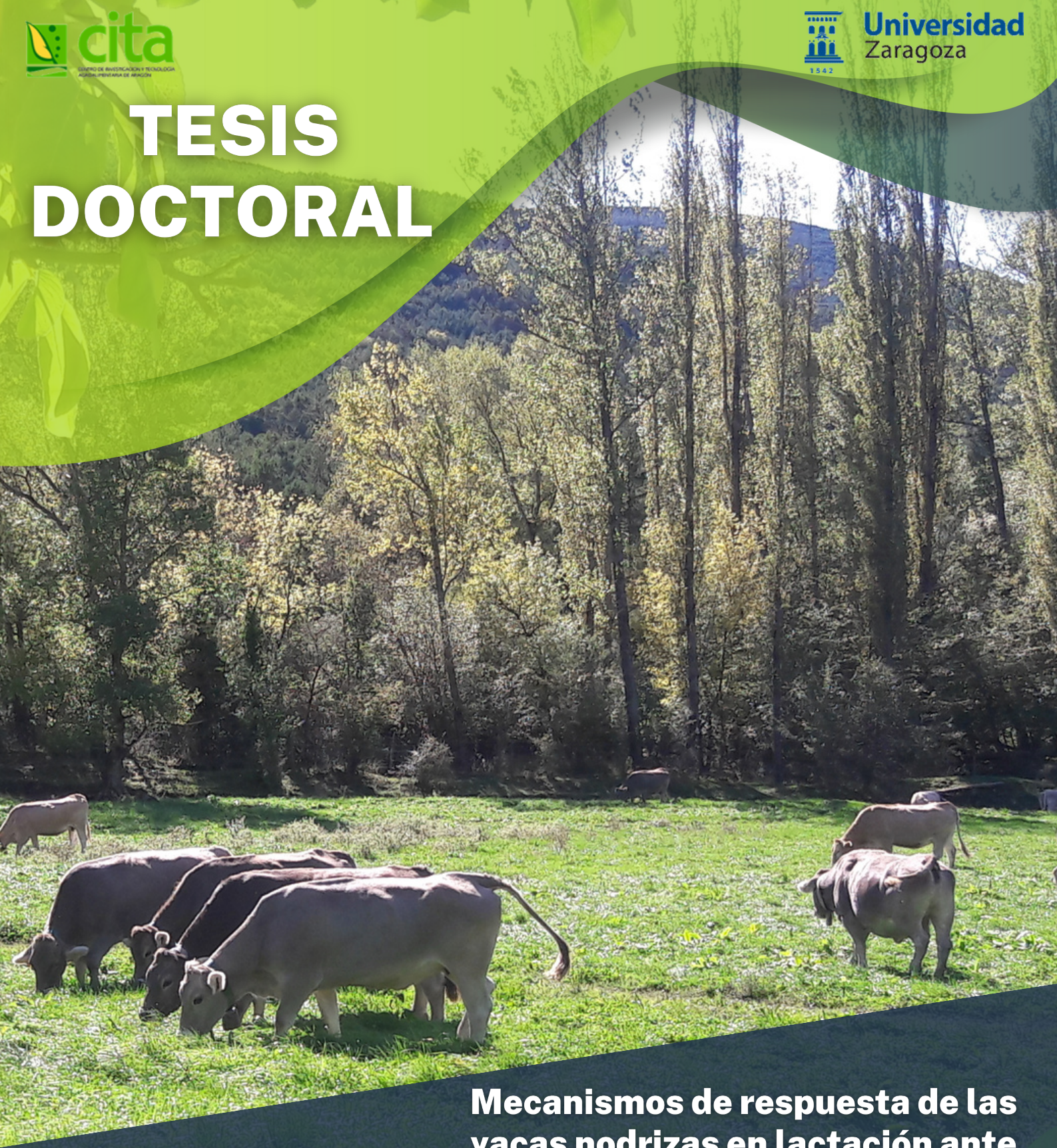
cita
CENTRO DE INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA DE ARAGÓN

Universidad Zaragoza
1542

TESIS DOCTORAL



Mecanismos de respuesta de las vacas nodrizas en lactación ante retos alimenticios breves

KARINA GABRIELA ORQUERA ARGUERO

Julio 2023

Facultad de Veterinaria
Departamento de Producción Animal y Ciencia de los Alimentos

TESIS DOCTORAL

Mecanismos de respuesta de las vacas nodrizas en lactación ante retos alimenticios breves

Desarrollada por

Karina Gabriela Orquera Arguero

Para optar al grado de Doctor por la Universidad de Zaragoza.

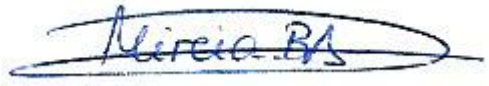
Mayo 2023

CERTIFICACIÓN DE LAS DIRECTORAS DE TESIS

La **Dra. Isabel Casasús Pueyo** y la **Dra. Mireia Blanco Alibés**, Investigadoras del Departamento de Ciencia Animal del Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA),

HACEN CONSTAR Que **Karina Gabriela Orquera Arguero** ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada "Mecanismos de respuesta de las vacas nodrizas en lactación ante retos alimenticios breves", que corresponde con el proyecto de Tesis aprobado por la comisión de Doctorado, y que cumple con los requisitos exigidos para optar al grado de Doctor por la Universidad de Zaragoza, por lo que autorizan su presentación para que pueda ser juzgada por el Tribunal correspondiente.

Lo que suscribimos como directoras del trabajo, en Zaragoza, a 03 de mayo de 2023.



Dra. Isabel Casasús Pueyo

Dra. Mireia Blanco Alibés

Esta Tesis Doctoral se ha realizado en el Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) en el marco del proyecto de investigación “GENomic management Tools to Optimize Resilience and Efficiency GenTORE” (Proyecto financiado por la Unión Europea dentro del Programa de Investigación e Innovación H2020, convenio de subvención Nº 727213, Investigadora responsable: Isabel Casasús Pueyo).

La doctoranda ha disfrutado de un contrato predoctoral financiado por el Gobierno de Aragón asociado al proyecto “Respuesta productiva y metabólica de vacas nodrizas ante una subnutrición breve e intensa al inicio de la lactación”, publicado en la convocatoria del de 20157(BOE). Convocatoria 2017-2021. Durante este período, ha formado parte del Grupo de Investigación “Sistemas agroganaderos alimentarios sostenibles (SAGAS)”, A14_17R y A14-20R, reconocido por el Gobierno de Aragón.

La publicación de los resultados de esta tesis se ha realizado con el apoyo del Grupo de Investigación “Investigación en Producción Agroganadera Sostenible y Saludable (INPASS)”, A25_23R.

Agradecimientos

Escribir este apartado lleva plasmados muchos sentimientos ya que esta Tesis doctoral ha sido el resultado de un largo camino que, aunque se veía lejano ha llegado a su fin. Mirar atrás y ver todo lo que tuve que recorrer para llegar aquí me llena de muchos sentimientos y emociones y sin duda alcanzarlo ha sido una mezcla de experiencias, vivencias y muchos "challenges". Sin embargo, haberme cruzado por el camino con varias personas ha sido fundamental para llegar a la meta. Por esto y más quiero agradecer:

A mis directoras Isabel y Mireia por su confianza, paciencia y sus cuidadosas revisiones. Aunque ha sido una época un tanto complicada en disponibilidad de tiempo, agradezco el esfuerzo y dedicación para concluir esta tesis. Indudablemente su guía profesional y personal a lo largo de todo este tiempo ha sido fundamental.

A todo el personal de la Garcipollera, a José Manuel, Nasko, Inazio, Javi, Miguel Angél y Einar por todo su trabajo para desarrollar el experimento. Pero gracias sobre todo por ser buenas personas, siempre amables y estar dispuestos a ayudarme en cualquier momento dentro y fuera de la finca. Sin su ayuda y compañía todo el tiempo vivido en Béscos no hubiese sido el mismo. Mención aparte tienen Tote y Elena gracias por todo el tiempo que compartimos juntos, por las comidas, cenas e "interrogatorios", pero especialmente gracias por hacerme sentir como en casa y que era parte de la familia. Siempre tendrán un lugar especial en mi corazón.

Al equipo de digestibilidad, a Angelines, Andrés y Juanra porque, aunque mi estancia en el laboratorio fue muy breve siempre estuvieron dispuestos a ayudarme profesional y personalmente y hacer que este trabajo fuese mucho más sencillo.

Al personal de la casa amarilla Begoña, Dani, Ernesto, Guillermo, Marga, Sandra, con quienes he coincidido en mayor o menor medida, pero de quienes me llevó los buenos momentos compartidos. Y en especial a Albi por su cercanía, disponibilidad a ayudarme en cualquier aspecto y sus abrazos reconfortantes.

A Dani Villalba de la Universidad de Lleida, por todo el trabajo y tiempo invertido para que "las curvas nos diesen respuesta". Por su disponibilidad cada vez que necesitaba ayuda, por su cercanía, amabilidad y por resolver TODAS mis dudas con R.

Mención especial tienen mis compañeros del CITA, que durante estos 4 años y más a algunos puedo decir que más que compañeros les considero amigos. Como dicen por ahí los amigos son la familia que uno escoge, y es lo que ellos han sido. Gracias por todos los momentos vividos, paseos, comidas, cenas, coffees, y solo unas cuantas cervezas compartidas en este tiempo. A Agustí, quién tiene un lugar especial para mí, por su cercanía y por compartir tantas aventuras, pero sobre todo por ser mi

“guía espiritual” en estos años y sacarme ciertos demonios. A Clàudia y a Enrique que entre horas de “terapia” y horas dentro de la casa amarilla han sido con quienes he coincidido y compartido más tiempo; recorrer, aprender y contar con su ayuda a lo largo de este camino ha sido muy importante. A Kenza, a quién he conocido más fuera del CITA, por ser compañera de inicio a fin en este largo viaje, al final parece que lo vamos a lograr. A María, aunque dentro del CITA coincidimos poco tiempo, ha estado en muchos momentos, por su amistad, buena vibra, locura y apoyo en todos los aspectos. Y a la nueva generación Alicia y Olaia, aunque hemos compartido poco tiempo ha sido maravilloso conocerlas y compartir cosas. A Leire, quién ha llegado en un momento importante, por su cercanía, por su vibra y su gran energía, pero sobre todo por sus acciones que me han hecho sentir muy bien. A Manjola, con quién el día a día es muy agradable y gracias por estar cuando lo he necesitado. Gracias a cada uno porque sin duda han hecho que esta etapa haya sido mucho más fácil de llevar y porque definitivamente este “desmierde” sin su compañía no hubiese sido igual.

Otra parte fundamental para este logro son los que están del otro lado del charco, separarme de ellos no ha sido nada fácil y dicen que el tiempo todo lo cura, sin embargo, mientras más pasa el tiempo el estar lejos de los tuyos duele más, pero al mismo tiempo valoro grandemente su presencia en mi vida.

A mis padres, Ramiro y Silvana gracias por todo su apoyo invaluable y ayudarme a abrir las alas y volar del nido, por enseñarme tantas cosas; llegar aquí y ahora no hubiese sido posible sin ellos. Gracias mami por ser ejemplo de lucha constante e incansable, ser inspiración, pero sobre todo por demostrarme tu infinito amor. A mi hermana y mi sobri, Pame y Ame, simplemente gracias por ser mi soporte, mi refugio y mi motivo para seguir adelante. A mi abuelita Lucita y a mi tía Nancy, gracias por tanto amor y por tener siempre palabras llenas de cariño y dulzura. Gracias a toda mi familia porque cuando yo no he sido capaz de confiar en mí ellos sí lo han hecho.

A mis amigos, algunos de los cuales ya llevamos caminando más de media vida juntos, gracias por tener palabras de apoyo y llenarme el corazón de buenos momentos, pero especialmente por hacerme sentir que, aunque la distancia cada vez pesa más, la amistad verdadera todo lo puede.

Y finalmente pero no menos importante a Marco, quizá el destino lo había planeado que coincidiéramos en tiempo y lugar. Gracias por estar de principio a fin en esta etapa y haber compartido y vivido tantísimas cosas. Gracias por ser mi diario confidente, por escucharme y hacerme ver las cosas desde otras perspectivas, pero sobre todo por la grande paciencia que a veces debes tener conmigo.

Resumen/Summary

RESUMEN

Los sistemas extensivos de producción de vacas nodrizas están expuestos a grandes variaciones en la disponibilidad y calidad de alimentos a lo largo del año. Esto puede verse agravado como consecuencia del cambio climático, ya que las alteraciones en temperatura y precipitaciones y una mayor frecuencia de fenómenos extremos pueden dar lugar a periodos de escasez de alimentos de manera más o menos repetida. En este contexto, se hace necesario la búsqueda de animales que afronten y se recuperen rápidamente de dichas restricciones, además de profundizar en el conocimiento de los mecanismos que desencadenan la respuesta adaptativa y los factores individuales de los que depende.

El objetivo de la presente tesis fue evaluar la resiliencia en vacas nodrizas lactantes, para lo que se estudió su respuesta de adaptación ante periodos cortos e intensos de restricción-realimentación en diferentes meses de la lactación. Para ello se alimentaron 32 vacas con una dieta formulada para cubrir el 100% de sus necesidades energéticas y proteicas desde el parto hasta el cuarto mes postparto, exceptuando las fases de restricción, en las que la dieta cubrió solo el 55% de dichas necesidades. En el segundo, tercer y cuarto mes de lactación las vacas se sometieron a periodos cortos de restricción (4 días) y realimentación (4 días), y se evaluó la repercusión del mes de lactación sobre sus rendimientos y su metabolismo. En el cuarto mes de lactación se realizaron tres retos nutricionales consecutivos (4 días) con 3 días de recuperación, para evaluar una potencial habituación ante una perturbación repetida.

Dada la elevada variabilidad individual, se agrupó a las vacas en función de su respuesta ante los cambios de dieta. Para ello se modelizaron las curvas de la producción de leche y la concentración plasmática de ácidos grasos no esterificados (NEFA) y β-hidroxibutirato (BHB) en las vacas sometidas a la restricción-realimentación en los tres meses de lactación y también en los tres retos consecutivos. De estas curvas se obtuvieron nuevas variables, a partir de las cuales se identificaron dos perfiles de respuesta metabólica: alta y baja.

Las vacas del grupo de alta respuesta metabólica tuvieron mayor producción de leche, concentración plasmática de NEFA y BHB y respondieron más intensamente a la restricción, lo que indicaría una mayor movilización lipídica, que las de baja respuesta. Esto sugiere que la respuesta fue impulsada por el potencial lechero de las vacas y que, a pesar de su mayor producción de leche, las primeras fueron capaces de activar las vías metabólicas adecuadas para responder y recuperarse del desafío. En los retos repetidos, las vacas del grupo de alta respuesta tuvieron mayor producción de leche,

con respuesta más rápida ante cambios en la dieta, y mayor concentración de NEFA y BHB en los dos primeros retos que las vacas de baja respuesta metabólica.

El mes de lactación afectó a todos los parámetros evaluados, observándose una disminución tanto en la producción de leche como en las concentraciones basales (previas a los retos) de algunos metabolitos plasmáticos a medida que avanzaba la lactación. En la mayoría de los parámetros el efecto de la restricción dependió del mes de lactación. En cuanto a la leche, la restricción alimenticia indujo una pérdida de producción, disminución del contenido en proteína y aumento de la urea, de diferente magnitud en los distintos meses. Las concentraciones plasmáticas de NEFA aumentaron con la restricción en los tres meses, mientras que las de BHB y urea sólo aumentaron en el mes 4, y recuperaron sus valores basales durante la realimentación. A pesar del escaso efecto en la cantidad de grasa de la leche, la restricción afectó a su composición en ácidos grasos. Se observó una disminución inmediata de los ácidos grasos saturados, *de novo* (C4 a C15:1) y mixtos (C16:0+C16:1), mientras que los ácidos grasos mono-, poli-insaturados y de movilización (>C17:0) aumentaron durante la restricción. Los cambios se revirtieron inmediatamente durante la realimentación. Estos cambios se correlacionaron estrechamente con las diferencias en el balance energético y la concentración plasmática de NEFA.

En cuanto a la respuesta ante los retos repetidos, la restricción disminuyó la producción de leche en mayor proporción tras el primer reto, mientras que la urea de la leche respondió por igual en todas las repeticiones, sin afectar de manera clara al resto de los componentes de la leche. Las concentraciones plasmáticas de NEFA, BHB (solo en las vacas de alta respuesta) y urea se incrementaron en los tres retos de manera similar durante la restricción. Al final del experimento, las vacas recuperaron los valores basales en todos los casos.

Estos resultados sugieren que las vacas de carne utilizan diferentes estrategias de adaptación para hacer frente a los retos nutricionales a medida que avanza la lactación, predominando la movilización de la grasa corporal al inicio y el catabolismo proteico en etapas posteriores. La respuesta metabólica no difirió entre retos repetidos, sin observarse signos de habituación ni de sensibilización. La identificación de vacas con diferentes perfiles de respuesta metabólica ante una reducción en el aporte de nutrientes puede ser útil tanto para la toma de decisiones en la explotación, como para la selección de animales más resilientes ante las variaciones en la disponibilidad de recursos alimenticios.

SUMMARY

Extensive suckler cow production systems are exposed to large variations in feed availability and quality throughout the year. This can be exacerbated as a consequence of climate change, because alterations in temperature and precipitation and a higher frequency of extreme events can lead to more or less repeated periods of feed shortage. In this context, it is necessary to identify animals that cope with and recover quickly from restrictions, as well as to determine the mechanisms that trigger this response and the individual factors on which it depends.

The aim of the present thesis was to evaluate resilience in lactating beef suckler cows, by analyzing their adaptive response to short and intense periods of restriction-refeeding in different months of lactation. For this purpose, 32 cows were fed a diet formulated to meet 100% of their energy and protein requirements from calving until the fourth month postpartum, except for the restriction periods, when the diet only met 55% of these requirements. In the second, third, and fourth months of lactation, cows were subjected to short periods of restriction (4 days) and refeeding (4 days), where the impact of the month of lactation on their productive performances and metabolism was evaluated. In the fourth month of lactation, three repeated nutritional challenges (4 days) with 3 days of recovery were performed to evaluate the potential habituation response to repeated disturbances.

Given the high individual variability, cows were grouped according to their response to dietary changes. The curves of milk yield and plasma concentration of nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) were modeled in the cows in response to feed restriction-refeeding both in the three months of lactation and during the three repeated challenges. New variables were obtained from these curves, from which two metabolic response profiles were identified: high and low.

Cows in the high metabolic response cluster had higher milk yield, NEFA, and BHB plasma concentration and responded more intensely to restriction, which would indicate a greater lipid mobilization, than those in the low metabolic response cluster. This suggests that the response was driven by the cows' milk potential and that, despite their higher milk yield, the former were able to activate the suitable metabolic pathways to respond to and recover from the challenge. During the repeated challenges, cows in the high metabolic response cluster had higher milk yield and responded more quickly to the diet, and also had higher concentrations of NEFA and BHB on the first two challenges than their counterparts of the low metabolic response cluster.

The month of lactation affected all the studied traits, a reduction in both milk yield and basal (pre-challenge) concentrations of some plasma metabolites being observed as lactation progressed. In most parameters, the effect of restriction depended on the month of lactation. Feed restriction induced a loss of milk yield, a decrease in protein and an increase in urea milk content, of different magnitude in the different months. Plasma concentrations of NEFA increased with restriction in all three months, while those of BHB and urea increased only in month 4, and recovered their basal values during refeeding. Despite the minor effect on milk fat content, restriction affected the milk fatty acid composition. An immediate decrease in saturated, *de novo* (C4 to C15:1) and mixed (C16:0+C16:1) fatty acid was observed, whereas mono-, poly-unsaturated and mobilization (>C17:0) fatty acid increased during restriction. The changes were immediately reversed during refeeding. These changes correlated closely with differences in energy balance and plasma NEFA concentration.

Regarding the response to repeated challenges, restriction decreased milk yield in a greater proportion after the first challenge, while milk urea responded similarly across all repetitions, with no clear effect on the rest of the milk components. Plasma concentrations of NEFA, BHB (only in the high metabolic response cows), and urea increased in the three challenges similarly during restriction. At the end of the experiment, cows recovered basal values in all cases.

These results suggest that beef cows use different adaptive strategies to cope with nutritional challenges as lactation progresses, with body fat mobilization predominating at the beginning and protein catabolism at later stages. The metabolic response did not differ between consecutive challenges, with no signs of habituation or sensitization to repeated exposure. The identification of cows with different metabolic response profiles to a reduced nutrient supply may be useful both to support decision making at the herd level and for the selection of animals which are resilient to variations in the availability of feed resources.

Índices

ÍNDICE DE CONTENIDOS

ÍNDICE DE TABLAS

I. ANTECEDENTES

III. MATERIAL Y MÉTODOS GENERALES

CAPÍTULO I:

CAPÍTULO II:

CAPÍTULO III:

CAPÍTULO IV:

ÍNDICE DE FIGURAS

I. ANTECEDENTES

III. MATERIAL Y MÉTODOS GENERALES

CAPÍTULO I:

CAPÍTULO II:

CAPÍTULO III:

CAPÍTULO IV:

LISTA DE ABREVIATURAS

ADF	acid detergent fiber
ADL	acid detergent lignin
a.s.l.	above sea level
AUC	area under de curve
BCS	body condition score
BE	balance energético
BHB	β- hydroxybutyrate
BW	body weight
CC	condición corporal
Cl	cluster
CP	crude protein
CV	coefficient of variation
d	day
Dim	dimension of the principal components
DIM	days in milk
DM	dry matter
DMI	dry matter intake
dpp	días postparto
EB	energy balance
EDTA	ethylenediaminetetra-acetic acid
EN	energía neta
FA	fatty acid
FAME	fatty acid methyl esters
FP	feeding period
IGF-1	factor de crecimiento similar a la insulina I
GLM	general linear model
M	month
MDA	malondialdehyde
MIXED	mixed linear model
MR	metabolic response

MS	materia seca
MUFA	monounsaturated fatty acid
MY	milk yield
NDF	neutral detergent fiber
NE	net energy
NEFA	nonesterified fatty acid
P	probabilidad de error
PCA	principal components analysis
PDI	true protein digestible in the small intestine
PUFA	polyunsaturated fatty acid
r	coeficientes de correlación de Pearson
ROS	reactive oxygen species
RSD	residual standard deviation
SCC	somatic cell count
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SFA	saturated fatty acid
UFL	unité fourragère lait

I. Antecedentes

1.1. La producción de vacuno de carne

1.1.1. Sistema productivo y manejo general

España se sitúa como el cuarto país en importancia en la Unión Europea en cuanto a la producción de vacuno de carne, detrás de Francia, Alemania e Irlanda, aportando el 8% del censo bovino y el 10% de la carne de vacuno producida (700 mil toneladas). Este sector representó a escala nacional alrededor del 5,7% de la Producción Final Agraria y el 15,3% de la Producción Final Ganadera en 2021, con un censo de 6.576.296 cabezas, distribuidas en 144.540 explotaciones (MAPA, 2022).

