weeks of lactation, as the introduction of glucogenic nutrients after ~40 days in milk will improve milk yield but will not improve the energy balance (Van Hoeij et al., 2017).

4 Conclusions

Based on the result of the four experiments presented in this thesis, there is a clear potential for nutritional and management strategies supporting metabolic adaptation to lactation in an early stage, precalving. Preparing the liver prepartum for the increased metabolic requirements of lactation improves metabolic capacity and may help to reduce the incidence of metabolic disturbances. Immediately postpartum, high yielding dairy cows show to be capable of handling large amounts of fatty acids mobilized daily from their body reserves. Small disturbances in lipid processing may, however, have strong effects on lipid accumulation, and hepatic TG concentrations varied widely between individuals and trials presented in this thesis. A better understanding of lipid processing and transport in ruminants is still required to find potential leverage points to further improve nutrition and management of transition cows. To optimize transition management, early identification of individual cows with a reduced adaptation capacity towards a new lactation is instrumental. Identification of cows requiring additional support may be realized by biomarkers in blood or milk, or information collected by sensors or imaging techniques introduced in dairy barns.

The current environmental challenges worldwide have their impact on the dairy sector and the environmental goals imposed on dairy farms will also affect nutrition. Nitrogen losses and greenhouse gas emissions need to be reduced, while moving to a more circular agri-food system, all affecting the composition and nutritional value of (transition cow) diets. A focus on one or few environmental goals may result in a loss in other fields, such as dairy cow health and lifespan. All interventions should, therefore, be considered in an integral approach, including possible side-effects on dairy sustainability including cow health and welfare – especially when it comes to the process of adaptation to lactation. Dairy farmers may require support from practical tools and technical advisors to define the optimal transition period management for their specific farm situation and individual cows, while taking into account the overall effects on sustainability of the dairy farm.

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CURRICULUM VITAE

Roselinde Goselink was born on 26 November 1983 in Hengelo (the Netherlands) and grew up on her family's dairy farm. Following her graduation from the Grundel Lyceum in Hengelo, she started studying Veterinary Medicine at Utrecht University in 2001. Prior to starting her internships, Roselinde participated in the Honours Programme (Excellent Trace), resulting in a thesis on "Oxidative stress and antioxidant defense in high yielding dairy cattle postpartum" and an MSc grade in Veterinary Research in 2006.

After achieving her veterinary degree in November 2008, Roselinde enjoyed a break travelling in Central America and engaging in some temporary jobs. In March 2009, she started as a researcher in ruminant physiology and health at the department of Animal Nutrition of Wageningen Livestock Research (formerly known as "Veehouderij BV") located in Lelystad. Here her primary responsibilities included acquiring and leading research projects focused on dairy cattle health and nutrition, often conducted on research farm De Waiboerhoeve. To strengthen ties with Wageningen University, the institute relocated to Wageningen Campus in 2015 while the research farm transformed into Dairy Campus in Leeuwarden. She continued her work as a researcher in various projects and public-private collaborations such as Feed4Foodure and Duurzame Zuivelketen. She was involved in the organization of various symposia and the 16th International Conference on Production Diseases in farm animals (ICPD).

Upon her return from maternity leave in 2020, Roselinde assumed the role of Head of the department of Animal Nutrition. The department currently holds a team of 45 employees working in ruminant, swine, poultry and fish nutrition, roughage and insect production for a sustainable food system.

Roselinde Goselink is the daughter of Herman Goselink and Agnes Goselink-Borre and the younger sister of Anton. The familial dairy farm is still active, in the hands of Roselinde and her partner Bart Withag. Together they are raising a family with their daughter Jolijn, born on 19 February 2020, and their son Niek, born 23 December 2022.

EDUCATION CERTIFICATE

Graduate School WIAS

A. The Basic Package (3 ECTS)	year
WIAS Introduction Day	2010
WGS Ethics in Animal Sciences course or alternative ethics course	2013

B. Disciplinary Competences (13 ECTS)	year
Preparing own PhD research proposal	2010
WIAS course Epigenesis and epigenetics, Wageningen (the Netherlands)	2011
PhD course Fatty acids in relation to product quality and health, Ghent University	2012
VLAG course Energy metabolism and body composition	2016
Wageningen Academy Cursus Rundveevoeding	2015
Wageningen Academy Cursus Levensduur	2015
Nutrition in High Yielding Dairy Cattle	2012-2016

C. Professional Competences (10 ECTS)	year
Course "Projectmanagement Basis"	2009
Commercial skills training, Kenneth Smit Training	2011
Wageningen UR Young Management Development course	2014
Brainbox Mediatraining	2015
CAN International Symposium on Dairy Cattle Nutrition	2011-2022

D. Societal Relevance (3 ECTS)	year
Koeien & Kansen workshop Voeding Melkvee, Lelystad	2011
Themadag "Voeding van hoogproductief melkvee", Wageningen	2008
159th Meeting of the NVWV "De wens van de pens", Hengelo	2011
Koeien & Kansen workshop Voeding Melkvee, Hengelo	2011

E. Presentation Skills (5 ECTS)	year
ANR Forum day (NVO), Roeselare (Belgium)	2013
15th International Conference on Production Diseases in Farm Animals (Sweden)	2012
ADSA ASAS Joint Annual Meeting, Phoenix AZ (USA)	2012
4th General Meeting DAIRYMAN, Teagasc, Moorepark (Ireland)	2011

F. Teaching competences (6 ECTS)	year
Supervision 2 BSc thesis students	2010-2011
Supervision 2 MSc thesis students	2011-2012
Teaching and preparation for various courses	2010-2022

Education and Training Total:	**40 ECTS**

One ECTS credit equals a studyload of approximately 28 hours

COLOPHON

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