La producción de vacuno de carne está conformada por dos subsectores: el de la vaca nodriza y el del cebo de terneros, los cuales son complementarios pero que presentan localizaciones y modelos de producción diferentes. Además de su importancia económica, el sector vacuno de carne tiene un relevante impacto social y ambiental. Las explotaciones de vacas nodrizas por lo general se encuentran en zonas desfavorecidas, por lo que esta actividad contribuye a generar empleo y a fijar población en el medio rural. Su desarrollo en sistemas extensivos o semi-extensivos permite el aprovechamiento de recursos naturales pastables, así como mantener la diversidad genética de la cabaña bovina con razas adaptadas a las condiciones climáticas y edafológicas de España (MAPA, 2021).

Las vacas nodrizas se ubican principalmente en la dehesa y pastizales del oeste y suroeste, en la cornisa cantábrica y en zonas de montaña como los Pirineos (MAPA, 2022). Su alimentación se basa principalmente en el aprovechamiento de forrajes ya sea directamente mediante pastoreo o por conservación de éstos para ser aportados en periodos de escasez. Estos recursos naturales dependen de varios factores como condiciones climáticas y las interacciones animal-planta-suelo, entre otras, que condicionan tanto su disponibilidad como su valor nutritivo. En particular, las zonas de montaña mediterráneas se caracterizan por sus marcadas diferencias estacionales, que se reflejan en el ciclo de crecimiento y la diversidad de las especies vegetales (Ruiz y Ruiz, 1986).

Los sistemas ganaderos tradicionales de montaña han adaptado los movimientos de los rebaños al ciclo natural de crecimiento de los pastos, pasando de los prados cercanos a la explotación a los pastos de montaña (Oteros-Rozas et al., 2013). El manejo de las vacas en estos sistemas de producción depende de la época del año, así durante la primavera las vacas se alimentan de los pastos producidos en media montaña y ascienden hacia los pastos en el puerto durante el verano. En otoño vuelven a zonas de media montaña y en invierno las vacas se estabulan (Blanco et al.,

2008; Casasús et al., 2002; Sanz et al., 2001). En el caso de las vacas nodrizas estabuladas durante la invernada, el suministro de alimento durante esta fase se suele simplificar adoptando un régimen de alimentación en el cual todas las vacas en un mismo estado fisiológico reciben la misma dieta independientemente de sus requisitos individuales (Pullar y Rigby, 1993; Manninen et al., 2004), lo que puede dar lugar a diferencias en el balance energético (BE) individual (Bocquier y González-García, 2010).

1.1.2. Uso de las reservas corporales en las vacas de cría

En los sistemas extensivos, las vacas nodrizas no siempre se alimentan al nivel de sus necesidades teóricas a lo largo de su ciclo productivo, pasando por fases de movilización de reservas corporales durante los periodos de escasez de alimentos y recuperación de las mismas durante las épocas de abundancia de alimentos en el pastoreo. La continuidad de este sistema se basa en la capacidad de los animales para adaptarse a las limitaciones nutricionales durante periodos más o menos largos (Blanc et al., 2006). En ganado vacuno esto incluye la capacidad de mantener tanto los niveles productivos (homeostasis metabólica, mantenimiento del peso, producción lechera y crecimiento de las crías) como reproductivos (ciclicidad, fertilidad, etc.) (Colditz y Hine, 2016).

La respuesta productiva de los animales ante diferentes niveles de alimentación se ha evaluado en diversas etapas y estados fisiológicos en vacuno de carne en condiciones de montaña. Estos estudios recogen los efectos de diversos planos nutritivos durante la gestación temprana (Noya et al., 2020, 2019) o tardía (Sanz et al., 2004), la lactación, los distintos periodos de recría de las novillas de reposición (Rodríguez-Sánchez et al., 2018, 2015), y las etapas de lactancia y cebo de los terneros criados para la producción de carne (Blanco et al., 2008), considerando siempre manejos alimenticios diferenciados durante periodos relativamente largos (al menos 3 meses). En dichos trabajos se describen los efectos sobre el desarrollo, el estado de reservas corporales, los rendimientos productivos y el metabolismo de los animales, observándose con frecuencia una compensación entre los manejos y rendimientos en los distintos periodos del ciclo productivo (Casasús et al., 2002).

En el contexto actual de cambio climático, la severidad y frecuencia de fenómenos meteorológicos extremos pueden afectar negativamente de forma directa o indirecta a los rendimientos, la salud y el bienestar animal (Lacetera, 2019). Entre los efectos directos del cambio climático están los asociados al aumento de la temperatura y la intensidad de las olas de calor que pueden causar alteraciones productivas, metabólicas, estrés oxidativo e inmunosupresión. Las repercusiones indirectas en los

sistemas ganaderos se asocian con cambios en la disponibilidad de los recursos alimenticios tanto en cantidad como en calidad (Deroche et al., 2020; Dumont et al., 2015) y por ende, el aumento de la volatilidad de los precios de los insumos y los productos (Lacetera, 2019).

Por lo tanto, ante este escenario de constantes cambios y desafíos se buscan animales que sean capaces de adaptarse a estas restricciones de alimento sin comprometer su rendimiento. Varios conceptos se han propuesto para definir la respuesta adaptativa de los animales: robustez, resiliencia, plasticidad, tolerancia son algunos de los términos usados para caracterizar animales en función de su capacidad para afrontar perturbaciones. Para König y May (2019), la resistencia, tolerancia y resiliencia son componentes de la robustez. La **robustez** es la capacidad de ajustar el rendimiento para la calidad media del entorno y, en particular, para adaptarse a entornos restrictivos mientras que la **resiliencia** es la capacidad del animal de recuperarse de una perturbación de corta duración (Friggens et al., 2022). Se considera que un animal es resiliente cuando tras verse afectado por una perturbación vuelve rápidamente a su estado original previo a la exposición (Colditz y Hine, 2016; Berghof et al., 2019). La **plasticidad** se define como la combinación de mecanismos fisiológicos con los cuales un animal hace frente a los desafíos del entorno (Friggens y Newbold, 2007), y puede mostrar propiedades de elasticidad y flexibilidad: La respuesta es elástica cuando la recuperación es completa y flexible cuando no se recupera plenamente el estado inicial (Blanc et al., 2010).

Existe una gran variabilidad en la respuesta de los animales frente a una perturbación, su capacidad de adaptación a la misma y su recuperación al cesar dicha perturbación. Como indicador de esta resiliencia, se ha propuesto evaluar la diferencia entre el rendimiento potencial y el observado en condiciones alteradas ya sea de forma natural o inducida (Adriaens et al., 2021; Berghof et al., 2019; Codrea et al., 2011). Para estudiarla, en vacas y cabras lecheras se han propuesto ensayos con retos alimenticios cortos, que consistieron en fases de restricción y realimentación en distintas etapas de la lactación (Billa et al., 2020; Bjerre-Harpøth et al., 2012; Friggens et al., 2016). Uno de los métodos para evaluar este carácter es el modelizado de curvas de respuesta que se ha propuesto como una herramienta útil para cuantificar las perturbaciones tanto en el rendimiento del animal como en la variación individual. Sin embargo, este método ha sido utilizado principalmente en vacas de leche para medir la reacción ante perturbaciones (Barreto-Mendes et al., 2022; Ben Abdelkrim et al., 2021b), pero este enfoque no se ha utilizado en vacas nodrizas.

1.2. Adaptación de las vacas lactantes al déficit energético

1.2.1. Inicio y mantenimiento de la lactación

Tras la gestación y el parto, el inicio de la lactación exige la coordinación de varios procesos metabólicos y adaptaciones fisiológicas en distintos tejidos corporales para atender la gran demanda de nutrientes. Durante este proceso se desencadenan complejos mecanismos homeostáticos y homeorréticos para mantener un equilibrio fisiológico y reorganizar la distribución de los nutrientes (Bell, 1995). El control homeostático implica el mantenimiento del equilibrio fisiológico del animal mientras que la homeorresis es el control coordinado del metabolismo de los tejidos corporales necesarios para mantener un estado fisiológico (Bauman y Currie, 1980).

Para garantizar un suministro adecuado de glucosa para apoyar la lactación, la regulación biológica implica una serie de cambios orquestados (Tabla 1) que incluyen el aumento de las tasas hepáticas de gluconeogénesis (Drackley et al., 2001), una reducción de la captación y el uso de la glucosa por el tejido adiposo y el músculo (van Knegsel et al., 2005), y un cambio en la oxidación de nutrientes de todo el cuerpo para que se utilice menos glucosa como fuente de energía. Para estimular la lipólisis y la gluconeogénesis entran en juego numerosas hormonas como la insulina, la hormona de crecimiento, el factor de crecimiento similar a la insulina I (IGF-1), las catecolaminas, las hormonas tiroideas, el cortisol, la leptina, etc., implicadas en los flujos de nutrientes entre los tejidos (Chilliard et al., 1998; Chilliard et al., 2000a; Rico y Razzaghi, 2023).

Durante la etapa de transición, desde el último estadio de la gestación al inicio de la lactación, se produce un aumento muy rápido de la producción de leche. En esta fase la capacidad de ingestión es reducida, por lo que el aporte de nutrientes es limitado, dando lugar a un BE negativo (Drackley, 1999; Ingvartsen, 2006; Drackley y Cardoso, 2014). En vacuno de leche, generalmente alimentado a voluntad, este desequilibrio es muy acusado porque tiene elevadas necesidades nutritivas por su gran producción lechera (Gross et al., 2011a). Este BE negativo también ocurre en vacuno de carne, aunque en menor medida porque si bien tiene menor producción de leche suele ser alimentado con dietas más restrictivas para reducir los costes de alimentación.

Tabla 1. Adaptaciones fisiológicas para favorecer la lactación en las vacas lecheras.

Proceso o tejido de respuesta	Respuesta
Tejido mamario	↑ del número de células secretoras
	↑ del uso de nutrientes
	↑ del suministro de sangre
Consumo de alimento	↑ cantidad
Tracto digestivo	↑ del tamaño
	↑ de la capacidad de absorción
	↑ de la tasa de absorción de nutrientes
Hígado	↑ del tamaño
	↑ de las tasas de gluconeogénesis
	↑ de la movilización de glucógeno
	↑ de la síntesis de proteínas
Tejido adiposo	↓ de la síntesis de grasa *de novo*
	↓ de la captación de ácidos grasos preformados
	↓ de la re-esterificación de ácidos grasos
	↑ de la lipólisis
Músculo esquelético	↓ de la utilización de la glucosa
	↓ de la síntesis de proteínas
	↑ de la degradación de las proteínas
Hueso	↑ de la movilización de calcio y potasio
Corazón	↑ del gasto cardiaco
Hormonas plasmáticas	↓ de la insulina
	↑ de la somatotropina
	↑ de la prolactina
	↑ de los glucocorticoides
	↓ de triyodotironina (T3) y tiroxina (T4)
	↓ del IGF-1

Fuente: (Bauman y Currie, 1980; Baumgard et al., 2017)

La capacidad que tiene el animal para adaptarse a los períodos de BE negativo depende de la activación de ciertos mecanismos endócrinos y metabólicos que ayudan a mantener la homeostasis (Chilliard et al., 1998). A nivel productivo uno de los efectos principales del BE negativo causado por una restricción alimentaria es una reducción en la producción de leche (Gross et al., 2011a; Leduc et al., 2021). Además, un BE negativo severo está relacionado con un mayor riesgo de trastornos metabólicos (Drackley, 1999)

así como con repercusiones negativas en reproducción y la fertilidad (Diskin et al., 2003; Sanz et al., 2004).

Ante situaciones de escasez de nutrientes se produce un reparto de los mismos hacia las distintas funciones productivas, conforme a las prioridades que dependen tanto del estado fisiológico como de la variabilidad individual de los animales (Friggens et al., 2013; Ollion et al., 2016), lo cual se refleja en diferentes estrategias metabólicas para hacer frente al desafío nutricional. Diversos estudios también han analizado los mecanismos y plazos para la recuperación de la normalidad cuando la restricción termina y los animales vuelven a alimentarse de acuerdo a sus necesidades (Agenäs et al., 2003; Bjerre-Harpøth et al., 2012; Gross et al., 2011a).

1.2.2. Metabolismo energético y proteico

La adaptación de los rumiantes a periodos de subnutrición implica numerosos cambios a nivel digestivo, metabólico, endocrino, etc., que se resumen en la Figura 1. Para hacer frente a la escasez de alimento se desencadenan regulaciones endocrinas que favorecen la movilización de reservas y el ahorro de glucosa, con una disminución de la insulina, IGF-1, leptina, glucagón y prolactinas; y un incremento de hormona de crecimiento, progesterona y cortisol (Leduc et al., 2021). Una vez instaurados estos mecanismos de ahorro de metabolitos limitantes, se produciría una reducción del metabolismo básico y del gasto ligado a la actividad (Blanc et al., 2006).

En cuanto a la **movilización de las reservas corporales** como fuente de energía, ésta se produce principalmente a partir de grasa corporal y en menor grado de proteína muscular (Chilliard et al., 2000a, 1998; van der Drift et al., 2012). En estos procesos, el hígado juega un papel clave en la coordinación del flujo de nutrientes, mediante la regulación de la oxidación de los ácidos grasos, la gluconeogénesis y de la síntesis de triglicéridos (Drackley et al., 2001; Wathes et al., 2021).

El **tejido adiposo** es la reserva energética principal del cuerpo. Durante los periodos de BE negativo los triglicéridos almacenados en el tejido adiposo son hidrolizados por las lipasas en forma de glicerol y de ácidos grasos no esterificados (NEFA, por su siglas en inglés), que son liberados a la circulación (Kuhla et al., 2016). Los NEFA pueden ser utilizados directamente como fuente de combustible por tejidos como el músculo, utilizados para la síntesis de grasa láctea por la glándula mamaria, o procesados por el hígado. Allí pueden oxidarse completamente para proporcionar energía, o solo parcialmente para producir cuerpos cetónicos como la acetona, ácido acetoacético y β-hidroxibutirato (BHB, por sus siglas en inglés), que actúan como

combustible alternativo para tejidos como el cerebro y el corazón (Herdt, 2000; McArt et al., 2013).

La movilización de tejido adiposo varía de acuerdo a la severidad de la subnutrición tanto en nivel como en duración de la misma, así como de las reservas iniciales (Chilliard et al., 2000a). Uno de los indicadores de la movilización de reservas corporales de tejido adiposo es la medida de la condición corporal (CC).

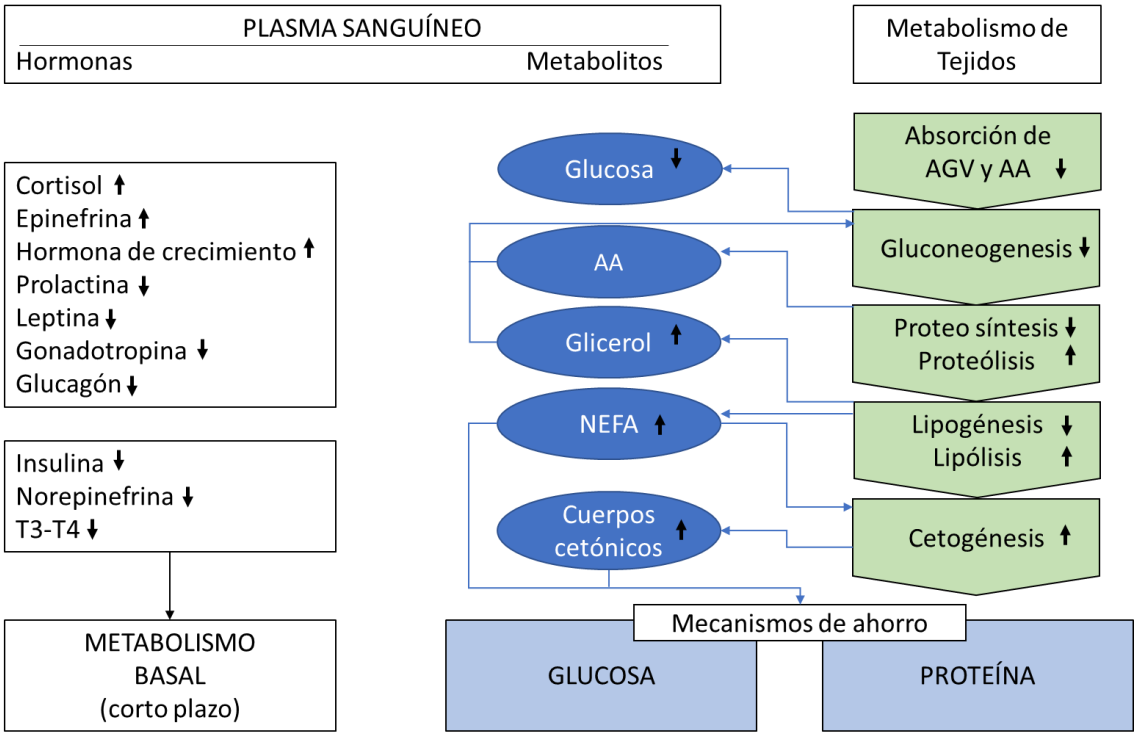


Figura 1. Adaptaciones metabólicas y endocrinas a la subnutrición en rumiantes. AGV: ácidos grasos volátiles, AA: Aminoácidos, NEFA: Ácidos grasos no esterificados (siglas en inglés). Fuente: adaptado de Chilliard et al. (1998).

Durante periodos de BE negativo también puede existir un proceso de **catabolismo proteico** con el fin de movilizar aminoácidos que contribuyan a la gluconeogénesis hepática y a la síntesis de proteína láctea en la glándula mamaria (Bauman, 2000). Los aminoácidos se utilizan principalmente para la síntesis de proteínas, pero también se metabolizan para obtener energía como fuentes de carbono para la síntesis de glucosa (Herdt, 2000). Este fenómeno se da especialmente al inicio de la lactación (Sadri et al., 2023), y supone un aporte energético limitado en relación a la movilización de grasa. Así, las vacas restringidas con buena CC pueden llegar a movilizar el 75% de sus reservas de grasa y el 22% de sus reservas de proteína durante la lactación (Chilliard, 1999). En el mismo sentido, las vacas lactantes movilizaron 54 kg de lípidos y 21 kg de proteína en las 5 primeras semanas de lactación, y 18 kg más de

lípidos sin más pérdida de proteína hasta la semana 12 (Komaragiri y Erdman, 1997). Estos cambios expresados en unidades de energía corporal suponen que el 93% de pérdidas son debidas a la grasa y el 7% a la proteína.

1.2.3. Factores que condicionan la respuesta ante un balance energético negativo

Los mecanismos biológicos involucrados en la adaptación ante un BE negativo, bien sea de origen natural o causado por una restricción alimentaria, pueden estar condicionados por diversos factores.

Por un lado, los mecanismos adaptativos pueden diferir entre animales de distintas **razas u orientación productiva**. El ganado vacuno de leche y el de carne difieren en la regulación endocrina de la partición de nutrientes debido principalmente a sus diferencias en el potencial lechero y la acumulación de masa corporal (Pareek et al., 2007). El vacuno de leche tiene elevada producción lechera y menor tasa de crecimiento, mientras que en el vacuno de carne ocurre a la inversa (Sapkota et al., 2020), lo cual puede generar diferentes respuestas adaptativas (Blanc et al., 2006). En vacuno de leche se ha analizado la respuesta ante restricciones alimentarias en lactación tanto a largo (Hervé et al., 2019; Vanbergue et al., 2018) como a corto plazo (Ferraretto et al., 2014; Kvidera et al., 2017), analizando también la respuesta tras la realimentación (Abdelatty et al., 2017; Bjerre-Harpøth et al., 2012; Gross et al., 2011a). En el vacuno de carne los estudios son menos abundantes y se han desarrollado sobre todo en el largo plazo (De La Torre et al., 2015; Fiems et al., 2009; Martin et al., 2022).

La respuesta adaptativa puede depender también del **tipo de restricción alimentaria**, que según Leduc et al. (2021) puede ser: cuantitativa si se limita la cantidad de alimento, o cualitativa cuando disminuye la densidad nutritiva de la ración, donde se pueden utilizar dietas bajas en energía o en proteína. En función de la intensidad o severidad de la restricción y de su duración, los mismos autores consideran que si ésta es mayor al 50% y con una duración de menos de una semana se puede considerar severa, mientras que una restricción menor al 50% es moderada.

La **etapa de la lactación** en que ocurre la restricción juega un papel fundamental en las diferentes estrategias que los animales emplean para enfrentarse a los periodos de restricción y realimentación (Bjerre-Harpøth et al., 2012; Gross et al., 2011a). Aunque las vacas tengan un BE similar y estén en la misma etapa de lactación pueden diferir en su estado metabólico, resultando en variaciones individuales en la respuesta (Kessel et al., 2008).

Existe una gran **variabilidad individual en la respuesta** a un BE negativo, parcialmente explicada por factores genéticos que influyen en la movilización de grasa y la partición de nutrientes (Friggens y Newbold, 2007). La resiliencia estaría condicionada por este componente genético: Berghof et al. (2019) indican que una mayor resiliencia se correlaciona genéticamente con menor producción de leche, mejor estado sanitario de la ubre y mayor longevidad. Para evaluar esta variabilidad individual se han modelizado las curvas de producción de leche y otros parámetros biológicos con el fin de medir la adaptación fisiológica en periodos de estrés (Kessel et al., 2008). Ante este abanico de respuesta individuales, agrupar los animales en función de la forma, la dinámica y el momento en que responden a un desafío y cómo se recuperan de este, podría ser interesante para simplificar ciertas prácticas de manejo (Barreto-Mendes et al., 2022; Ben Abdelkrim et al., 2023, 2021a). En vacuno de leche, se ha clasificado a los animales según su rendimiento productivo y metabólico (de Koster et al., 2019; Heirbaut et al., 2022; Tremblay et al., 2018), indicando que identificar vacas con perfiles productivos similares facilitaba la toma de decisiones a nivel de rebaño. Por lo tanto, realizar un agrupamiento de animales con pautas de respuesta similares podría ser interesante para aplicar manejos específicos en función de estos perfiles.

La mayoría de los estudios de restricción han valorado el efecto de un solo reto o de retos espaciados entre sí, efectuados en distintos momentos. Sin embargo, es posible que ante **desafíos nutricionales repetidos de manera consecutiva** se activen mecanismos adaptativos que permitan al animal habituarse a la perturbación recurrente. La habituación se entiende como la disminución de la capacidad de respuesta a un estímulo como resultado de una exposición repetida (Alvarenga et al., 2023). En ganado vacuno se ha analizado la respuesta de habituación a cambios de manejo (Veissier et al., 2001) o de dieta (Rauch et al., 2021). Ante desafíos repetidos de acidosis ruminal, se ha descrito un efecto acumulativo en el que la respuesta empeora en los retos sucesivos Dohme et al. (2008), mientras que se ha observado el efecto contrario, una mitigación ante sucesivas repeticiones Nagata et al. (2018). En aspectos de comportamiento, Schütz y Cox (2014) no observaron un mayor efecto a la exposición repetida a diferentes tipos de revestimiento de suelo, mientras que Stockman et al. (2011) analizando el estrés de terneros durante el transporte describen una respuesta atemperada tras varios viajes sucesivos.

1.2.4. Efecto de un balance energético negativo sobre la producción y composición de la leche

El uso de nutrientes para la síntesis de la leche requiere una regulación integrada del metabolismo de la glándula mamaria y otros tejidos corporales (Bauman, 2000), siguiendo una jerarquía de la distribución de nutrientes donde la galactogénesis es prioritaria (Baumgard et al., 2017). En vacuno de leche, su producción lechera al inicio de la lactación está relacionada con la magnitud de la utilización de las reservas corporales durante la fase de BE negativo. La movilización de grasa corporal podría aportar más de un tercio de la leche producida durante el primer mes de lactación (Bauman y Currie, 1980). En las vacas nodrizas, su potencial lechero depende de la genética, optimizado por ciertas prácticas de manejo, pero la producción real está regulada por el amamantamiento de la cría (frecuencia e intensidad) (Sepchat et al., 2017). Por ello, la curva de lactación de vacuno de carne es algo diferente a la del vacuno de leche (Sapkota et al., 2020), dada la diferente evolución en el tiempo de los aportes y necesidades nutricionales.

Mecanismos de acción

Durante la lactación temprana la glándula mamaria tiene una alta tasa metabólica y, por lo tanto, al enfrentarse a periodos de restricción alimentaria la glándula mamaria debe adaptarse. Las bajas concentraciones de glucosa e insulina en plasma durante el BE negativo podrían reflejar la alta prioridad de la glándula mamaria para la glucosa (Bauman, 2000). Esta priorización está bajo un control genético, regulado por mecanismos homeostáticos y homeorréticos tal y como se ha explicado anteriormente (Bauman y Currie, 1980; Friggens y Newbold, 2007). Ante una restricción energética en lactación puede modificarse la actividad secretora y/o el número de células secretoras de la glándula mamaria y a su actividad enzimática, sin observarse deformaciones permanentes (Boutinaud et al., 2019; Hervé et al., 2019).

Efecto sobre la producción de leche

En una revisión reciente sobre los efectos de la restricción nutricional en **vacuno de leche,** Leduc et al. (2021) describieron un amplio rango de reducción en la producción de leche en distintos estudios (-7% a -71%). El efecto dependería de la duración e intensidad de la restricción, así como de la etapa de lactación. La producción de leche puede volver a su rendimiento normal una vez que los animales regresan a su dieta estándar, en un plazo de tiempo que también estaría condicionado por la duración e intensidad de la restricción previa. Así con una restricción de la alimentación de 5 días

al 50% (Billa et al., 2020) o 4 días al 60% (Bjerre-Harpøth et al., 2012) la producción de leche puede volver a los valores basales dentro de los 8 días tras finalizar la restricción.

La producción de leche ha sido un parámetro menos estudiado en **vacas de carne**, dado que no se mide directamente de manera habitual. La producción de leche en vacas nodrizas aumenta lentamente después del parto hasta alcanzar un pico máximo entre el primer y tercer mes de lactación. Este pico es más tardío que en vacas de leche (Sapkota et al., 2020; Sepchat et al., 2017), ya que depende del equilibrio entre la capacidad de ingestión del ternero y el potencial de producción de la vaca. Trabajos previos en vacas de raza Parda de Montaña reportan datos de la producción de leche y calidad de leche, y de su relación con el nivel de alimentación de las vacas (Alvarez-Rodríguez et al., 2010; Cortés-Lacruz et al., 2017; Dervishi et al., 2017). Las vacas de raza Charolesa tuvieron una pérdida de leche de -12% con una restricción alimentaria del 50% aplicada durante 4 ó 10 días, independientemente de la duración de la restricción, aunque el tiempo de recuperación tras la realimentación fue más largo cuando la restricción fue más prolongada (De La Torre et al., 2022).

El efecto de la restricción puede depender de la **etapa de la lactación** en la que se aplica, tal y como se ha mencionado con anterioridad. Según Adriaens et al. (2021), los efectos de la restricción son más severos cuando la perturbación ocurre al principio o a la mitad de la lactación, se desarrollan más rápido y se recuperan más lentamente que en las etapas posteriores de la lactación. Sin embargo, otros estudios no encontraron diferencias en la pérdida de producción, en torno al -30 % de los valores previos al desafío, en la lactación temprana, media y tardía (Bjerre-Harpøth et al., 2012) o en su recuperación durante la realimentación (Codrea et al., 2011). Según Elgerma et al. (2018), las vacas lecheras con menos fluctuaciones en la producción de leche bajo perturbaciones naturales son más resistentes, ya que una menor variación en el rendimiento se correlaciona genéticamente con una mejor salud y longevidad. Dado que las curvas de lactación difieren entre el vacuno de leche, con 305 días en lactación, y el vacuno de carne, con destetes más tempranos (Grossman y Koops, 2003; Dematawewa et al., 2007; Blanco et al., 2009), el efecto de la restricción según la etapa de lactación podría ser diferente entre ambos tipos de ganado.

Efecto sobre la composición de la leche

La literatura refiere resultados heterogéneos respecto a los efectos de una restricción nutricional sobre los principales componentes de la leche. El contenido de **grasa** está influido por la dieta (Bauman y Griinari, 2003), la fase de lactación y el potencial genético (Bauman y Griinari, 2003; Rico y Razzaghi, 2023). Ante una

restricción energética, Leduc et al. (2021) describen un amplio rango de variación de este parámetro (+6% a +129%), ya que los NEFA liberados en la lipomovilización pueden ser oxidados en el hígado o incorporarse directamente a la grasa de la leche, siendo este efecto más intenso bajo una restricción severa.

Otros componentes de la leche podrían verse afectados, aunque en menor medida. Leduc et al. (2021) señalaron que la restricción provoca una disminución en el contenido de **proteína** (-3% a -17%) y de **lactosa** (-2% a -20%) y un aumento del conteo de **células somáticas**, mientras que la **urea** presenta un comportamiento muy variable. La proteína de la leche parece verse afectada tanto por la energía como por la proteína de la dieta. Con dietas con alto contenido proteico aumentaría la producción de leche y su contenido en proteína y urea (Broderick, 2003). Ante un déficit energético, se reduce la producción de leche y su tasa proteica, pero la urea incrementa en respuesta a la menor necesidad de aminoácidos para una menor secreción de leche (Bittante, 2022; Broderick, 2003).

Ante una restricción del 50%, Gross et al. (2011a) no encontraron diferencias en el contenido de grasa en leche pero sí una reducción en el contenido de proteína, mientras que Bjerre-Harpøth et al. (2012) reportaron un aumento en la grasa y una disminución de la proteína de la leche durante la restricción, que en ambos casos regresarían a sus valores normales dentro de 3 - 4 días tras finalizar la restricción. Adicionalmente, en este último estudio se observó un efecto de la etapa de lactación sobre la respuesta de la proteína de la leche a la restricción, que disminuyó durante en lactación temprana y media pero no en la lactación tardía.

El contenido de lactosa en la leche se mantiene constante a lo largo de la lactación y casi no se ve afectado por la escasez de energía (Gross et al., 2011a). La lactosa es un componente altamente osmótico, que regula el volumen de leche producido, cuyo principal precursor es la glucosa (Guinard-Flament et al., 2006). Por otro lado los cambios en el conteo de células somáticas estaría más bien asociado a la pérdida de integridad del epitelio mamario (Leduc et al., 2021).

En los últimos años, se han examinado diversos componentes de la leche como indicadores no invasivos del estado nutricional de vacas lecheras (Gross y Bruckmaier, 2019; Billa et al., 2020; Pires et al., 2022) ya que pueden medirse de forma sencilla a partir de muestras diarias de leche que se recogen y analizan rutinariamente en las máquinas de ordeño (Mäntysaari et al., 2019). Entre dichos indicadores, el **perfil de ácidos grasos** de la leche puede reflejar el estado metabólico de los animales, por lo que en vacas lecheras se han propuesto como marcadores potenciales para

diagnosticar un BE negativo (Dórea et al., 2017; Khiaosa-ard et al., 2020; Mann et al., 2016). Los ácidos grasos de la leche pueden originarse a partir de 4 vías principales: directamente de la dieta, por la síntesis *de novo* en la glándula mamaria, por la formación en el rumen por biohidrogenación o por la degradación bacteriana y liberación de las reservas de grasa corporal (Chilliard et al., 2000b). Grummer (1991) sugiere que los ácidos grasos C4:0 a C14:0, y aproximadamente la mitad de los C16:0 de la leche se sintetizan *de novo* en la glándula mamaria, mientras que el resto de los C16:0 y todos los ácidos grasos de cadena larga derivan de la captación mamaria de triglicéridos y NEFA circulantes, ácidos grasos preformados procedentes de la movilización del tejido adiposo (Chilliard et al., 2000a; Palmquist, 2009).

Los efectos de una restricción nutricional pueden reflejarse en una disminución de los ácidos grasos *de novo*, y en incrementos de los ácidos grasos preformados, especialmente C17:1 cis-9, C18:0 y C18:1 cis-9 derivados de la movilización de grasa corporal y efectos variables en los ácidos grasos de origen mixto (C16:0) (Billa et al., 2020; Gross et al., 2011b). Cuando los animales vuelven a su dieta habitual, la mayoría de estos cambios desaparecen a los 4 días (Gross et al., 2011b). Según Churakov et al. (2021) el contenido de C18:1 cis-9 en la leche podría considerarse incluso mejor indicador de un BE negativo que las concentraciones plasmáticas de NEFA y BHB. Adicionalmente se propone el uso de ratios entre los ácidos grasos *de novo* y los preformados, como el ratio C18:1 cis-9/C15:0, como potencial indicador del diagnóstico de cetosis en vacas lecheras (Jorjong et al., 2014), o el ratio C17:0/C15:0, que podría estar relacionado con una mayor movilización de grasa (Dórea et al., 2017).

1.2.5. Indicadores plasmáticos de un balance energético negativo

Ciertos metabolitos plasmáticos implicados en las rutas metabólicas se han utilizado como herramienta para el estudio y diagnóstico del estado nutricional y de la respuesta del animal a los cambios de dieta (Chilliard et al., 1998; Agenäs et al., 2003; Puppel y Kuczyńska, 2016).

La **glucosa**, asociada al metabolismo energético, tiene un rol central durante la lactación porque su absorción por la glándula mamaria es esencial para la síntesis de lactosa de la leche (Bell y Bauman, 1997). Su utilización en tejidos y órganos durante la lactación está regulada por las hormonas pancreáticas, insulina y glucagón (Drackley et al., 2001), siendo la insulina un mediador para mantener el equilibrio metabólico frente a variaciones a corto plazo en el suministro y la demanda de nutrientes. Por ejemplo interviene en el hígado (inhibición de la gluconeogénesis), tejido adiposo (síntesis de grasa), músculo esquelético (captación de glucosa) y cuerpo entero (oxidación de

glucosa) (Bauman, 2000). Sin embargo, Pareek et al. (2007) observaron que las concentraciones plasmáticas de insulina pueden ser más altas en vacas de carne en comparación con las vacas lecheras. Pese a las variaciones de la glucosa observadas durante una restricción alimenticia (-31% y +5%), la glucosa sería considerada un indicador pobre del estado nutricional (Leduc et al., 2021). Su variación estaría determinada básicamente por la intensidad de la restricción, ya que con una subnutrición moderada (≤ 50%) el contenido de glucosa permanecería estable (Hervé et al., 2019; Laeger et al., 2012).

Las **concentraciones de NEFA y BHB** en plasma son indicadores de la movilización de las reservas de grasa corporal, que es la primera respuesta para cubrir el déficit energético durante un período de BE negativo (Overton y Waldron, 2004). En caso de restricción severa, debido a la llegada masiva de NEFA al hígado, éste es incapaz de oxidarlos por completo, resultando en la formación de cuerpos cetónicos como el BHB. Leduc et al. (2021) reportaron que la mayoría de los estudios de restricción nutricional en vacas de leche, la concentración de NEFA en plasma puede incrementarse en un amplio rango (+14% a +3475%) mientras que el BHB puede incrementar, pero en menor magnitud (+26% a +721%) y con mayor efecto en etapas tempranas de la lactación. Las concentraciones plasmáticas de ambos metabolitos tienen una respuesta inmediata a la realimentación, ya que pueden volver a sus valores iniciales dentro de las 24-48 h posteriores a la restricción (Billa et al., 2020; Bjerre-Harpøth et al., 2012).

El **contenido plasmático de urea** refleja el estado proteico del animal (Caldeira et al., 2007), ya que el nitrógeno ureico en sangre es el principal producto final del metabolismo del nitrógeno en los rumiantes, y sus altas concentraciones son indicativas de una utilización ineficiente del nitrógeno dietético (Nousiainen et al., 2004). La urea plasmática está influenciada por una gran variedad de parámetros interrelacionados, como la ingesta de proteínas, su composición de aminoácidos, la cantidad de carbohidratos y el catabolismo del tejido muscular (DePeters y Ferguson, 1992; Bell, 1995). La urea se forma principalmente en el hígado, y luego se transporta en el plasma sanguíneo y llega a otros fluidos en el cuerpo, como la leche (Spek et al., 2013; Van Saun, 2006), existiendo una alta correlación entre las concentraciones de urea en sangre y leche (Bastin et al., 2009; Kessler et al., 2020).

Diversos metabolitos, entre ellos el **malondialdehido** (MDA), son biomarcadores del estado oxidativo, que se produce durante períodos de alta demanda metabólica. Los efectos adversos del BE negativo durante el periodo de transición se han relacionado con los efectos proinflamatorios de los ácidos grasos liberados durante la lipólisis masiva al principio de la lactación (Castillo et al., 2006; Sordillo y Aitken, 2009). El exceso de lípidos circulantes y el bajo estado antioxidante se asocian a este proceso, que ocurre cuando los radicales libres provocan reacciones en cadena, originando especies reactivas del oxígeno (ROS) que favorecen la inflamación (Bradford et al., 2015). Estas sustancias aumentan durante la última semana de gestación y hasta un máximo en el primer mes postparto, y se relacionan con las concentraciones plasmáticas de NEFA y BHB (Bernabucci et al., 2005).

De acuerdo con todo lo descrito, podemos concluir que cuando las vacas se enfrentan a una perturbación nutricional ocurren numerosos cambios a nivel fisiológico, metabólico y productivo con el fin de sobrellevar tal desafío. Estos efectos han sido evaluados con diferentes métodos, especialmente en el ganado vacuno de leche, pero la información en vacas nodrizas es escasa, y la respuesta podría ser diferente.

II. Objetivos

El objetivo principal de esta tesis doctoral fue estudiar los factores que influyen en la resiliencia (capacidad de respuesta productiva ante perturbaciones ambientales) a escala del animal en sistemas producción de vacuno de carne. Para abordar este objetivo general se plantearon los siguientes objetivos parciales:

- Analizar los factores fisiológicos y metabólicos ligados al animal que influyen en su capacidad de respuesta, o resiliencia durante la restricción-realimentación.
- Determinar la repercusión de una restricción energética y proteica y posterior realimentación en diferentes momentos de la lactación sobre el metabolismo y los rendimientos productivos de las vacas nodrizas, con el fin de determinar los mecanismos mediadores de la respuesta adaptativa.
- Evaluar el efecto de una restricción energética y proteica repetida y posterior realimentación como indicador de habituación ante una exposición continua.
- Encontrar biomarcadores que permitan la identificación de los animales más aptos para desenvolverse en ante una perturbación de tipo nutricional.

III. Material y Métodos Generales

El Comité de Ética Animal del Centro de Investigación aprobó los procedimientos experimentales (protocolo n.º CEEA- 03-2018-01), que siguieron las directrices de la Directiva de la UE 2010/63 relativa a la protección de los animales utilizados para experimentación y otros fines específicos (EU, 2010). Los experimentos se desarrollaron en la Finca Experimental de la Garcipollera perteneciente al Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). La finca se ubica en Béscos de la Garcipollera (Jaca, 42°37' N, 0°30' O), a 945 m de altitud, y presenta una temperatura media de 10,2 ºC y precipitación anual de 1059 mm.

3.1. Manejo de las vacas y terneros

Para este experimento se utilizaron 32 vacas multíparas de raza Parda de Montaña (7,5 ± 2,91 años) y sus terneros, siendo una pareja retirada del estudio debido a una lesión física. Las vacas se distribuyeron tras el parto de forma aleatoria en corrales (7 u 8 vacas/corral, 10 x 20 m) equipados con comederos individuales para el forraje y estaciones automáticas de distribución de pienso ALPRO (DeLaval, Tumba, Suecia). Los terneros se alojaron en cubículos, adyacentes a los parques de sus madres, con cama de paja y se les permitió mamar de sus madres dos veces al día durante aproximadamente 30 minutos a las 06:00 y a las 14:00.

Desde el parto hasta el inicio del experimento todas las vacas fueron alimentadas con la misma dieta calculada para cubrir el 100% de los requerimientos energéticos y proteicos netos para el mantenimiento y lactación de una vaca media, considerando un peso vivo de 615 kg y una producción de leche de 8,5 kg/d. Dicha dieta estaba compuesta por 8,0 kg de heno y 3,0 kg de pienso en materia fresca. En la Tabla 2 se describe la composición de materias primas del pienso suministrado a las vacas.

Tabla 2. Materias primas del pienso.

Ingredientes	
Harinilla de maíz (%)	25,0
Residuos de destilería (%)	20,0
Cebada (%)	15,0
Maíz (%)	10,0
Trigo (%)	10,0
Salvado de trigo fino (%)	9,0
Harina de extracción de soja (%)	5,8

En la Figura 2 se muestran los retos nutricionales consistentes en tres periodos consecutivos (Basal, Restricción, Realimentación) realizados en el segundo, tercer y cuarto mes de la lactación. Se consideró como día 0 el primer día del periodo de Restricción coincidiendo con el día 31, 58 y 87 postparto en el segundo, tercer y cuarto mes de la lactación, respectivamente. Durante cada reto, las vacas recibieron una dieta que cumplía con el 100% de las necesidades energéticas y proteicas durante cuatro días (día -4 a -1, período Basal). En los siguientes cuatro días recibieron una dieta formulada para cubrir el 55% de las necesidades (día 0 a 3, período de Restricción). Finalmente, volvieron a ser alimentadas con la dieta formulada para cubrir el 100% de sus necesidades durante los siguientes 4 días (día 4 a 7, período de Realimentación) excepto en el cuarto mes que por el diseño del experimento solamente tuvieron 3 días de Realimentación (día 4 a 6).

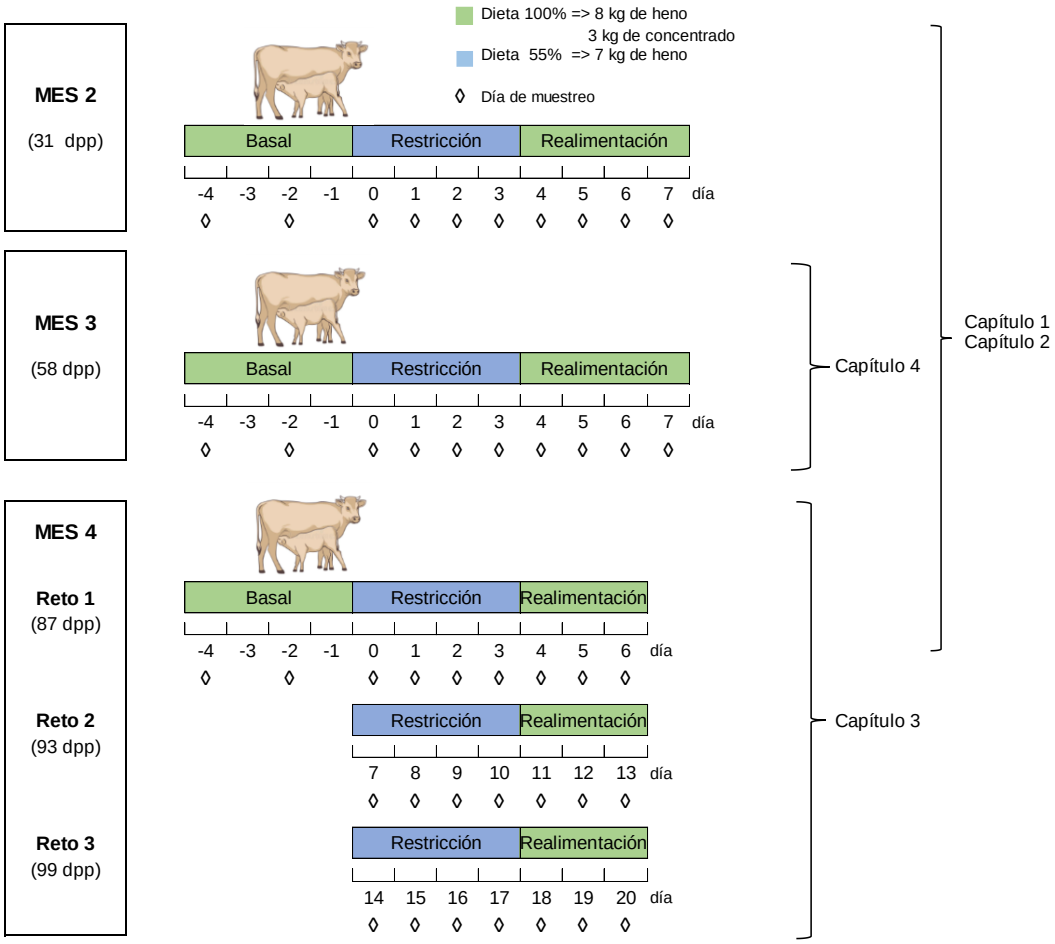


Figura 2. Esquema representativo de la cronología de los retos nutricionales lo largo de la lactación, y capítulos derivados de esta tesis asociados a los distintos periodos.
dpp: días postparto en el momento de inicio de la restricción (día 0).

Para estudiar la habituación, las vacas fueron sometidas a retos consecutivos en el cuarto mes de la lactación (retos 1, 2, 3). El diseño de este experimento consistió en un periodo Basal (día -4 a -1), descrito con anterioridad, tras el cual las vacas fueron sometidas a tres retos nutricionales repetidos consistentes en 4 días con dieta para cubrir el 55% de sus necesidades (Restricción) seguidos por 3 días con la dieta para cubrir el 100% de sus necesidades (Realimentación).

Las vacas recibieron diariamente 8,0 kg de heno durante los periodos Basales y de Realimentación y 7,0 kg de heno durante los periodos de Restricción. El heno se ofreció individualmente a las 08:00 en una sola comida en cajas de fibra de vidrio de 200 L ubicadas tras una cornadiza con autobloqueo. Las vacas permanecieron atadas durante un periodo de 2 horas aproximadamente hasta que terminaban su ración. El pienso se suministró en estaciones de alimentación automáticas ALPRO, controladas por el software de gestión del rebaño ALPRO 7.0 para Windows (DeLaval). Las estaciones de alimentación se programaron para ofrecer diariamente 3,0 kg/día de pienso a todas las vacas durante los periodos Basales y de Realimentación, mientras que las vacas no recibieron pienso durante los periodos de Restricción. Todos los animales tuvieron libre acceso al agua y a bloques de vitaminas y minerales durante todo el experimento.

3.2. Medidas

3.2.1. Ingestión

Desde el parto hasta el fin del ensayo, se controló diariamente el alimento ofertado a las vacas y su rehusado para estimar la ingestión de las vacas. En una báscula se pesaba la ración diaria de heno asignada a las vacas (7 u 8 kg, según el periodo). La ingestión de pienso se registró diariamente con el programa de la estación de alimentación.

3.2.2. Peso de vacas y terneros

Las vacas se pesaron en una báscula digital al parto, un mes postparto, y diariamente durante los días de muestreo arriba indicados en la Figura 2. Las vacas se pesaron por la mañana a las 7:00, tras amamantar a los terneros y antes de recibir la dieta asignada. Los terneros se pesaron antes y después de los periodos de amamantamiento de la mañana y de la tarde, constatándose que los terneros mamaban de sus madres hasta la saciedad.

3.2.3. Condición corporal de las vacas

La CC se registró al parto, al mes postparto y en cada reto: un día del periodo basal y un día del periodo de realimentación. Esta medición fue realizada por una persona entrenada, siguiendo el método descrito por Lowman et al. (1976), cuya escala va de 0 a 5 puntos y se determina mediante la palpación de la zona de las apófisis transversas de las vértebras lumbares y de la base de la cola. También se midió el grosor de la grasa subcutánea mediante ecografía en tres puntos: en la grupa, en el punto P8 y en la decimotercera vértebra torácica (Schröder y Staufenbiel, 2006). Se utilizó un ecógrafo equipado con una sonda multifrecuencia (7.5 MHz; Aloka SSD-900, Aloka, Madrid, España).

3.2.4. Producción lechera

La producción de leche se estimó en el primer mes postparto (días 30 y 31 postparto) y diariamente durante los días de muestreo anteriormente indicados en la Figura 2, utilizando la técnica de doble pesaje del ternero (Le Neindre y Dubroeucq, 1973). A partir del peso de los terneros antes y después de los dos periodos de amamantamiento diarios se calculó la leche consumida.

3.3. Muestreos

Los siguientes muestreos se realizaron diariamente en los mismos días arriba indicados para el registro de la ingestión, peso y producción de leche. La toma de muestras se realizó por la mañana (7:00), tras el amamantamiento del ternero y antes de que las vacas recibieran alimentos.

3.3.1. Alimentos

Se tomaron muestras de heno y pienso al momento de suministrar la ración diaria. Las muestras se tomaron cada dos días y se hizo un pool por cada periodo de cada reto, se identificaron y posteriormente se liofilizaron (liofilizador Genesis Freeze Dryer 25, Hucoa Erlöss, SA/Thermo Fisher Scientific, Madrid, España) hasta el análisis de composición química.

3.3.2. Leche

En el primer mes postparto y diariamente durante los periodos experimentales se recogieron muestras de leche tras el amamantamiento y retirada del ternero. Para ello, se administró a las vacas una inyección intramuscular de oxitocina (40 UI, Facilpart, Laboratorios Syva, León, España) para facilitar la bajada de la leche residual. Tras 5 minutos de espera, se recogió manualmente una muestra de leche por vaca de los

cuatro pezones, tras descartar 3 chorros de leche por pezón. Se obtuvieron 2 muestras de leche determinar la composición química (100 ml) y el perfil de ácidos grasos (40 ml) de la leche. La primera muestra fue conservada con azida sódica (PanReac, Barcelona, España) a 4° C hasta el análisis y la segunda muestra se liofilizó (liofilizador Genesis Freeze Dryer 25) y se mantuvo a -20° C hasta el análisis de ácidos grasos.

3.3.3. Sangre

Las vacas se sangraron para determinar el perfil metabólico. Las muestras de sangre se obtuvieron de la vena coccígea utilizando tubos con heparina (BD Vacutainer Becton-Dickenson and Company, Plymouth, Reino Unido) para las determinaciones de BHB, MDA y progesterona y tubos que contenían K2 EDTA (BD Vacutainer Becton-Dickenson and Company, Plymouth, Reino Unido) para el análisis de glucosa, NEFA y urea. Inmediatamente después de la extracción, las muestras de sangre se centrifugaron a 3500 rpm durante 20 minutos a 4° C; el plasma se recogió y se congeló a -20° C hasta su posterior análisis.

3.4. Análisis químicos

3.4.1. Alimentos

La composición química de los alimentos se determinó en el Laboratorio de Valoración Nutritiva del Departamento de Ciencia Animal del CITA. Se analizó la materia seca del heno y del pienso poniendo las muestras en una estufa de ventilación forzada a 60º C durante 48 h hasta peso constante. Las muestras se molieron en un molino rotatorio (ZM200 Retsch, Alemania) con el tamiz de 0,2 mm para la determinación de proteína y el de 1 mm para el resto de la composición química. Todos los análisis de los alimentos se realizaron por duplicado y se utilizaron los métodos oficiales para la determinación de materia seca (método 934.01), cenizas (método 942.05) y proteína bruta (método 968.06) (AOAC, 2000). El contenido de nitrógeno se determinó siguiendo el Procedimiento Dumas (índice nº 968.06) con un analizador de nitrógeno (Modelo NA 2100, CE Instruments, Thermoquest SA, Barcelona, España). Los contenidos de fibra neutro detergente, fibra ácido detergente y lignina ácido detergente se analizaron siguiendo los métodos de Van Soest et al. (1991) utilizando un analizador de fibras (modelo Ankom 200/220, Ankom Technology Corporation, Fairport, NY, EE.UU.). En las muestras de pienso se analizó la fibra neutro detergente con α-amilasa termoestable. La lignina se analizó en el residuo de fibra ácido detergente mediante la solubilización de la celulosa con ácido sulfúrico. Todos los valores se corrigieron con el contenido libre de cenizas. El contenido energético bruto de los alimentos se determinó con una bomba de calorimetría (Model Parr 1341, Parr Instrument Company, Moline, IL, EE. UU.). Los

valores nutritivos se calcularon a partir de la composición química de las dietas utilizando ecuaciones del INRA (INRA, 2007). La composición química y valor nutritivo del heno y pienso se presenta en la Tabla 3.

Tabla 3. Composición química y el valor nutritivo de los alimentos (media ± desviación estándar).

Parámetro	Heno	Pienso
Composición Química		
Materia seca (MS), g/kg	920 ± 0,3	887 ± 2,1
Ceniza, g/kg MS	85,4 ± 0,33	68,5 ± 0,03
Proteína cruda, g/kg MS	94,4 ± 0,39	168 ± 0,1
Fibra neutro detergente, g/kg MS	584 ± 1,0	253 ± 0,4
Fibra ácido detergente, g/kg MS	330 ± 0,5	114 ± 0,3
Lignina ácido detergente, g/kg MS	33,1 ± 0,19	30,1 ± 0,17
Valor nutritivo		
Energía neta, MJ/kg MS	5,4 ± 0,03	7,5 ± 0,07
Proteína metabolizable, g PDI[1]/kg MS	73 ± 12,1	121 ± 2,9

[1] proteína verdadera digerible en el intestino delgado.

Los ácidos grasos de los alimentos liofilizados fueron determinados como ésteres metílicos de ácidos grasos (por sus siglas en inglés FAMEs, fatty acid methyl esters), mediante cromatografía de gases con detector de ionización de llama en el Laboratorio de Valoración Nutritiva del CITA. Los ácidos grasos fueron metilados y extraídos según lo propuesto por Sukhija y Palmquist (1988). La determinación se realizó con un cromatógrafo de gases Bruker Scion 436-GC (Bruker, Billerica, EE.UU.) equipado con un automuestreador CP-8400 (Bruker, Billerica, EE.UU.), una columna capilar de poli(biscianopropil siloxano): SP-2560 (100 m x 0,25 mm ID x 0,20 µm) (Sigma-Aldrich, Sant Louis, EE.UU.) y el software Compass CDS (Bruker, Billerica, EE.UU.). La identificación de los FAMEs se realizó usando los estándares comerciales GLC-532, GLC-401, GLC-643, GLC-642, GLC-463, C18:1 cis-11, C19:0 (Nu-Chek-Prep Inc., Elysian, EE.UU.). La cuantificación se realizó según lo descrito en la norma UNE-EN ISO 12966-4:2015 y se expresó en porcentaje de cada FAME individual con respecto a la cantidad total de FAMEs identificados.

3.4.2. Leche

Se analizaron los contenidos de grasa, proteína, lactosa, urea y células somáticas con un escáner infrarrojo (Milkoscan 7 RM, Foss Electric Ltd., Hillerød, Dinamarca). En la muestra de leche liofilizada, los ácidos grasos se metilaron y

extrajeron como se describe en Kramer et al. (1997). La determinación de los FAMEs se realizó usando la misma instrumentación que en el caso de los alimentos, pero el cromatógrafo de gases se equipó con una columna capilar de poli (biscianopropil siloxano) de mayor longitud: SP-2560 (200 m x 0,25 mm ID x 0,20 µm) (Sigma-Aldrich, Sant Louis, EE.UU.). La identificación de los FAMEs se realizó usando los estándares comerciales GLC-532, GLC-401, GLC-643, GLC-642, GLC-463, C18:1 cis-11, C18:1 trans-11, C19:0, C23:0 (Nu-Chek-Prep Inc., Elysian, EE.UU), mezcla BR1, mezcla BR4 (Larodan Research Grade Lipids, Solna, Suecia) y mediante tiempos de retención relativos observados en la bibliografía (Kramer et al., 1997; Shingfield et al., 2003; De la Fuente et al., 2015). La cuantificación se realizó según lo descrito en la norma UNE-EN ISO 12966-4:2015 y se expresó en porcentaje de cada FAME individual con respecto a la cantidad total de FAMEs identificados. Por último, los ácidos grasos se agruparon según su grado de saturación como ácidos grasos saturados por sus siglas en inglés (SFA), ácidos grasos mono-insaturado (MUFA) y ácidos grasos poli-insaturado (PUFA). También se agruparon según su origen como de síntesis *de novo* (C4:0 - C15:1), de origen mixto (C16:0 - C16:1) y de movilización (≥ C17:0), de acuerdo con Palmquist (2009). Adicionalmente se calculó la proporción de C18:1 cis-9 /C15:0.

3.4.3. Plasma

Las concentraciones plasmáticas de glucosa (método enzimático-colorimétrico) y urea (método cinético) se determinaron con un analizador automático (Gernon, RAL S.A, Barcelona, España). Las concentraciones de BHB (método cinético enzimático) y NEFA (método colorimétrico) se determinaron utilizando kits Randox (Randox Laboratories Ltd, Country Antrim, Reino Unido).

El estado oxidativo se determinó utilizando MDA como biomarcador de peroxidación lipídica según lo descrito en Yonny et al. (2016). Este indicador se determinó mediante cromatografía líquida y detector de fluorescencia, utilizando un cromatógrafo líquido de ultra-alta resolución Acquity UPLC H-Class (Waters, Milford, Massachusetts, EE.UU.), equipado con una columna de fase reversa de PFP/fluorofenilo ligada a base de sílice (Acquity UPLC HSS PFP, 100 mm × 2,1 mm × 1,8 µm, Waters), un detector de absorbancia (detector Acquity UPLC Photodiode Array PDA eλ, Waters) y un detector de fluorescencia (2475 Multi λ Fluorescence Detector, Waters) y todo ello controlado mediante el software Empower 3 (Waters). La cuantificación de MDA se realizó mediante detección de fluorescencia a λexcitación = 530 nm y λemisión = 550 nm siguiendo las condiciones cromatográficas descritas en Bertolín et al. (2019).

El análisis de la progesterona se realizó mediante un ensayo de inmunoadsorción ligado a enzima (ELISA), utilizando un kit específico para bovinos (Ridgeway Science, Lydney, Reino Unido). Cuando la concentración era mayor a 1 ng/mL, se consideró a las vacas cíclicas.

3.5. Cálculos y análisis estadístico de los datos

3.5.1. Cálculo del balance energético de las vacas

Se utilizó el contenido energético bruto de los alimentos para calcular su contenido energético neto (EN). El BE de las vacas se calculó utilizando las ecuaciones del sistema INRA (INRA, 2007) que a continuación se describen:

$$\mathrm{BE(UFL)} = \mathrm{EN\ ingesta} - \mathrm{EN\ mantenimiento} - \mathrm{EN\ producción}$$

$$\mathrm{EN\ ingesta\ (UFL)} = \frac{\mathrm{UFL}}{\mathrm{kg\ MS}} \times \mathrm{consumo\ MS(kg)} - \mathrm{E}$$

$$\mathrm{E} = (0{,}00063 \times \%\ \mathrm{Pienso}^2) - (0{,}017 \times \mathrm{UFL\ ingeridas}) + (0{,}002 \times \mathrm{UFL\ ingeridas}^2)$$

$$\mathrm{EN\ mantenimiento\ (UFL)} = 0{,}041\mathrm{x} \times \mathrm{PV}^{0{,}75} \times 1{,}1$$

$$\begin{aligned}\mathrm{EN\ producción\ (UFL)} &= \mathrm{Producción\ de\ leche\ (kg)} \\ &\times \{0{,}44 + [0{,}0055 \times (-40 + \mathrm{contenido\ grasa;\ g/kg})] \\ &+ [0{,}0033 \times (-31 + \mathrm{contenido\ proteina;\ g/kg})]\}\end{aligned}$$

UFL : “unité fourragère lait”, 1 UFL = 7,12 MJ.
E: corresponde a la “interacción digestiva”.
% de Pienso, en base de materia seca.

3.5.2. Análisis estadístico de datos

Los análisis estadísticos se detallan en los capítulos presentándose aquí de manera general. Los datos obtenidos durante el experimento se analizaron con los programas estadísticos SAS v 9.4 (SAS Institute Inc., Cary, NC, EE. UU) y R (R Development Core Team, 2021).

Agrupamiento de vacas

Se hizo un estudio de curvas de respuesta frente a los retos nutricionales realizados en distintos meses de la lactación (capítulo I) y en los repetidos (capítulo III). Las curvas predichas para las variables de producción de leche, NEFA y BHB en plasma se modelizaron utilizando la metodología de “natural cubic splines” (Perperoglou et al., 2019). Los splines se obtuvieron utilizando el comando ns en la librería splines de R.

Este modelo permitió obtener nuevas variables de respuesta para cada parámetro, para cada vaca y en cada reto nutricional. Las nuevas variables de la curva fueron *1) línea base*: valores estimados previos a la restricción alimentaria por la interpolación lineal desde el periodo basal hasta el de realimentación; *2) pico y 3) días hasta el pico*: en el caso de la producción de leche, el pico fue la máxima pérdida diaria de leche, mientras que para los NEFA y BHB el "pico" era el máximo incremento diario comparado con los valores de referencia. Los "días hasta el pico" eran los días transcurridos desde el inicio de la restricción hasta que se alcanzaron esos valores máximos; *4) área bajo la curva durante la restricción y 5) área bajo la curva durante la realimentación*: la pérdida total de leche estimada o el contenido extra de NEFA o BHB durante la restricción y la pérdida total de leche estimada o el contenido extra de NEFA o BHB durante la realimentación hasta que se recuperan los valores basales; *6) días para recuperar la línea base*: los días desde el inicio de la restricción hasta que la producción de leche y los contenidos de NEFA o BHB alcancen de nuevo el valor basal. Estos parámetros se analizaron mediante un análisis multivariante utilizando el paquete estadístico Factor Mine del software R, y se realizó un análisis de componentes principales (función PCA) para identificar las variables que explicaban la mayor parte de la variabilidad de la respuesta entre individuos. Posteriormente se llevó a cabo un análisis de conglomerados jerárquico (en inglés conocidos como clusters) sobre estas componentes principales (función HCPC), para agrupar las vacas con un patrón de respuesta similar. En este caso las vacas se agruparon en distintos **clusters de respuesta metabólica**.

El estudio del perfil de ácidos grasos de la leche se emplearon datos obtenidos en el tercer mes de lactación (capítulo IV) por lo que el agrupamiento de vacas no se podía hacer de la manera realizada en los capítulos I y III. En el capítulo IV además de los datos recogidos durante el periodo experimental (mes 3), se disponía de los siguientes datos previos al experimento: peso y CC al parto; peso, CC, producción de leche y BE en el día 30 y 31 de lactación. Por ello se utilizó un método de agrupación diferente asignándose las vacas a grupos en función de su semejanza en términos de distancia euclidiana calculada a partir de los datos previos al experimento. Se realizó una agrupación no jerárquica mediante el método k-means (procedimiento FASTCLUS). La selección del número óptimo de conglomerados se basó en criterios de conglomeración cúbica. Se obtuvieron dos **clusters según su estado nutricional** previo al experimento. Se realizó un análisis de la varianza de las variables de clasificación utilizando un modelo lineal general (procedimiento GLM) y tomando el conglomerado de estatus como efecto fijo.

Estudio de la variabilidad de los datos en los retos repetidos

Se realizó un análisis exploratorio de variabilidad de los datos de producción de leche, y concentración de NEFA y BHB en plasma por cada periodo de alimentación en los retos repetidos (capítulo III). Para ello se analizó la frecuencia de distribución de los datos, representada gráficamente mediante "gráficos de violín" usando el paquete estadístico ggplot2 de R. En este tipo de gráficos, la región de mayor anchura indica la mayor densidad de los datos mientras que los puntos superior e inferior representan los valores máximo y mínimo de los datos. Para comparar las diferencias entre la variabilidad de datos se utilizó la prueba F para comprobar si las varianzas de los distintos retos y periodos son iguales.

Análisis de los parámetros productivos y metabólicos

Los parámetros productivos (peso, producción y composición de la leche) y metabolitos en plasma, se analizaron mediante análisis de varianza de medidas repetidas (PROC MIXED). Dependiendo del capítulo se consideraron distintos efectos fijos: el cluster de respuesta metabólica (capítulos I y III, respuesta alta vs. baja) o el cluster de estado nutricional (en equilibrio vs. en desequilibrio, capítulo IV); el día (múltiples valores según capítulo) o periodo de manejo (Basal, Restricción y Realimentación); el mes de lactación (mes 2, 3 y 4; capítulos I y II) o la identificación del reto sucesivo (1º, 2º y 3º; capítulo III), y sus posibles interacciones. El animal se consideró como efecto aleatorio. Los grados de libertad se ajustaron con la corrección de Kenward-Roger para tener en cuenta los valores faltantes, en caso necesario. La estructura de las componentes de la varianza se seleccionó en función de los criterios de información de Akaike y Bayesiano más bajos.

Se estimaron las medias de mínimos cuadrados y se obtuvieron comparaciones por pares de las medias mediante la probabilidad de diferencia ajustada con la corrección de Tukey. La relación entre variables se determinó a través de los coeficientes de correlación de Pearson (r) usando el paquete CORRPLOT de R. El nivel de significación para todos los análisis fue $P < 0,05$ y se discutieron las tendencias cuando $0,05 \leq P < 0,10$.

IV. Publicaciones

Capítulo I

“Modelización de la respuesta individual de las vacas de carne a una restricción nutricional corta en diferentes estados de lactación”

Correspondiente al artículo:

Modelling beef cows’ individual response to short nutrient restriction in different lactation stages.

Karina G. Orquera-Arguero; Daniel Villalba; Mireia Blanco; Javier Ferrer; Isabel Casasús. 2022.

Animal. 16: 100619. https://doi.org/10.1016/j.animal.2022.100619

1. Introduction

Beef cattle managed under extensive conditions depend on the local availability of feed resources, which vary throughout the year in quality and quantity terms. This results in seasonal mobilisation patterns and the replenishment of body reserves, which might limit animal performance in critical physiological stages (Noya et al., 2019). The fact that cows face perturbations prevents them from fully expressing their production potential, with wide variability in individual coping strategies. In temperate climates, beef herds are housed in the winter (Blanco et al., 2008), and management is often simplified by group-feeding cows with a single diet irrespectively of their individual requirements. In these circumstances, animals' ability to cope with a nutritional challenge is particularly relevant.

This individual variability has been addressed in cows by testing different models to quantify the gap between the potential and disturbed performance that natural or induced perturbations cause (Adriaens et al., 2021; Bjerre-Harpøth et al., 2012; Codrea et al., 2011; De La Torre et al., 2022) as an indicator of not only animals' resilience, but also their capacity to be minimally affected by perturbations and to rapidly return to the previous state (Berghof et al., 2019). When disturbances happen during lactation, complex homeostatic and homeorhetic mechanisms concur to maintain a physiological equilibrium while redirecting nutrient partitioning towards milk production (Bauman and Currie, 1980). In dairy cows, the major source of milk yield variation in animals lies in their ability to partition nutrients towards the mammary gland (Baumgard et al., 2017). This process is mediated by the somatotropic axis, with increased growth hormone and decreased insulin production in higher-yielding cows, which promotes glucose-sparing mechanisms and the mobilisation of body reserves in peripheral tissue (Knight et al., 2004). Pareek et al. (2007) found differences in this endocrine regulation of nutrient partitioning between dairy and beef breeds in relation to their different milk secretion and body mass accretion potentials.

To ensure adequate nutrient supply for milk production, lipolysis releases non-esterified fatty acids (NEFA) from adipose tissue, which can be oxidised in the liver into ketone bodies like β-hydroxybutyrate (BHB) (Bell, 1995). Both metabolites have been proposed to assess the degree and effects of a negative energy balance (EB) in ruminants (Gross et al., 2011a; Kessel et al., 2008) whereas BW and body condition score (BCS) changes are poor indicators in dairy cattle (Pedernera et al., 2008). With feed restriction, negative EB is associated with decreased milk yield and higher NEFA

and BHB concentrations, and the magnitude of these effects depends on the lactation stage, and also on restriction severity and duration (Leduc et al., 2021).

The joint analyses of milk yield dynamics and other traits are useful for analysing the drivers of their concomitant changes (Ben Abdelkrim et al., 2021b). Multitrait clustering in different lactation phases has been used to identify distinct strategies to cope with metabolic challenges (de Koster et al., 2019; Friggens et al., 2016). In the long term, this has provided data to characterise dairy cows according to their ability to prioritise nutrient use among different life functions (Ollion et al., 2016), but this approach has not been used in beef cows. Therefore, the objectives of this study were to: (1) model beef cows' response of milk yield and plasma NEFA and BHB concentrations to short feed restriction and refeeding in three lactation stages; (2) cluster cows according to their metabolic response (MR); (3) determine differences between groups of cows and lactation stages. We hypothesised that beef cows would respond differently to restriction depending on their potential milk yield, and eventually on their size and fat reserves, and different coping strategies would be elicited as lactation advanced.

2. Material and methods

2.1. Experimental design

This experiment was conducted at the CITA La Garcipollera Research Station (Spain, 42°37'N, 0°30'W, 945 m a.s.l.). It involved 31 Parda de Montaña lactating beef cows [626 ± 47.7 kg BW, 2.8 ± 0.22 BCS and 7.5 ± 2.91 years at calving]. Cow-calf pairs were loose-housed in straw-bedded pens (7 or 8 cows/pen, 10x20 m) equipped with individual feeders for forage and ALPRO automatic concentrate feeding stations (Alfa Laval Agri, Tumba, Sweden). Calves were penned in cubicles and allowed to suckle twice daily for 30 minutes at 06:00h and 14:00h. The study consisted of three feeding periods repeated over the second, third and fourth lactation months. During each lactation month, cows received a diet that was calculated to meet 100% of their requirements for 4 days (d-4 to d-1, basal period), then they were restricted for 4 days (d0 to d3, restriction period) with a diet that met only 55% of their requirements and were returned to the 100% energy diet for 4 days (d4 to d7, refeeding period). On the first day (d0) of the restriction period, cows were in milk for 31, 58 and 87 (± 5.5) days (DIM; months 2, 3, and 4 of lactation, respectively) (Figure 1).

Cows were fed a flat-rate regime during lactation. Diets were calculated by considering the net energy and metabolisable protein requirements for the maintenance and lactation of a standard cow (BW 615 kg, milk yield 8.5 kg/d) using INRA equations (INRA, 2007). During the basal and refeeding periods, all the cows received 8.0 kg of hay (as a fed basis) daily, and only 7.0 kg of hay during the restriction period, offered daily at 08:00h as a single meal in individual feeders. Cows were tied up for approximately 2 h until they finished their ration. The ALPRO feeding stations were programmed to offer 3.0 kg (as fed)/day of concentrate to all the cows during the basal and refeeding periods. The individual intake was recorded daily. Animals had free access to water and mineral blocks.

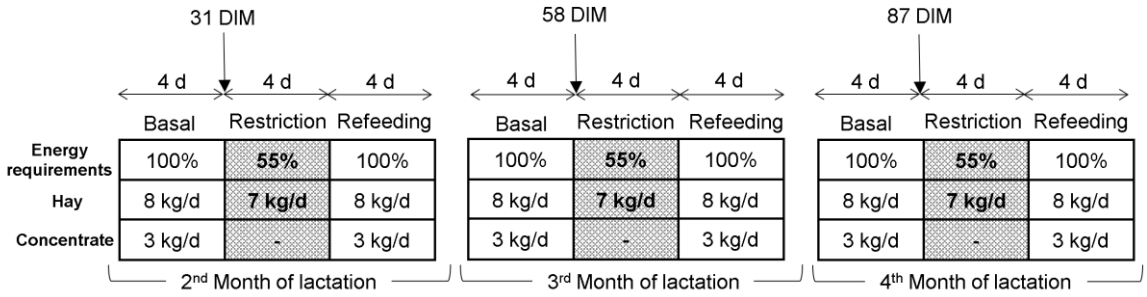


Figure 1. Schematic representation of the timeline of three short nutritional challenges of the beef cows throughout lactation. DIM: days in milk.

2.2. Measurements, sampling and chemical analyses

Samples of the offered feedstuffs were collected daily to determine their chemical composition and nutritive value (Table 1). All the analyses of feedstuffs were run in duplicate. Official methods were used to determine the contents of DM, ash and CP (Nitrogen analyser, Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain) (AOAC, 2000). The methods of Van Soest et al. (1991) were followed to obtain the contents of NDF, ADF and ADL using a fibre analyser (model Ankom 200/220, Ankom, Macedon, NY, USA). In the forage samples, NDF was assayed with heat stable amylase. Lignin was analysed on ADF residue by the solubilisation of cellulose with sulphuric acid. All the values were corrected for ash-free content. The feed values were calculated from the measured chemical composition of diets using INRA equations (INRA, 2007).

In the three months of lactation, during the basal period (d-4 and d-2) the BCS was assessed on scale from 0 to 5 based on the estimation of fat covering loin, ribs and tailhead. Milk yield was estimated (d-4, d-2 and daily from d1 to d7) by the weigh-suckle-weigh technique (Le Neindre and Dubroeucq, 1973), calculated using the milk consumed by the calf during both daily sucklings. Cows were weighed and bled on the same days

at 07:00h, after suckling and before hay was offered. Blood samples were collected from the coccygeal vein using test tubes with EDTA and heparin (BD Vacutainer, BD, Plymouth, UK) for the NEFA analysis and the BHB analysis, respectively. They were immediately centrifuged (3 500 rpm for 20 min at 4 °C). Plasma was collected and frozen at -20 °C until further analyses. Randox kits (Randox Laboratories Ltd, Crumlin, UK) were used to determine the BHB plasma concentration (kinetic enzymatic method, sensitivity: 0.100 mmol/L) and the NEFA concentration (colorimetric method, sensitivity: 0.072 mmol/L). The mean intra- and interassay CVs were 6.8% and 6.8% for BHB and 4.0% and 4.9% for NEFA, respectively.

Table 1. Chemical composition and nutritive value (mean ± SD) of the feedstuffs received by the beef cows during each month of lactation.

	Month 2	Month 3	Month 4
Hay			
Chemical composition			
DM, g/kg	919 ± 12.1	922 ± 11.7	918 ± 10.5
Ash, g/kg DM	98 ± 12.7	86 ± 24.4	78 ± 3.9
CP, g/kg DM	97 ± 25.7	109 ± 18.3	85 ± 8.1
NDF, g/kg DM	558 ± 59.2	570 ± 52.4	614 ± 21.2
ADF, g/kg DM	334 ± 33.5	324 ± 32.9	333 ± 15.9
Lignin, g/kg DM	41 ± 4.0	35 ± 12.8	28 ± 4.1
Nutritive Value			
Net energy, MJ/kg DM	5.4 ± 0.54	5.5 ± 0.54	5.4 ± 0.54
Metabolizable protein, g PDI[1]/kg DM	81 ± 17.9	79 ± 12.8	59 ± 5.7
Concentrate			
Chemical composition			
DM, g/kg	907 ± 2.4	906 ± 4.0	911 ± 11.1
Ash, g/kg DM	68 ± 1.3	68 ± 1.4	69 ± 2.1
CP, g/kg DM	173 ± 3.5	167 ± 4.7	169 ± 4.2
NDF, g/kg DM	246 ± 17.4	256 ± 23.2	254 ± 18.2
ADF, g/kg DM	102 ± 4.5	114 ± 11.1	120 ± 10.5
Lignin, g/kg DM	25 ± 7.5	29 ± 8.8	33 ± 6.6
Nutritive Value			
Net energy, MJ/kg DM	7.5 ± 0.34	7.3 ± 0.34	7.5 ± 0.34
Metabolizable protein, g PDI[1]/kg DM	123 ± 2.4	119 ± 3.3	120 ± 3.0

[1] true protein digestible in the small intestine.

2.3. Calculations and statistical analysis

The statistical analysis involved three steps:

Step 1: Modelling the individual response. The curve predicted for each trait (milk yield, NEFA, BHB) on the day of the experiment was modelled using natural cubic splines. A natural cubic spline with *K* knots is represented by *K* basis functions. Each

basis function is a third-degree polynomial specified in the Hermite form. Compared to other splines, a natural cubic confers additional constraints; i.e., function is linear beyond boundary knots. This frees up four degrees of freedom, which can be spent more profitably by sprinkling more knots in the interior region (Perperoglou et al., 2019). Each parameter that defines the natural cubic spline basis with eight knots was estimated for each cow within each month using a non-linear mixed model with the random effect of the cow. The basal level of each cow within a month was also modelled with a mixed model, which included only the intercept, the linear random regression coefficients and the data from the basal and refeeding periods. Splines were obtained using command ns in the library splines of R (R Development Core Team, 2014). Mixed models were solved using command nlme in library lme4 of R.

The new response variables obtained from the fitted curve for milk yield and plasma metabolites (NEFA and BHB) are depicted in Figure 2a and 2b, respectively. These response variables were: *1) baseline:* estimated values without feed restriction according to a linear interpolation from the basal to the refeeding period; *2) peak:* the maximum difference between the actual daily value and the baseline value. For milk yield, the peak was the maximum daily milk loss, whereas it was the maximum daily increment compared to baseline values for NEFA and BHB; *3) days to peak:* days from the start of restriction until the peak values were reached; *4) area under the curve (AUC) during restriction:* the estimated total milk loss or the extra NEFA or BHB contents during restriction compared to the baseline values; *5) days to regain baseline:* days from the start of restriction until the milk yield, and the NEFA or BHB contents reached the baseline again. *6) AUC during refeeding:* the estimated total milk loss or extra NEFA or BHB contents during refeeding until the baseline values were regained.

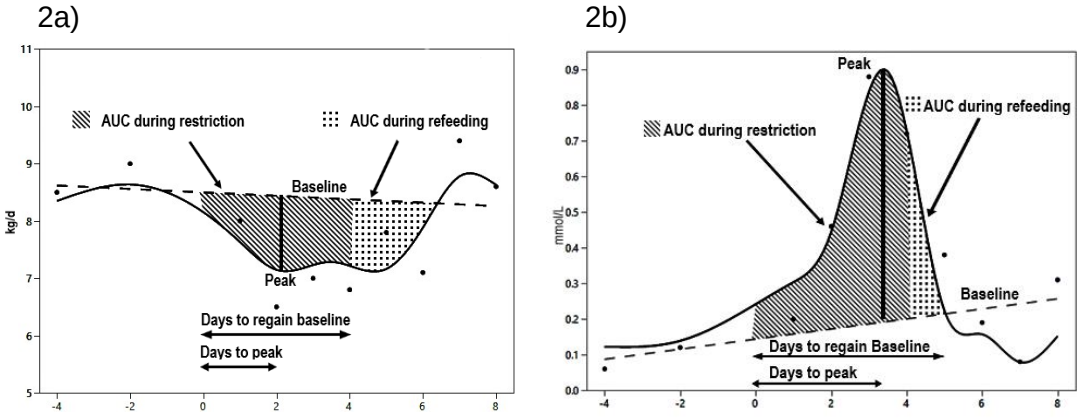


Figure 2. Schematic representation of the piecewise model for describing the variables of the milk yield (2a) and non-esterified fatty acids and β-hydroxybutyrate (2b) beef cows' response curves to a 4-day restriction and a 4-d refeeding period. AUC: area under the curve.

Step 2: Multivariate analysis. The new response variables obtained in step 1 for each trait, individual cow and month were employed to perform a multivariate analysis using the Factor Mine statistical package of the R software. First of all, a principal component analysis (PCA function) was used to identify the variables which accounted for most of the variability in the response among individuals. Then hierarchical clustering on these principal components (HCPC function) was carried out to group the cows with a similar response pattern. The optimum number of clusters was calculated automatically by the algorithm.

Step 3: Effect of cluster and lactation stage on performance and metabolic response. The phenotypic values and the new response variables during the three lactation months were studied according to the clusters obtained in the previous step using the SAS statistical package v 9.4 (SAS Institute Inc., Cary, NC, USA). Mixed linear models (MIXED procedure) were employed after taking cluster, month, and their interaction as fixed effects, and cow as the random effect. The least square means and associated SE were obtained and multiple comparisons were adjusted with Tukey correction. The Pearson correlations (r) between the response variables were obtained following the CORR procedure. The results were considered significant when $P < 0.05$, and trends were discussed when $0.05 \leq P < 0.10$.

3. Results

The first three principal components (PC) obtained in the PCA accounted for 48% of total variance. The first one (Dim 1, 25.6% of variance) was positively associated with the peaks and AUCs of NEFA, and negatively with the peaks and AUCs of milk yield during restriction (Figure 3a). The second PC (Dim 2, 12.5% of variance) was associated positively with the AUCs of NEFA during both restriction and refeeding, and negatively with peaks and AUCs of milk yield and BHB during restriction. Finally, the third PC was associated positively with the peak and AUC of milk yield in months 2 and 4, and with days to regain the baseline values of all the traits in month 2, and negatively with peak and AUC of BHB in month 4 (data not shown). The clustering analysis generated two clusters which differed in their MR, named Low MR (n=16) and High MR (n=15) (Figure 3b). The cows in the Low MR cluster had lower energy requirements and a less negative EB, and showed a poorer response to restriction in terms of milk yield and plasma NEFA and BHB concentrations. The cows in the High MR cluster showed a stronger response (Figure 4).

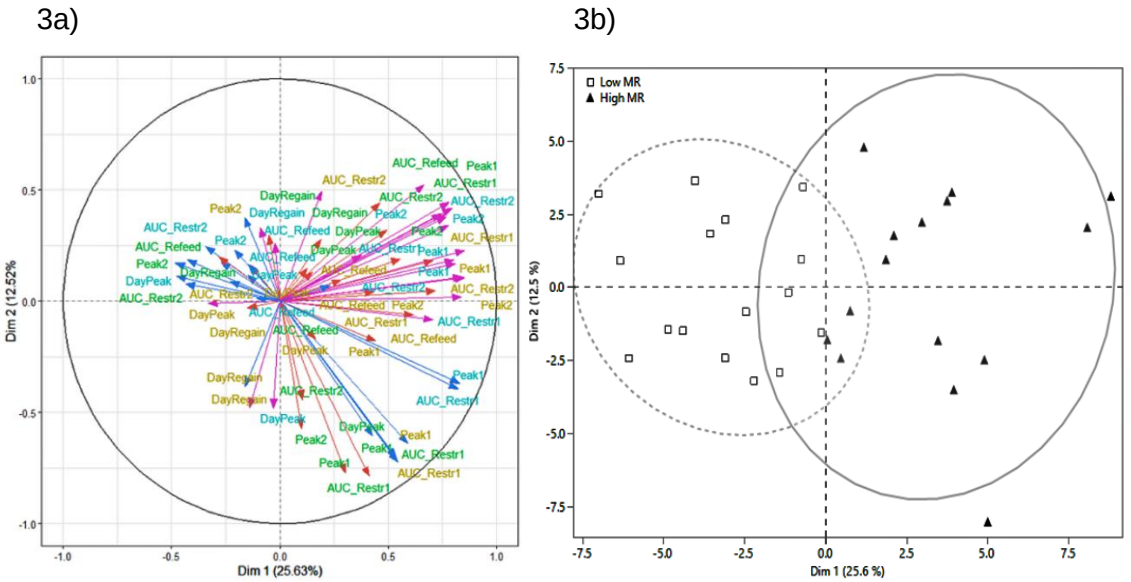


Figure 3. Variable factor map of the first two Principal Components (3a) and Distribution of the cows into the two generated metabolic response (MR) clusters (3b). Variables related to milk yield (blue arrows), plasma non-esterified fatty acids (pink arrows) and plasma β-hydroxybutyrate (red arrows), and months of lactation 2, 3, and 4 for yellow, green, and blue text labels, respectively.

Considering individual DM intake, on average diets met 91%, 61% and 93% of the net energy requirements and 100%, 58% and 103% of the metabolisable protein requirements during the basal, restriction and refeeding periods, respectively. Cow BW and BCS during the basal period did not differ between MR clusters (591 vs. 590 kg in the Low MR and the High MR, respectively, P = 0.91; 2.80 vs. 2.70 BCS points, respectively, P = 0.18). Both traits were affected by lactation stage, and were higher in month 2 than thereafter (599, 588 and 584 kg in months 2, 3 and 4, respectively, P < 0.001; 2.81, 2.73 and 2.71 respectively, P < 0.001). The milk yield response to feed restriction and subsequent refeeding according to the MR cluster and the month of lactation is shown in Table 2. The MR cluster affected the baseline values and the response to restriction ($P \leq 0.04$), but not the recovery pattern in the refeeding phase. The High MR cows had a higher baseline milk yield and AUC values during restriction, and tended to have greater peak milk loss. The month of lactation affected all the response variables during restriction ($P \leq 0.02$), but not during refeeding. A lower baseline yield was observed in month 4, and peak loss was greater in month 3 than in month 4, with intermediate values in month 2. The peak was reached more quickly, and total milk loss (AUC during restriction) was greater in month 3, with similar values in months 2 and 4.

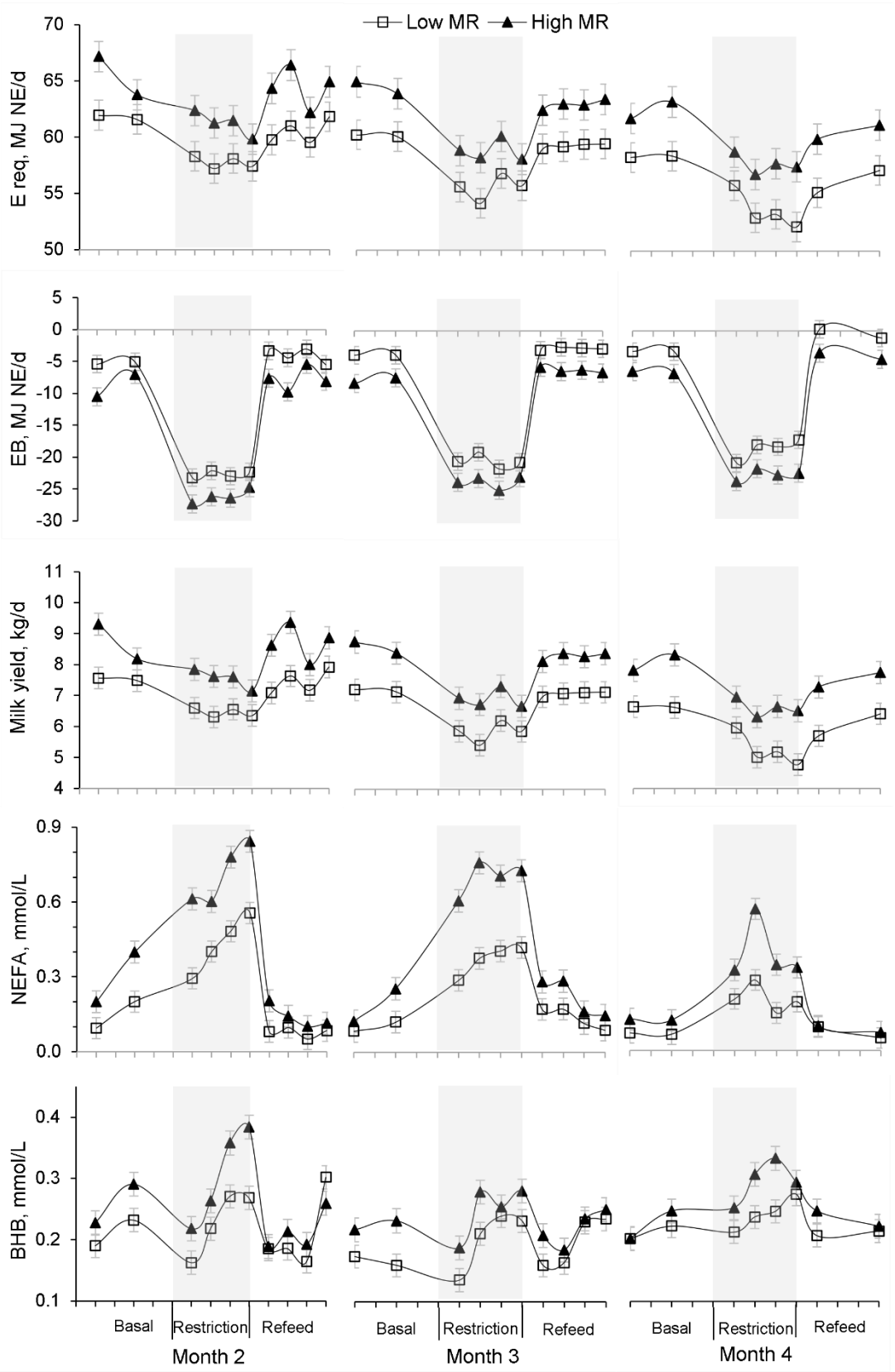


Figure 4. Energy requirements (E req), energy balance (EB), milk yield, and plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) concentrations of Low and High metabolic response (MR) beef cows during the experiment. Means are plotted and the vertical bars indicate the SE.

Table 2. Effect of metabolic response (MR) cluster and month of lactation on the milk yield response of beef cows to a 4-day restriction and a 4-day refeeding period.

	MR Cluster (Cl)		Month (M)				*P*-values[1]	
	Low MR	High MR	2	3	4	RSD	Cl	M
Baseline, kg/d	6.94^{y}	8.27^{x}	8.10^{a}	7.80^{a}	6.92^{b}	0.584	0.002	0.001
Peak*, kg/d	-1.32	-1.56	-1.45^{ab}	-1.61^{b}	-1.27^{a}	0.463	0.068	0.020
Days to peak, d	2.57	2.63	2.80^{a}	1.78^{b}	3.22^{a}	0.990	0.813	0.001
AUC$^{\dagger}_{restriction}$*, kg	-3.80^{y}	-4.81^{x}	-4.01^{a}	-5.21^{b}	-3.70^{a}	1.656	0.036	0.002
Days to regain baseline, d	5.93	5.74	5.65	5.98	5.87	0.935	0.326	0.376
AUC$^{\dagger}_{refeeding}$*, kg	-0.83	-0.74	-0.68	-0.82	-0.86	0.798	0.644	0.647

Within a variable, least square means with different superscript ($^{x, y}$) differ between MR clusters with $P < 0.05$; least square means with different superscripts ($^{a, b, c}$) differ among months with $P < 0.05$.

[1] the interaction was not significant for any variable ($P > 0.05$).

† area under the curve; *deviation from baseline.

The response of the plasma NEFA and BHB concentrations is shown in Table 3. For NEFA, the MR cluster affected the baseline values, peak and AUC during restriction ($P \leq 0.001$), with higher values obtained by the High MR cows. No differences were observed in the days to peak or to regain the baseline. All the NEFA response variables were affected by the month of lactation ($P \leq 0.04$). The baseline values were lower in month 4 compared to the other two months. Peak concentrations during restriction decreased significantly from lactation month 2 to lactation month 4, and were reached more quickly in month 4 than in the others. The days to regain baseline were also affected by month, with faster recovery in months 2 and 4 than in month 3. Only the AUC during refeeding was affected by the interaction between the MR cluster and the month of lactation (Figure 5a). Regarding the BHB response, the baseline values and the AUC during restriction were higher in the High MR than in the Low MR cluster ($P \leq 0.02$). The month of lactation affected both parameters and the AUC during refeeding, which were lower in month 3 ($P \leq 0.03$), and tended to affect the days to regain the baseline ($P = 0.06$). Finally, the peak was affected by the interaction between the MR cluster and the month of lactation ($P = 0.03$), and the differences between the MR clusters were only significant in month 2, but not thereafter. Furthermore, the peak BHB in the Low MR cows remained stable throughout lactation, whereas the values in their High MR counterparts were higher in month 2 than later (Figure 5b).

Table 3. Effect of metabolic response (MR) cluster and month of lactation on plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) response of beef cows to a 4-day restriction and a 4-day refeeding period.

	MR Cluster (Cl)		Month (M)				*P*-values[1]	
	Low MR	High MR	2	3	4	RSD	Cl	M
NEFA								
Baseline, mmol/l	0.09^{y}	0.15^{x}	0.13^{a}	0.15^{a}	0.08^{b}	0.049	0.001	0.001
Peak*, mmol/l	0.26^{y}	0.51^{x}	0.54^{a}	0.38^{b}	0.24^{c}	0.129	0.001	0.001
Days to peak, d	2.94	3.05	3.38^{a}	3.09^{a}	2.51^{b}	0.583	0.453	0.001
AUC†$_{restriction}$*, mmol x d/l	0.68^{y}	1.42^{x}	1.36^{a}	1.17^{a}	0.62^{b}	0.396	0.001	0.001
Days to regain baseline, d	5.74	5.74	5.55^{b}	6.08^{a}	5.59^{b}	0.869	0.991	0.036
AUC†$_{refeeding}$*, mmol x d/l	0.13^{y}	0.21^{x}	0.24^{a}	0.23^{a}	0.04^{b}	0.094	0.001	0.001
BHB								
Baseline, mmol/l	0.220^{y}	0.248^{x}	0.238^{ab}	0.222^{b}	0.243^{a}	0.031	0.024	0.026
Peak, mmol/l	0.07^{y}	0.11^{x}	0.12^{a}	0.07^{b}	0.08^{b}	0.068	0.002	0.003
Days to peak, d	3.20	3.11	3.29	3.08	3.09	0.815	0.574	0.540
AUC†$_{restriction}$*, mmol x d/l	0.04^{y}	0.13^{x}	0.10^{a}	0.02^{b}	0.13^{a}	0.135	0.011	0.006
Days to regain baseline, d	5.30	5.21	4.91	5.29	5.56	1.064	0.662	0.062
AUC†$_{refeeding}$*, mmol x d/l	-0.003	0.01	0.01^{a}	-0.02^{b}	0.02^{a}	0.045	0.175	0.001

Within a variable, least square means with different superscript ($^{x, y}$) differ between MR clusters with $P < 0.05$; least square means with different superscripts ($^{a, b, c}$) differ among months with $P < 0.05$.

[1] the interaction was significant for NEFA AUC$_{refeeding}$ ($P = 0.01$) and BHB Peak ($P = 0.03$).

† area under the curve; *deviation from baseline.

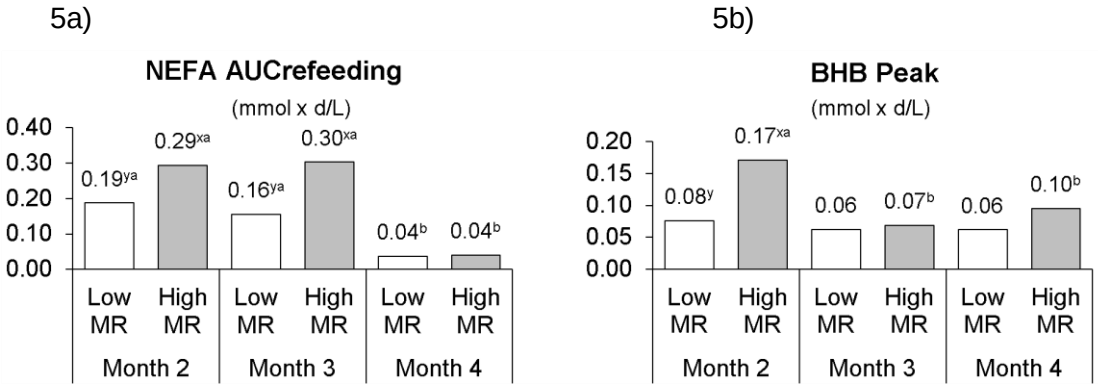


Figure 5. Effect of the metabolic respose (MR) cluster and month of lactation on non-esterified fatty acids (NEFA) AUC$_{refeeding}$ (5a) and β-hydroxybutyrate (BHB) peak (5b) in beef cows in response to a 4-d restriction and a 4-d refeeding period.

For each response variable, means with different superscript ($^{x, y}$) differ between MR clusters within month ($P < 0.05$) and with different superscripts ($^{a, b}$) differ among months within MR clusters with ($P < 0.05$).

AUC$_{refeeding}$: area under the curve during the refeeding period.

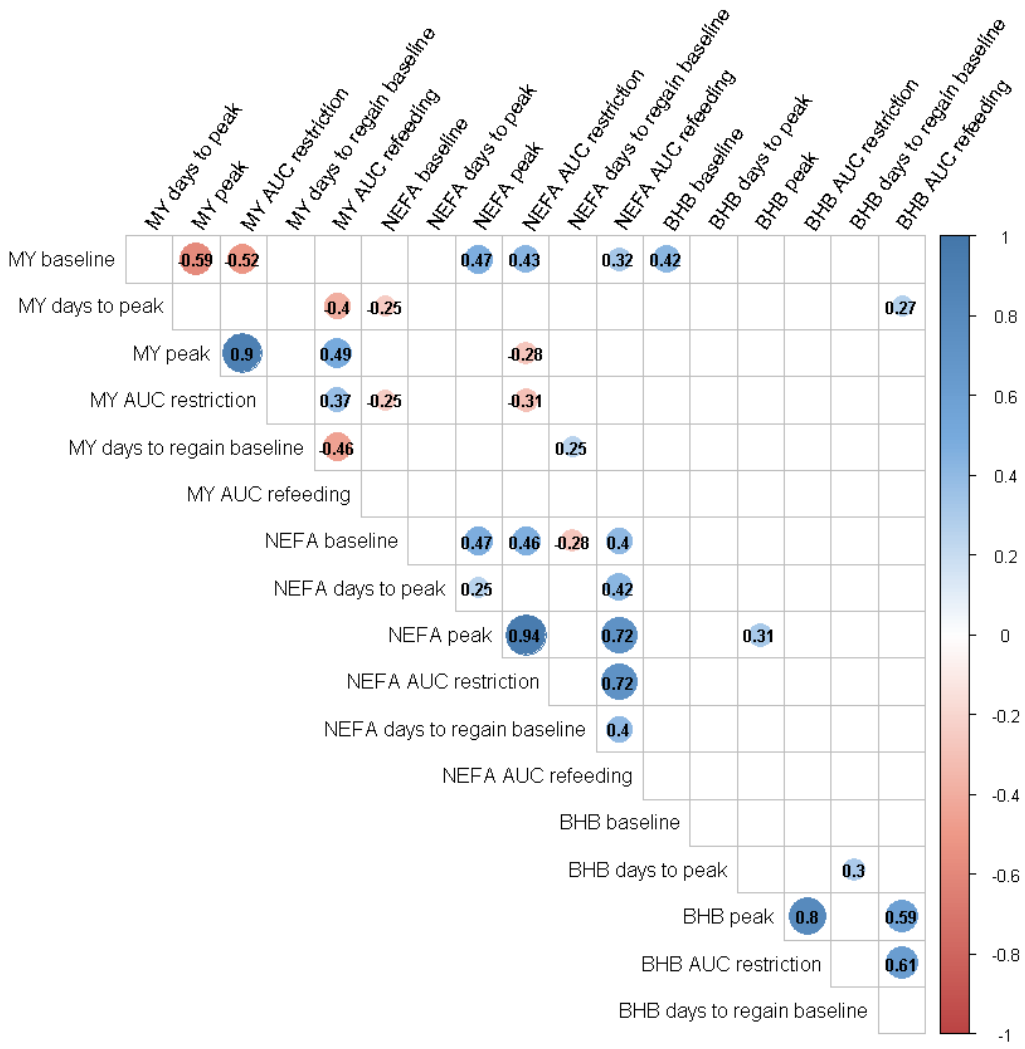


Figure 6. Significant Pearson correlations between the response variables of milk yield (MY) and the plasma non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) concentrations in beef cows. AUC: area under the curve.

The significant correlations among the response variables of the milk yield, NEFA and BHB concentrations, all months considered, are shown in Figure. 6. Within trait, the AUC during restriction correlated strongly with the peak ($P < 0.001$), but not with the days to peak. For milk yield, the baseline values correlated negatively with the peak and AUC during restriction ($P < 0.001$). Milk loss (AUC) during refeeding correlated positively with the peak and AUC during restriction, but negatively with days to peak and to regain baseline ($P < 0.001$). For NEFA, the baseline values correlated positively with the peak and AUCs during restriction and refeeding ($P < 0.001$). The AUC during refeeding correlated strongly with the peak and AUC during restriction, and only moderately with days to peak and regain the baseline ($P < 0.001$). Regarding BHB, the AUC during refeeding correlated positively with the peak and AUC during restriction ($P < 0.001$). In the three traits, the correlations between days to peak and days to regain baseline were

not significant. Across traits, the milk yield baseline values correlated moderately with the NEFA peak and AUC during restriction and the BHB baseline values, and weakly with the NEFA baseline values and the BHB peak ($P \leq 0.03$). The NEFA peak correlated weakly with the BHB peak and negatively with the milk yield peak ($P \leq 0.03$), but the milk yield and BHB peaks did not correlate. The AUCs of milk yield and NEFA during restriction were negatively correlated ($P \leq 0.003$), but not with those of BHB.

4. Discussion

4.1. Response curves

Different mathematical models have been used to characterise milk yield in dairy cows, from traditional models describing the shape of the lactation curve to individually adjusted polynomial curves based on well-established statistical models (Harder et al., 2019). Fewer modelling studies have been conducted on beef cattle because it is not routinely measured in common practice (Cortés-Lacruz et al., 2017; Sapkota et al., 2020; Sepchat et al., 2017). Animal performance can be affected by perturbations caused by climate, management or diseases, which can compromise both animal nutrition and welfare. Several studies have evaluated the response of ruminant females to natural (Adriaens et al., 2021; Poppe et al., 2020) or induced (Barreto-Mendes et al., 2022; Codrea et al., 2011; Friggens et al., 2016) perturbations, and found wide interindividual variations. They have analysed deviations from a theoretical unperturbed lactation curve (Ben Abdelkrim et al., 2021a), which corresponds to the baseline in our study, and they have described the response while conducting challenges and in the recovery phase. Although most studies have modelled milk yield, this methodology could be extrapolated to other biological time-series data (Codrea et al., 2011), which are increasingly available with the rise of in-line measurement technologies. Ben Abdelkrim et al. (2021b) used a similar model to simultaneously predict the dynamics of milk yield and BW response over time, and to explore the relation between them, as we do herein with plasma NEFA and BHB.

4.2. Effect of the metabolic response cluster

The clustering analysis identified two distinct groups of cows that differed mainly in terms of their milk yield and NEFA response, and less markedly in their BHB response to nutritional challenges. Both BW and the BCS were similar in the two clusters throughout lactation, which implies that body size or fat reserves did not affect the response, which would be driven mainly by milk yield and the concomitant metabolic effort to sustain it. These findings are similar to those reported by Pedernera et al. (2008), who found that BW and BCS changes did not accurately reflect the extent of mobilisation

of dairy cows' body reserves in early lactation. Schuh et al. (2019) reported that the BCS affected reserve mobilisation intensity, with higher NEFA and BHB serum concentrations in the cows with a high BCS, but this was not the case in our study. Breed or parity (Adriaens et al., 2021; Ben Abdelkrim et al., 2021a) can also influence individual responses to perturbations, but they did not differ between the MR clusters.

The size of the response was related to basal performance. All the basal values were higher in the High MR than in the Low MR profile, which coincides with Friggens et al. (2016). At the individual level, significant correlations were observed between the basal values and the response during restriction (peaks and AUCs) for milk yield and NEFA, but not for BHB. Berghof et al. (2019) have also indicated that high-performing animals can be more sensitive to perturbations. Interestingly, these differences were only observed in the magnitude of the response, but not in the time taken to react and recover, which reflects the plasticity of cows' response.

The impact of feed restriction on milk yield can widely range (from -7% to -71%) depending on restriction severity and duration, and also on lactation stage (Leduc et al., 2021). Here the absolute milk loss was higher in the High MR than in the Low MR cows, but peak milk loss in relative terms was 19% of the basal milk yield for both groups. When comparing Holstein and Montbéliarde cows, with different pre-challenge milk yields, Billa et al. (2020) also observed a similar relative response to a 6-day 50%-feed restriction between them. The MR cluster did not affect the time taken to reach the peak here (mean 2.6 days) or to regain the baseline (5.8 days), which implies that responses were larger, but not faster, in the High MR than in the Low MR cows. Both reaction times were shorter than those observed in natural (Adriaens et al., 2021) or induced (Bjerre-Harpøth et al., 2012) perturbations in dairy cows, which is likely due to the lower milk yield and the associated metabolic load of beef cows.

Homeorhetic controls regulate different metabolic adaptations to support lactation. Of them, growth hormone and insulin are key mediating factors responsible for the partition of nutrients away from body storage and towards the mammary gland (Baumgard et al., 2017; Knight et al., 2004). Although the hormones involved in this partitioning were not herein investigated, we observed significant effects of feed restriction on the plasma metabolites that result from their action, which were more evident in NEFA than in BHB. With poor nutrient supply, cows mobilise adipose tissue by releasing circulating NEFA so they are either converted into milk triglycerides in the udder or oxidised in the liver as an energetic substrate (Bell, 1995). All the NEFA response variables had almost doubled in the High MR than in the Low MR cluster, which

denotes that the cows with higher milk yields had greater basal fat mobilisation and were able to further increase lipolysis during the nutritional challenge. Excessive lipid mobilisation can surpass the liver's metabolic capacity to oxidise NEFA and ketone bodies such as BHB are produced (Mann et al., 2016). Thus the High MR cows also had a higher BHB peak and AUC during restriction than the Low MR cows. Threshold values of 0.60 mmol NEFA/L (Jorjong et al., 2014) and 1.2 mmol BHB/L (Li et al., 2012) are associated with the risk of clinical ketosis in dairy cows. Regarding NEFA, they were reached only by the High MR cows during the peaks of months 2 and 3, but not by the Low MR cows, and never for BHB, which suggests that circulating NEFA supplied enough energy to meet the metabolic demands induced by nutrient restriction.

The response profiles observed herein suggest that the High MR cows had a higher potential milk yield and were able to efficiently partition more nutrients towards milk synthesis than the Low MR cows. Elgersma et al. (2018) considered that dairy cows with fewer milk yield fluctuations under natural perturbations were more resilient because minor variance in performance genetically correlated with better health and longevity. Conversely, we can conclude that the High MR cows were able to establish homeorhetic mechanisms in the short term (Bauman and Currie, 1980) with sufficient intensity to ensure that, despite their more negative EB, they continued to display better lactation performance and recovered after the challenge. Ollion et al. (2016) have described different profiles in dairy cows depending on their lactation performance, reproduction and ability to maintain their reserves, the most determinant life functions among which trade-offs have often been identified. They found that milk yield was an important driver of these profiles, as we observed in the present work, but not the only one given the wide individual variability in the strategies to prioritise nutrient allocation to these life functions.

4.3. Effect of the lactation stage

Previous studies have analysed the adaptations of lactating ruminants to feed restriction in different phases. Within-animal responses are repeatable between early- and mid-lactation in dairy cows (Gross and Bruckmaier, 2015), between consecutive lactations in dairy goats (Friggens et al., 2016) and between two consecutive feeding challenges of different duration in beef cattle (De La Torre et al., 2022), which indicate that variability may be genetically driven. Here we clustered cows according to their response throughout lactation and analysed the month of lactation separately, finding a strong effect on most response variables. The general lack of interactions between MR cluster and month confirmed the validity of our approach.

To the best of our knowledge, no comparable studies are available on beef cows in different lactation stages. As stated above, the lactation curves of beef breeds are less well-known than those of dairy cattle. Sepchat et al. (2017) have described slow increases in milk production after calving, which peaked between the first and third lactation months. The curve was flatter than in dairy cows due to the balance between a calf's ability to drink milk and the dam's production potential. A recent meta-analysis by Sapkota et al. (2020) described earlier peak milk yields dairy-beef crosses (4-6 weeks) compared to pure beef cows (5-8 weeks), the latter showing a better persistency. The basal milk yield here was similar in months 2 and 3, which suggests that the peak was reached before week 8, and then decreased in month 4. The basal values agreed with previous observations in multiparous Parda de Montaña cows, as in Blanco et al. (2008), regardless of suckling management, calf sex or supplementation (Cortés-Lacruz et al., 2017).

The impact of feed restriction on milk yield was higher in month 3 than in months 2 and 4, as shown by the greater peak loss (in both absolute and relative terms, 21% vs. 18%), which was attained more quickly, and the total milk loss. With an induced short-term feed restriction, Bjerre-Harpøth et al. (2012) found a similar milk loss in relation to pre-challenge values (30%) in early-, mid- and late-lactation with dairy cows, unlike our results. In response to natural perturbations, effects were severer, developed more quickly and recovered more slowly in early- to mid-lactation than in later stages (Adriaens et al., 2021). Conversely, we found that lactation stage did not affect the recovery rate during refeeding, as observed by Codrea et al. (2011).

Whereas the milder effect of nutrient restriction in later stages (i.e. in month 4) was supported by the abovementioned literature, the stronger impact in month 3 than in month 2 was not expected given the similar energy and protein intake. We hypothesise that, as the basal milk yield was similar, but both BW and the BCS were lower in month 3, these beef cows' coping strategy in month 3 was not sufficient to buffer the effect of feed restriction on milk production. The basal NEFA concentrations were similar in months 2 and 3, and were higher than those of month 4, but the peak values of NEFA and BHB decreased steadily, and were reached more quickly for NEFA, as lactation progressed. All this indicates decreasing lipid mobilisation. Apparently, despite the metabolic demand for milk yield still being high in month 3, these beef cows' response to homeorhetic controls was not sufficient to ensure adequate nutrient supply to support milk synthesis under the feed restriction. Baumgard et al. (2017) indicated that when a negative EB occurs, the dairy cows selected for higher milk yield are able to partition more nutrients away from storage and towards mammary utilisation. The opposite would

be the case in our study, where that response would be less intense in beef cows with a lower genetic capacity for milk production. This is supported by the findings of Pareek et al. (2007), who compared the response to a metabolic challenge between breeds of different genetic merits for milk yield, and found that dairy cows had lower insulin levels, a lower EB, but greater milk production efficiency than beef cows which, in turn, had a higher potential for body energy and protein accretion.

Regarding the BHB peak, the interaction between month and the MR cluster implied that lipid mobilisation was insufficient only in month 2 for the High MR cows, and the ketogenesis from NEFA resulted in a greater BHB peak in response to feed restriction in early lactation. The higher metabolic load in earlier lactation stages has been described in dairy cows, with natural NEFA peaks 1-2 weeks postpartum and a delayed response in BHB peaks at 2-3 weeks (Gross et al., 2011a; Kessel et al., 2008), which decrease thereafter. In Parda de Montaña beef cows fed at 75% (Alvarez-Rodríguez et al., 2009) or 100% (Noya et al., 2019) of their requirements, NEFA peaked at 0.27 to 0.35 mmol/l up to week 5 postpartum and then decreased to reach 0.08 mmol/l in month 4, whereas BHB contents remained constant (approx. 0.20 mmol/l) throughout lactation (Rodríguez-Sánchez et al., 2018).

This effect of month on the basal values could condition the coping strategies which cows apply to face undernutrition in different stages. Bjerre-Harpøth et al. (2012) found decreasing basal NEFA concentrations from early to late lactation, and high BHB contents only in early lactation. When short-term energy deficit was induced, the relative changes in NEFA during restriction increased throughout lactation, while BHB only responded in early lactation. Other studies report that plasma NEFA concentrations are less responsive to feed restriction in late lactation (Carlson et al., 2006; Gross et al., 2011a), when even a drastic energy restriction may not increase the BHB concentration if there are not sufficient NEFA for ketogenesis. According to our results, in a recent review on the effects of feed restriction on dairy cows, Leduc et al. (2021) found that NEFA increased (+14% to +3475%) in most studies, while the effect on BHB was less consistent (+26% to +721% in only 14 of the 23 studies).

5. Conclusion

Changes in the performance and plasmatic indicators of lipolysis and ketogenesis of beef cows in response to short-term feed restriction can be modelled using spline curves, which allows different metabolic response profiles to be established. The extent, but not the speed, of the individual response was driven primarily by basal milk yield, but adaptation strategies changed as lactation advanced, and as the nutrient demand for milk production and concomitant fat mobilisation decreased. Although long-term performance should also be evaluated, identifying animals that can respond to a nutritional challenge by establishing mechanisms to minimise the impact on their performance is key to develop breeding programmes for enhanced beef cows' resilience.

Capítulo II

“Rendimiento y respuesta metabólica de las vacas de carne a una restricción nutricional corta en diferentes meses de lactación”

Correspondiente al artículo:

Beef cows’ performance and metabolic response to short nutritional challenges in different months of lactation

Karina G. Orquera-Arguero; Isabel Casasús; Javier Ferrer; Mireia Blanco. 2023
Research in Veterinary Science 159: 26-34.
https://doi.org/10.1016/j.rvsc.2023.04.002

1. Introduction

Beef cows managed in temperate grassland systems depend very much on forage availability and quality during the grazing season, and also in the winter when they are usually group-fed preserved forages. Under these conditions, they face a dynamically changing nutrient supply, which can be inadequate to meet their requirements during some key physiological periods (Mulliniks and Beard, 2019). Projected climate changes, including more frequent extreme weather events, will further affect the quantity and nutritive value of the feed available throughout the production cycle (Henry et al., 2018). To successfully cope with these challenges, effective strategies need to be developed at both the animal and farm levels (Blanc et al., 2006).

Lactating cows respond to limiting nutritional environments with the mobilisation of body tissues and a range of behavioural and physiological mechanisms that involve modifications in nutrient allocation towards the different metabolic functions, whose priority differs depending on lactation stage (Bjerre-Harpøth et al., 2012; Murrieta et al., 2010). In order to disentangle the mechanisms that determine this metabolic flexibility in response to environmental change, the nutritional perturbations involving both short- and long-term feed restriction-refeeding cycles have been widely studied in dairy cows (Abdelatty et al., 2017; Gross et al., 2011a; Pires et al., 2019). In beef cattle, several papers have assessed cows' performance and metabolic response to long-term underfeeding (Alvarez-Rodríguez et al., 2009; Fiems et al., 2015), but adaptation to short-term nutrient restrictions has only been recently considered (De La Torre et al., 2022; Orquera-Arguero et al., 2022). Animals' ability to respond to and recover after short-term disturbances, defined as resilience (Friggens et al., 2022), is key for their performance in variable environments.

In dairy cows, the adaptive response to underfeeding usually implies reduced milk yield, and milk composition may, or may not, be affected depending on the length and intensity of restriction, among other factors (Boutinaud et al., 2019; Kvidera et al., 2017; Leduc et al., 2021). In order to overcome the negative energy balance (EB), cows will mobilise their body reserves, including both fat and protein. The mobilisation of body fat releases non-esterified fatty acids (NEFA) into the blood stream, which can be oxidised in the liver into ketone bodies, such as β-hydroxybutyrate (BHB), as energy fuel (Bell, 1995). Complementary, NEFA can be esterified to triglycerides and accumulate in the liver, or taken up by the mammary gland, where they account for a significant fraction of milk fat synthesis. When the oxidative metabolism is altered, excessive reactive oxygen species (ROS) production leads to oxidative stress (Abuelo et al., 2015), for

which malondialdehyde (MDA), a degradation product of lipid peroxidation, has been proposed as a biomarker (Castillo et al., 2006). The catabolism of the protein mainly from the skeletal muscle yields glucogenic amino acids, and affects plasma glucose and urea concentrations (Ingvartsen et al., 2003). In *ad libitum*-fed dairy cows, body protein catabolism starts in the transition period (from 3 weeks before calving) and extends up to 5 weeks after calving, while fat reserves are mobilised up to 12 weeks postpartum, when feed intake matches milk yield requirements and endocrine status limits mobilisation (Sadri et al., 2023). This period can be shorter in lower milk-yielding breeds (Jorge-Smeding et al., 2022). When faced with temporary nutrient restriction, lactation stage plays a key role in the physiological adaptive response because the priority and requirements of the mammary gland change as lactation evolves by modifying the allocation of nutrients to milk synthesis (Boutinaud et al., 2019; Gross and Bruckmaier, 2019). Furthermore, when cows are refed, the post-challenge recovery rate can be faster in later lactation stages (Bjerre-Harpøth et al., 2012). This information is not available in beef cows, where the influence of lactation stage on nutrient allocation may differ from that of dairy cows due to their lower milk yield and different feeding management because they are rarely fed to appetite and are often placed in limited nutrient environments (Mulliniks and Beard, 2019).

The aim of this experiment was to determine lactating beef cows' response to short-term feed restriction and refeeding periods in three different months of lactation both on the productive and physiological levels. We hypothesised that cows would respond to nutritional perturbations by reducing their milk yield and modifying their lipid and protein metabolism differently as lactation progressed.

2. Material and methods

The Animal Ethics Committee of the research centre approved all the experimental procedures (protocol no CEEA-03-2018-01), which followed the EU Directive 2010/63 guidelines on the protection of animals used for experimental and other specific purposes.

2.1. Animal management, experimental and diet design

The experiment was conducted at CITA La Garcipollera Research Station in the Pyrenees mountain area (Spain, 42°37' N, 0°30' W, 945 m a.s.l.) using 31 lactating Parda de Montaña beef cows [body weight (BW) (mean ± SD): 626 ± 47.7 kg; body condition score (BCS): 2.8 ± 0.22 (0-5 scale); age: 7.5 ± 2.91 yr]. Cows were randomly allocated in pens (7 or 8 cows/pen, 10 x 20 m) equipped with individual feeders for forage and automatic feeding stations (ALPRO, Alfa Laval Agri, Tumba, Sweden) for concentrate.

Calves were stocked in straw-bedded cubicles adjacent to their dams. They were allowed to suckle their dams daily for two 30-minute periods at 06:00h and 14:00h. All the cows received the same ration, which was composed of different quantities of hay and concentrate. The chemical composition and nutritive value of feedstuffs are presented in Table 1 (for detailed information see Orquera-Arguero et al., 2022). Diets were calculated by considering the net energy and metabolisable protein requirements for the maintenance and lactation (INRA, 2007) of a standard cow with a BW of 615 kg and a milk yield of 8.5 kg/d. From calving to the end of the experiment all the cows were fed a diet that met 100% standard cow energy and protein requirements, except for 3 restriction periods when they were fed a diet to meet 55% standard cow energy and protein requirements. The experiment consisted of three consecutive 4-day feeding periods, which were repeated over months 2, 3 and 4. Every month, the trial started with 4 days on which cows had access to the abovementioned diet, which met 100% of their requirements (basal period). For the next 4 days, they were fed a diet that met 55% requirements (restriction period). On the last 4 days, once again they received the formulated diet to meet their 100% requirements (refeeding period). On the first day of restriction periods, cows were in milk for 31 (month 2), 58 (month 3), and 87 (month 4) days.

Table 1. Chemical composition and nutritive value (mean ± standard deviation) of the feedstuffs offered to the beef cows.

	Hay	Concentrate
Chemical composition		
Dry matter (DM), g/kg	920 ± 10.9	908 ± 6.7
Ash, g/kg DM	87.5 ± 17.3	68.3 ± 1.6
Crude protein, g/kg DM	97.1 ± 20.5	170 ± 4.7
Neutral detergent fibre, g/kg DM	581 ± 51.0	252 ± 19.2
Acid detergent fibre, g/kg DM	330 ± 27.3	112 ± 11.5
Lignin, g/kg DM	34.9 ± 9.30	29.3 ± 8.10
Nutritive Value		
Net energy, MJ/kg DM	5.4 ± 0.13	7.4 ± 0.36
Metabolizable protein, g PDI[1] /kg DM	73 ± 12.1	121 ± 2.9

[1] true protein digestible in the small intestine.

The diet fed to meet 100% energy and protein requirements was composed of 7.4 kg dry matter (DM) hay and 2.7 kg DM concentrate. During restriction, cows received 6.4 kg DM hay to meet 55% of their energy and protein requirements. Throughout the experiment, water and mineral blocks were supplied *ad libitum*. Hay was offered daily as a single meal at 08:00h in individual feeders with cows tied up for approximately 2 h until they had finished their ration. The ALPRO feeding stations were programmed to offer concentrate to all the cows during the basal and refeeding periods. The individual hay and concentrate intakes were recorded daily.

2.2. Measurements and samplings

All the cow measurements were taken daily in the morning before hay-feeding, and during each feeding period (basal, restriction, refeeding) in experiment months 2, 3 and 4. Cows were weighed on an electronic scale. Milk yield was estimated by the weight-suckle-weight technique of the calf (Le Neindre and Dubroeucq, 1973) as the sum of the milk consumed in both sucklings. After the morning suckling, a composite 50-mL milk sample was manually collected per cow from all four teats, after discarding 3 streams of milk per teat. After calf removal, cows were administered an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) 5 min before the manual extraction to facilitate the letdown of residual milk. Milk samples were preserved with sodium azide (PanReac, Barcelona, Spain) and refrigerated at 4ºC until further analyses. Cow blood samples were collected from the coccygeal vein in heparinised tubes (BD Vacutainer Becton-Dickenson and Company, Plymouth, UK) to determine BHB and MDA, and in tubes containing EDTA (BD Vacutainer Becton-Dickenson and Company) to analyse glucose, NEFA and urea concentrations. Immediately after collection, blood samples were centrifuged at 3,500 rpm for 20 min at 4ºC, and plasma was frozen at -20ºC until further analyses.

2.3. Chemical analyses

In milk samples, lactose, fat, protein and urea contents, and somatic cell count, were determined with an infrared scan (Milkoscan7 RM, Foss Electric Ltd., Hillerød, Denmark). Randox kits (Randox Laboratories Ltd., Country Antrim, UK) were employed to determine the plasma concentrations of NEFA (colorimetric method, sensitivity: 0.072 mmol/L) and BHB (kinetic enzymatic method, sensitivity: 0.100 mmol/L). An automatic analyser (Gernon, RAL S.A., Barcelona, Spain) was used to measure the plasma concentrations of glucose (enzymatic-colorimetric method, sensitivity: 0.06 mmol/L) and urea (kinetic method, sensitivity: 0.056 mmol/L). The mean intra- and interassay

coefficients were for NEFA: 4.0% and 4.9%, BHB: 6.8% and 6.8%; glucose: 2.2% and 2.4%; urea: 4.4% and 5.5%.

The plasma concentration of MDA, used as an indicator of oxidative status, was determined by liquid chromatography as described in Bertolín et al. (2019). An Acquity UPLC H-Class liquid chromatograph (Waters, Milford, Massachusetts, USA), equipped with a silica-based bonded phase column (Acquity UPLC HSS PFP, 100 mm × 2.1 mm × 1.8 µm, Waters), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ detector, Waters) and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters), were utilised. The intra- and interassay coefficients of variation were 4.6% and 7.3% for MDA, respectively.

2.4. Calculations and statistical analyses

The INRA system (INRA, 2007) was used to estimate the individual EB as the difference between inputs (net energy (NE) intake) and outputs (NE for maintenance and NE for lactation). The NE intake was estimated from the individual DM intake (DMI) and feedstuffs' energy contents. The NE for maintenance was calculated from the individual metabolic BW, and the NE for production was obtained using the milk yield, fat, and protein contents in milk.

Statistical analyses were performed by the SAS statistical package v 9.4 (SAS Institute Inc., Cary, NC, USA) and the R software. Normal data distribution was assessed with the Shapiro-Wilk test ($P > 0.05$). Normality could not be confirmed for the somatic cell count values. Therefore, analyses were run on the log-transformed data. Parameters were analysed with mixed models by taking feeding period (basal, restriction, refeeding), lactation month (months 2, 3 and 4), and their interaction, as fixed effects, and cow as the random effect. Degrees of freedom were adjusted with the Kenward-Roger correction. The least square means and associated standard errors were obtained and multiple comparisons were adjusted with Tukey correction. The Pearson's correlations between variables were obtained and presented on heatmaps for all the data and separately per feeding period using the CORRPLOT package of R (R Development Core Team, 2021). The level of significance for all the tests was $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$.

3. Results

The interaction between feeding period and lactation month affected all the parameters ($P < 0.05$ to $P < 0.001$), except milk yield, which only tended to be affected by this interaction ($P < 0.10$) and somatic cell count ($P > 0.05$). For each parameter, the basal values during the three lactation months, and then the effects of restriction and refeeding during the three lactation months are presented.

3.1. Cow performance

On average, 91%, 61% and 93% of the net energy requirements and 100%, 58% and 103% of the metabolisable protein requirements were met during the basal, restriction and refeeding periods, respectively. Cows' EB, BW, milk yield and milk composition are depicted in Figure. 1 according to feeding period and lactation month. The calculated basal EB improved progressively from month 2 to month 4 ($P < 0.01$). According to the experimental design, cows' EB was more negative during restriction than during the basal period in the three lactation months ($P < 0.001$). During refeeding, the EB returned to basal values in lactation months 2 and 3, but went even higher, close to a neutral EB, in lactation month 4 ($P < 0.001$). Basal BW decreased between months 2 and 4 ($P < 0.001$). BW diminished with restriction in the three lactation months (by -2.3%, -2.0% and -1.7% in months 2, 3 and 4, respectively). During refeeding, BW lowered by a further 1% in month 2 ($P < 0.001$), but remained unchanged in months 3 and 4 ($P > 0.05$).

The basal milk yield was higher in months 2 and 3 than in month 4 ($P < 0.05$ to $P < 0.001$). Milk yield decreased with restriction in the three lactation months by -14%, -19% and -20% in months 2, 3 and 4, respectively ($P < 0.001$). Milk yield increased during refeeding and reached the basal values in months 2 and 3, but stayed below the basal values in lactation month 4 (by -8%; $P = 0.03$). Regarding milk composition in the basal phase, lactose, fat and urea contents were not affected by lactation month ($P > 0.05$), whereas protein content was higher in month 2 than in the subsequent months ($P < 0.001$), and somatic cell counts were lower in month 2 than thereafter (99, 135 and 131 x 10^3 cells/mL in months 2, 3 and 4, respectively, $P < 0.05$).

Feed restriction did not affect milk lactose in month 2, but lowered in months 3 and 4 (by -1.9 and -1.5%, respectively) and then increased during refeeding in the three lactation months ($P < 0.001$). Milk fat content was similar regardless of feeding periods ($P > 0.05$). Protein content lowered with restriction in months 2 and 3 (by -5% and -4%, respectively; $P < 0.001$), but was not affected in month 4 ($P > 0.05$). It remained stable during refeeding in months 2 and 4, but increased to reach the basal values in month 3.

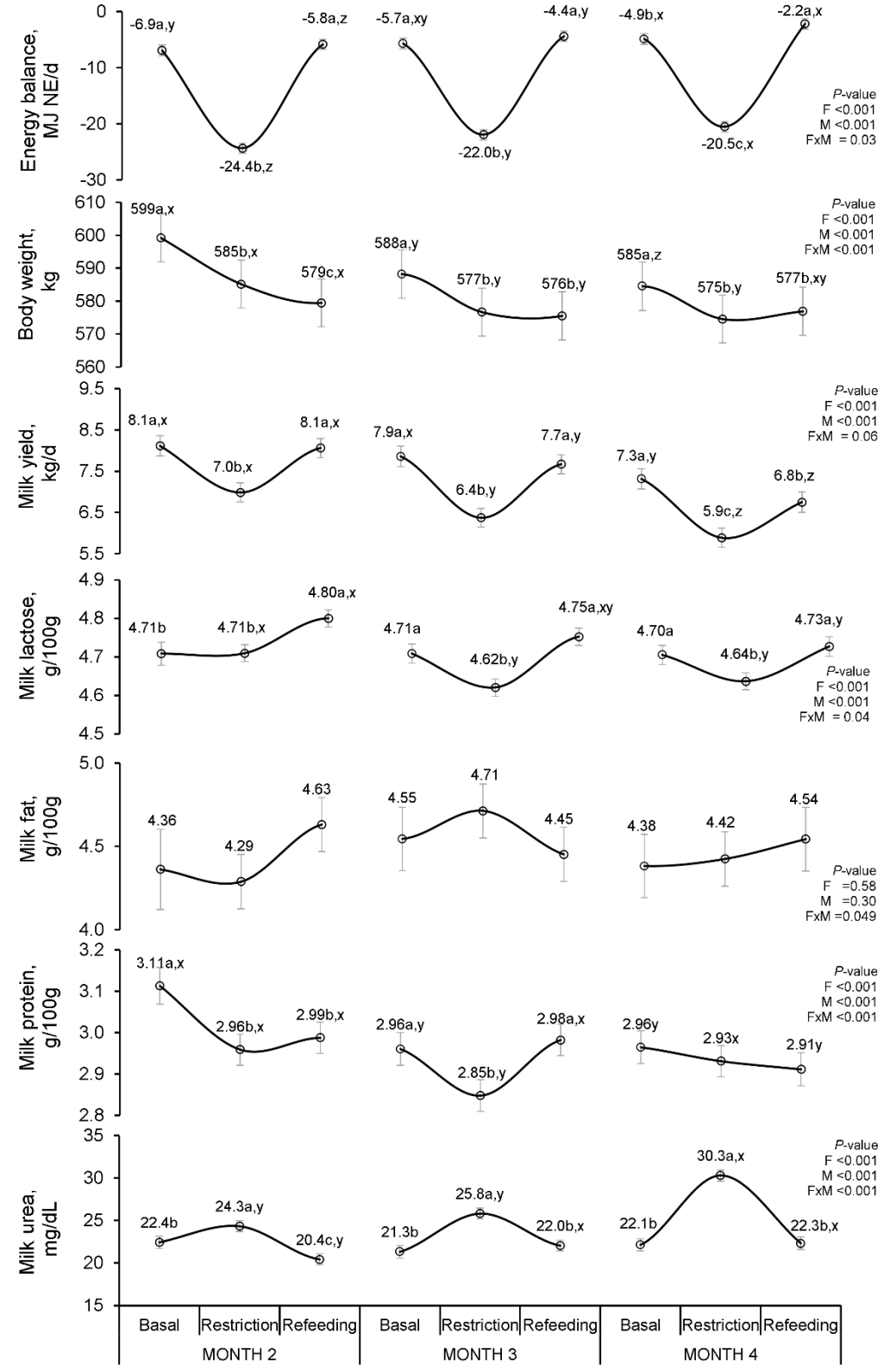


Figure 1. Effect of feeding period (F: Basal, Restriction, Refeeding) and lactation month (M: 2, 3, 4) on energy balance (EB), BW, milk yield and milk composition.

Within a parameter and month, the means with a different letter (a,b,c) indicate differences due to feeding period ($P < 0.05$). Within a parameter and feeding period, the means with a different letter (x,y,z) denote differences due to lactation month ($P < 0.05$).

Milk urea content increased during restriction in the three months by +8%, +21%, and +37% in months 2, 3 and 4, respectively ($P < 0.05$), and decreased during refeeding, even below the basal values in month 2 and to the basal values in months 3 and 4 ($P < 0.001$). The highest somatic cell counts were obtained during refeeding (128, 159 and 186 x 10^3 cells/mL in the basal, restriction and refeeding period, respectively, $P < 0.05$).

3.2. Plasma metabolic profile

The plasma concentrations of NEFA, BHB, glucose, urea and MDA are presented in Figure. 2. Lactation month did not affect the basal concentrations of BHB and urea ($P > 0.05$), but affected those of NEFA, glucose and MDA ($P < 0.001$). The basal NEFA concentrations were higher in month 2 than in month 4 ($P < 0.001$). The basal glucose concentrations were lower in month 3 than in months 2 and 4 ($P \leq 0.001$). The basal MDA concentrations were higher in month 2 than in the subsequent months ($P < 0.001$).

Regarding the effect of feeding period, NEFA concentrations increased to different extents due to restriction in the three months (by +157%, +269% and +212% in months 2, 3 and 4, respectively; $P < 0.001$), whereas refeeding lowered NEFA concentrations to below the basal value in month 2 ($P < 0.001$) and to basal values in months 3 and 4. The BHB concentration rose with restriction in the three months, but only significantly in month 4, by +14% ($P = 0.11$), +17% ($P = 0.11$) and +23% ($P < 0.001$) in months 2, 3 and 4, respectively. During refeeding, BHB decreased and reached basal values in months 2 and 4. Glucose concentration dropped during restriction in month 2 ($P = 0.01$), with no changes thereafter ($P > 0.05$). During refeeding, it decreased in month 2 ($P < 0.001$), increased in month 3 ($P < 0.001$) and remained unchanged in month 4 ($P > 0.05$). The urea concentration rose significantly during restriction, but only in lactation month 4 (by +18%; $P < 0.001$), and lowered during refeeding below the basal values in months 2 and 4 ($P < 0.01$). The MDA concentration did not change with restriction and was only affected by refeeding in month 4, with higher values than during the basal period ($P = 0.03$).

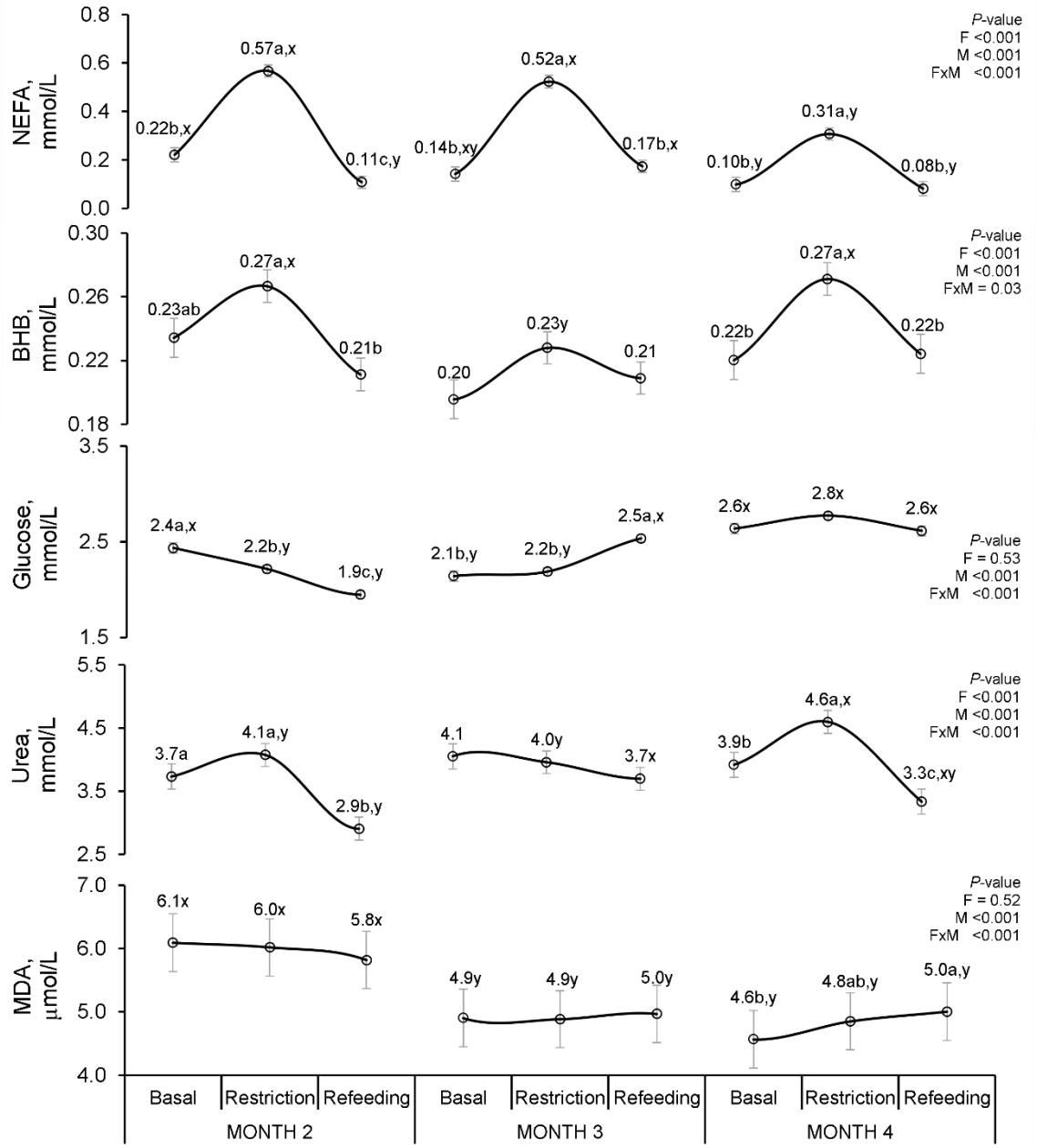


Figure 2. Effect of feeding period (F: Basal, Restriction, Refeeding) and lactation month (M: 2, 3, 4) on plasma concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose, urea and malondialdehyde (MDA).
Within a parameter and month, the means with a different letter (a,b,c) indicate differences due to feeding period ($P < 0.05$). Within a parameter and feeding period, the means with a different letters (x,y,z) denote differences due to lactation month ($P < 0.05$).

The significant overall correlations with r ≥ 0.25 between the performance parameters and plasma metabolites are shown in Figure. 3, whereas the correlations during each feeding period are depicted in Suppl. Figure.1. The overall correlations were weak (r = 0.25 to 0.39) or moderate (r = 0.40 to 0.59), but were were strong within feeding periods (r = 0.60 to 0.79) and very strong (r ≥ 0.80) ($P < 0.001$). BW correlated positively with milk yield and negatively with the EB. Milk urea content correlated negatively with

milk yield and the EB (P < 0.001). Within the basal, restriction and refeeding periods, correlations were moderate between BW and the EB (r = -0.52 to -0.64), and were very strong between milk yield and the EB (r = -0.87 to -0.93). The plasma NEFA concentration correlated negatively with the EB and milk protein content, and positively with milk urea content (P < 0.001). The BHB concentration correlated negatively with the EB and positively with milk urea and the plasma concentrations of glucose, urea and MDA (P < 0.001). The plasma urea concentration correlated negatively with the EB and positively with milk urea content and plasma glucose concentration (P < 0.001). Within feeding periods, the plasma urea concentration correlated positively with BW, milk yield and milk protein during the basal period and with milk protein during the refeeding period (P < 0.001). The plasma MDA concentration correlated positively with BW, milk yield, milk protein content and plasma urea concentration, and negatively with the EB (P < 0.001).

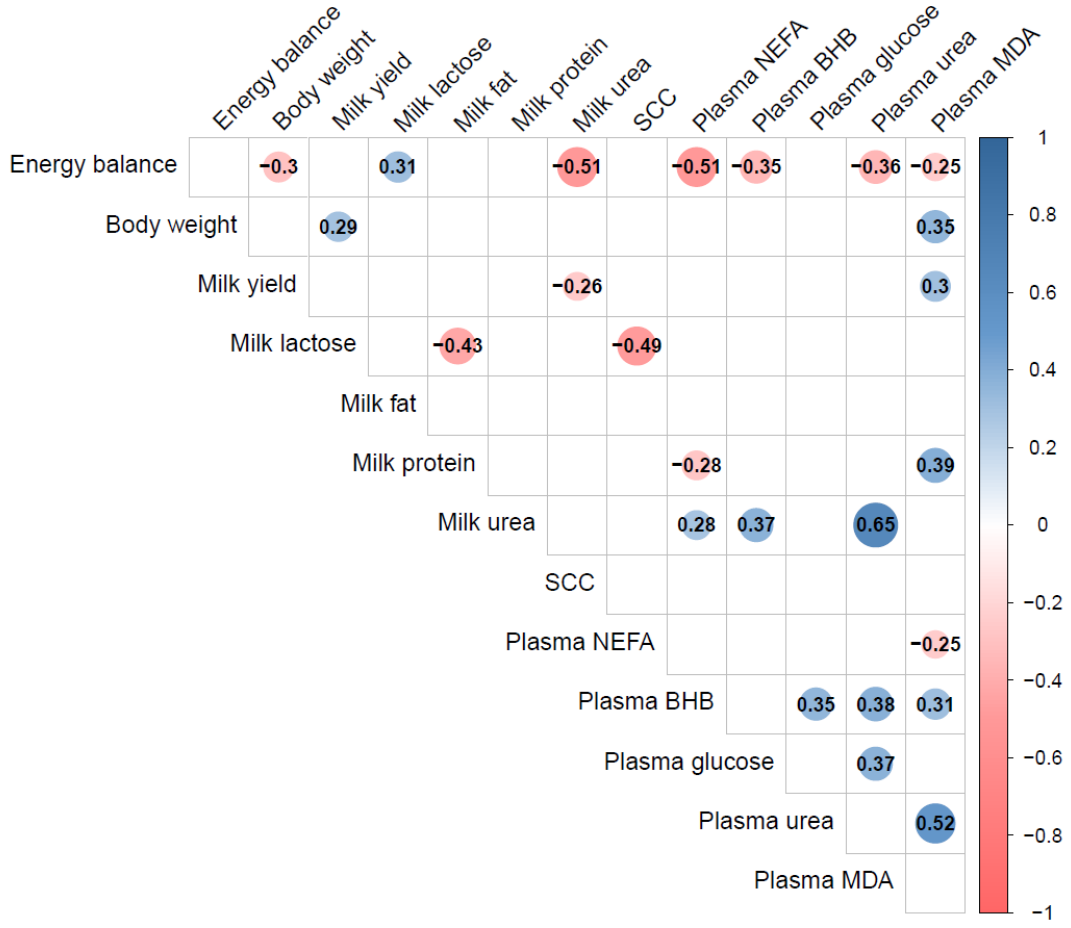


Figure 3. Significant Pearson's rank correlations[1] between cow performance and plasma metabolites in all the lactation months.

[1]Only the significant correlations (P < 0.05) are presented and the correlations between equal variables are omitted. SCC: somatic cell count; NEFA: non-esterified fatty acids; BHB: β-hydroxybutyrate; MDA: malondialdehyde.

4. Discussion

In the present experiment, restriction implied reductions of -36% in DMI, -42% in net energy intake and -47% in protein intake on average. The restriction herein applied could be considered moderate according to the review by Leduc et al. (2021) because the reduction in DMI was less than 50%. Basal cow performance and some plasma metabolites differed among the three lactation months, as did their patterns of response to restriction and refeeding. This scenario suggests a change in the metabolic priority of different biological functions as lactation advanced.

4.1. Cow performance

BW loss between months 2 and 4 agrees with previous experiments with lactating Parda de Montaña cows (Blanco et al., 2009). Beef cows are rarely fed according to their theoretical requirements (Blanc et al., 2006). During lactation, they have to rely on the mobilisation of their body reserves to produce milk. In the present experiment, BW was only mildly affected by a short feed restriction, similarly to the -4 to -5% BW loss reported after a 4-day 50% DMI restriction in beef cows (De La Torre et al., 2022) and dairy cows (Ferraretto et al., 2014; Kvidera et al., 2017). This BW loss could be linked with the decrease in DMI, gut fill loss and mobilisation of body reserves (Gross et al., 2011a; Laeger et al., 2012). Incomplete BW recovery in the 4-day refeeding phase implies that a longer recovery period is needed; e.g. 10 days in beef cows after a similar restriction to that herein applied (De La Torre et al., 2022) or at least 1 to 2 weeks in dairy cows with severer restrictions that cause greater BW loss (-10%; Billa et al., 2020; Pires et al., 2019).

The lower basal milk yield values with progressing lactation agree with previous data on Parda de Montaña cows (Casasús et al., 2004; Dervishi et al., 2017), and suggest that the peak milk yield had already been reached at the start of the experiment, in month 2, as described by Sapkota et al. (2020) for beef cows. In the present study, the reduced milk yield caused by feed restriction falls in line with those reported by other studies of comparable lengths and restriction severities in beef cows (-12%; De La Torre et al., 2022) and dairy cows (-13 to -20% in Abdelatty et al., 2017; Laeger et al., 2012; Nielsen et al., 2003). The milk loss magnitude was lower in month 2, when cows displayed the most negative EB, than thereafter. Several homeorhetic mechanisms involved in nutrient partitioning regulation concur to maintain milk yield during feed restriction periods or metabolic imbalance, e.g. decreased glucose use, increased body lipids use and the mobilisation of protein reserves as energy sources (Bauman and Currie, 1980; Ingvartsen et al., 2003). However, these regulation processes are stage-

dependent and the adaptive response diminishes with advancing lactation (Blanc et al., 2006). Our results indicate that the metabolic priority of the mammary gland in feed-restricted beef cows decreased after month 2. This would be supported by the shift in nutrient partitioning away from the udder towards subcutaneous adipose tissue, as observed on 60 d postpartum in beef cows by Murrieta et al. (2010). The milk yield response to refeeding was fast, with full recovery occurring within 4 days in months 2 and 3, but not in month 4. The lower milk synthesis priority in this later stage may increase the necessary recovery time. A quick response to refeeding has also been reported in low-producing beef cows (2 days for full recovery; De La Torre et al., 2022), but more days are required for full recovery with high-producing dairy cows in early lactation (7 to 8 days; ; Bjerre-Harpøth et al., 2012; Pires et al., 2019).

Concerning milk composition, the basal milk protein and lactose contents were similar, but fat content was higher than those previously reported in Parda de Montaña cows with a similar milk yield (Casasús et al., 2004; Dervishi et al., 2017). This difference was probably related to the sampling method. In this study, milk samples were manually obtained after calves had suckled (alveolar milk). In the above-mentioned studies, they were collected by machine milking before calves had access to their dam (cisternal milk). The fat concentration in cisternal milk is lower than in alveolar milk, whereas milk protein content is minimally affected (Sarikaya et al., 2005). The basal milk composition was similar in the three months, except for the higher protein content in early lactation. In dairy cows, lactose regulates milk osmolality and generally remains constant throughout lactation, while milk fat and protein tend to decrease from peak lactation in response to improved nutritional status and lower milk yield (Gross and Bruckmaier, 2019). All this was confirmed in our experiment for lactose and protein, but not for fat. This was probably due to the smaller differences in the EB and milk yield among months here than those observed in high-producing dairy cows. Furthermore, the stable basal milk urea throughout lactation agrees with the results reported in beef cows in the first three months of lactation (Wiseman et al., 2019) and in early-, mid- and late-lactating dairy cows (Bjerre-Harpøth et al., 2012).

Milk composition was affected by nutritional perturbation to different extents. Lactose content lowered with restriction and increased during refeeding, which agrees with previous reports in dairy cows that only needed 2 days to recover basal values after restriction had ended (Bjerre-Harpøth et al., 2012; Hervé et al., 2019; Sigl et al., 2013). The negative correlation herein observed between lactose content and somatic cell count has been associated with inflammatory reactions in milk secretory cells (Cinar et al.,

2015). However in our study, somatic cell count was always below the threshold for subclinical mastitis (200 $x10^3$ cells/mL; Dervishi et al., 2017).

Milk fat originates from either dietary or mobilisation fatty acids, which are taken up from the bloodstream, or by *de novo* synthesis in the mammary gland (Chilliard et al., 2000b). Here milk fat content was not affected by feed restriction, which is consistent with previous results in dairy cows restricted at 50-60% during 4-5 days with 10-22% milk yield loss (Abdelatty et al., 2017; Carlson et al., 2006; Gross et al., 2011a). Other experiments with 30-50% milk loss report increases in milk fat content during feed restriction (Agenäs et al., 2003; Bjerre-Harpøth et al., 2012), which are associated with an increment in the long-chain fatty acids that arise from body fat mobilisation (Gross et al., 2011b). Apparently fat mobilisation and the concurrent rise in circulating NEFA would not have been enough to increase milk fat content in our study, but could have made the proportion of long vs. short- and medium-chain fatty acids higher, as observed by Orquera-Arguero et al. (2023).

Milk protein may decrease with feed restriction, but changes in milk urea depend on the nature of restriction (Leduc et al., 2021) given the influence by feed intake, but also by urea transfer from blood to milk, and *vice versa* (Spek et al., 2016). Here we observed reductions in milk protein (in months 2 and 3) and increments in milk urea contents in response to simultaneous reduction in dietary energy and protein supply. These findings agree with other experiments with 50% nutritional restriction, e.g. -7% milk protein and +21% milk urea content in Carlson et al. (2006), -5.6% milk protein in Gross et al. (2011a). The higher milk urea content during restriction, especially in month 4, and its negative correlation with the EB suggests that protein catabolism took place in this phase to compensate for reduced energy intake, and this adaptation mechanism was more intense in later lactation stages. Body protein mobilisation to obtain glucose as an energy substrate increases circulating urea, which can be diffused from the blood stream to mammary glands (Spek et al., 2016). When restriction ended, basal values were regained after four refeeding days in most cases, except for milk protein in month 2. This suggests quicker recovery than that observed in high-producing dairy cows (Billa et al., 2020; Bjerre-Harpøth et al., 2012; Pires et al., 2019).

4.2. Plasma metabolic profile

Plasma metabolites have commonly been used as indicators of energy, protein and oxidative status (Castillo et al., 2006; van Knegsel et al., 2007). The basal values herein observed were similar to those reported in lactating Parda de Montaña cows fed their 100% requirements in the case of NEFA, BHB and urea (Alvarez-Rodríguez et al., 2009), but were lower than those of glucose (Rodríguez-Sánchez et al., 2018). The fact that basal NEFA decreased from month 2 to month 4 indicates that the lipid mobilisation needed to support the energy demand for milk yield decreased throughout lactation, as shown in dairy cattle (Gross et al., 2011a; Jorge-Smeding et al., 2022). Basal BHB remained stable, as noted by Rodríguez-Sánchez et al. (2018) in beef cows, but were unlike the results of Bruckmaier and Gross (2017) in Holstein cows, where BHB peaked between 2-3 weeks postpartum and decreased thereafter, which suggest more metabolic stress for dairy cows in early lactation.

Feed restriction in months 2 and 3 increased the plasma NEFA concentrations to more than 2-fold their basal values, which came close to the compromised metabolic status threshold in dairy cows (0.57-0.60 mmol/L; Ospina et al., 2010), but induced a milder response in month 4. This supports the high priority of nutrient partitioning towards the mammary gland in response to reduced energy supply in earlier lactation stages, when body fat is largely mobilised and NEFA are released to provide energy for milk synthesis. Orquera-Arguero et al. (2022) observed wide variability in this response among beef cows, with more marked increments in cows' BW and milk yield. Plasma BHB responded to reduced nutrient intake to a much lesser degree (+15 to 20%), and only significantly so in month 4, and remained far below the risk threshold for subclinical ketosis (>1.2 mmol/L) (Benedet et al., 2019). The greater increments in NEFA than in BHB concentrations in response to reduced feed supply agree with previous studies with similar restrictions in dairy cows (Kvidera et al., 2017; Moyes et al., 2009; Pires et al., 2019), but they did not even change in Charolais cows with lower milk yield BHB (De La Torre et al., 2022). Both metabolites reacted quickly to refeeding, and basal values had recovered within 4 days, which agrees with other studies in beef (De La Torre et al., 2022) and dairy cattle (Abdelatty et al., 2017; Gross et al., 2011a), regardless of lactation stage (Billa et al., 2020; Bjerre-Harpøth et al., 2012).

The response of plasma glucose to diet changes was not consistent across lactation stages in the present study because it only decreased with restriction in month 2. The stronger effect on early lactation has been ascribed by Bjerre-Harpøth et al. (2012) to greater physiological imbalance, and could be driven by higher mammary

glucose uptake for lactose synthesis (Gross et al., 2011a). In beef cows, no relevant changes were observed when feed was reduced at 54 or 75 days from calving (De La Torre et al., 2022). The literature reports conflicting results on the effect of moderate feed restrictions on glycaemia, which may decrease or remain stable, and has been considered a poor indicator of energy status in cows because gluconeogenesis can balance its concentration (Leduc et al., 2021).

Plasma urea is influenced by a wide variety of interrelated factors, such as dietary protein intake and muscle tissue breakdown when energy supply is insufficient (Puppel and Kuczyńska, 2016). Protein mobilisation from skeletal muscle releases glucogenic amino acids, which are used to supply glucose (Ingvartsen et al., 2003) and to generate urea during the process (Agenäs et al., 2006). The concentrations herein noted fell within the range reported for adequately nourished cows (1.8 to 7 mmol/L; Agenäs et al., 2006), and basal values remained stable throughout lactation, as observed by Bjerre-Harpøth et al. (2012) in early-, mid- and late-lactating cows. The lack of effect of feed restriction in months 2 and 3 agrees with previous reports in beef (De La Torre et al., 2015) or dairy cows (Hervé et al., 2019; Laeger et al., 2012), although other authors have found reduced blood urea in feed-restricted cows (Kvidera et al., 2017). The fact that restriction elicited a rise in the plasma urea concentration in month 4, when protein intake did not differ from previous months, implies that a certain degree of protein catabolism took place during restriction. This resulted in stable glycaemia in this month, as observed by Fiems et al. (2007) in energy-restricted beef cows. Apparently in late lactation, cows rely less on the mobilisation of fat reserves and more on the mobilisation of lean mass as a strategy to cope with a short-term nutritional challenge.

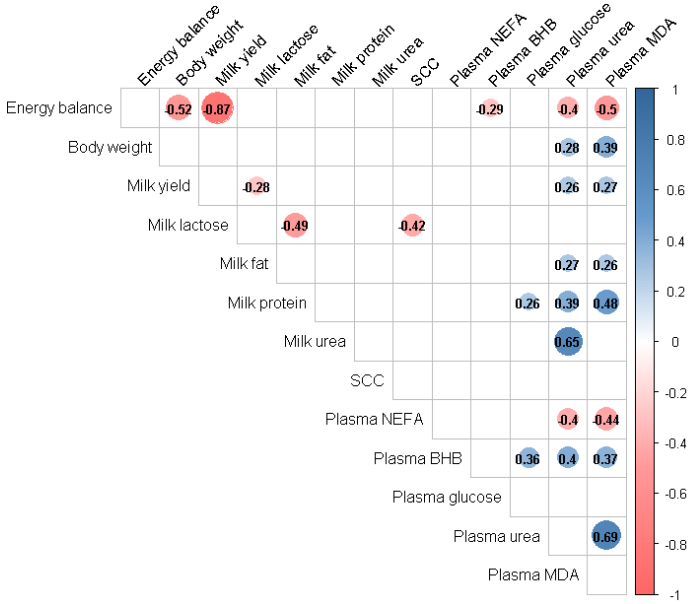
The metabolic adaptation to a negative EB can intensify the NEFA oxidation processes in the liver, and can result in both increased ROS production and oxidative stress developing (Turk et al., 2008), which occur with an imbalance between ROS production and antioxidant availability (van Knegsel et al., 2014). The values obtained in the present experiment are far below the concentrations reported by Castillo et al. (2006) for Holstein cows, which lowered from 69 to 29 µmol/l in the 8 first weeks of lactation. In our case, the higher MDA concentrations in early lactation (month 2) than thereafter, as observed by Castillo et al. (2006) in dairy cows, are likely the consequence of the higher plasma NEFA concentrations available for oxidation (Abuelo et al., 2015; Shi et al., 2015), with which they correlated.

5. Conclusions

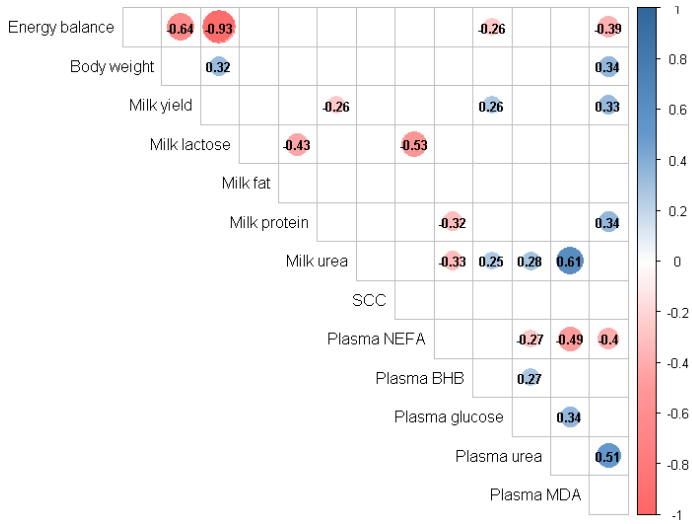
Short-term restriction-refeeding periods resulted in both productive and metabolic adaptations in lactating beef cows. The most relevant responses to feed restriction were a drop in milk yield and an increase in the plasma NEFA concentrations, although their magnitude of change decreased as lactation advanced. In early postpartum, the mobilisation of fat reserves partially buffered the impact of a moderate feed restriction on milk yield. In later stages, when priority for milk production decreased, body protein reserves were also mobilised and longer recovery times were needed to compensate for a less effective response. Our results show that beef cows use different metabolic strategies to face nutritional perturbations depending on lactation stage.

Supplemental material

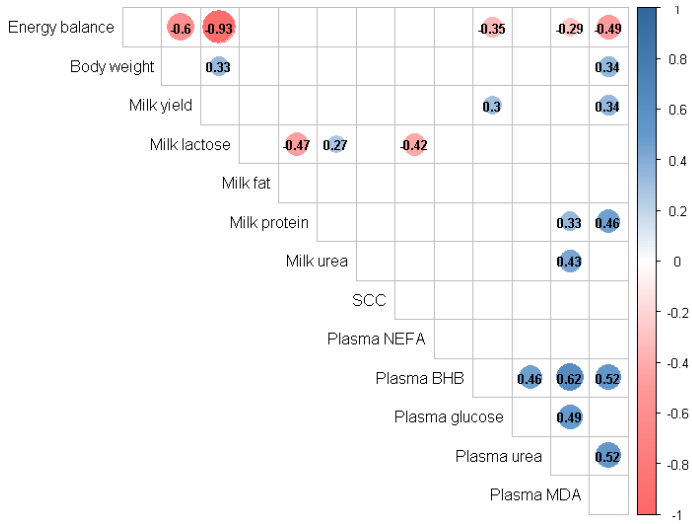
Basal



Restriction



Refeeding



Capítulo III

“Respuesta adaptativa de las vacas de carne a retos nutricionales repetidos”

Correspondiente al artículo:

Adaptive response of beef cows to successive nutritional challenges

Karina G. Orquera-Arguero, Isabel Casasús, Daniel Villalba, Javier Ferrer, Mireia Blanco.

(Finalizando redacción)

1. Introduction

Despite the uncertainty in climate change projections, alterations in environmental conditions are expected to negatively affect animal performance, health and welfare in direct and indirect ways (Lacetera, 2019). The direct effects, primarily associated with temperature rise and increased frequency and severity of heat weaves, may impair animal productive and reproductive performance and cause metabolic alterations, oxidative stress and immune suppression (Lacetera, 2019; Nardone et al., 2010). Amongst the indirect effects, changes in the availability, quality and seasonality of feed resources can result in restricted nutrient supply (Rojas-Downing et al., 2017). Additionally, feed shortage either caused by climate change or by the high price of feed due to the current global energy scarcity (Benoit and Mottet, 2023) may lead to a reduction of the feedstuffs provided to livestock by farmers. Furthermore, the frequency of extreme weather events is predicted to increase due to climate change, and as a consequence livestock could be more frequently exposed to repeated restriction periods.

Under restricted nutrient supply, a range of physiological adaptation mechanisms have been described in lactating cows, including body fat and protein mobilization and a reduction of milk yield (Agenäs et al., 2003; Bauman and Currie, 1980; Bell, 1995). The ability of animals to 'bounce back' from a relatively short-term disturbance is defined as resilience (Friggens et al., 2022); when the initial state is totally recovered after a challenge, the response is considered elastic, otherwise it is flexible (Blanc et al., 2010). The individual response to undernutrition may differ among individuals, and therefore identifying groups of cows with similar response profiles could be of interest for herd management (de Koster et al., 2019).

The response could also be affected by repeated exposure to feed restrictions. Habituation was defined as decreased responsiveness to repeated stimuli whereas sensitization implies increased responsiveness (Blumstein, 2016). Habituation studies have been performed in cattle using repeated exposures to stressors such as management (Veissier et al., 2001), acidosis challenges (Dohme et al., 2008; Nagata et al., 2018) or oscillations in diet quality (Rauch et al., 2021), with responses that either decreased, did not change, or were more acute over time. Here, we hypothesized that under successive nutritional challenges, beef cows would respond differently and reach a habituation state after repeated exposure. Therefore, the aims of this experiment were (1) to cluster lactating beef cows according to their metabolic response to three repeated short nutritional challenges and subsequent refeeding, and (2) analyze the effect of the

metabolic response profile and repeated feeding challenges on performance parameters and plasma metabolites indicative of energy and protein status.

2. Material and methods

The experimental procedures (protocol no. CEEA-03-2018-01) that follow the guidelines of the Directive 2010/63 EU on the protection of animals used for experimental and other specific purposes were approved by the Animal Ethics Committee of the research center.

2.1. Diets and animal management

This study was performed in at La Garcipollera Research Station (Spain, 42°37' N, 0°30' W, 945 m a.s.l.). Thirty-one multiparous lactating Parda de Montaña beef cows were involved in the experiment, which started when cows were 83 (± 5.4) days in milk, their body weight (BW) was 585 ± 40.0 kg, and their body condition score (BCS, 0 to 5 scale) was 2.6 ± 0.12. After calving, cows had been randomly assigned in pens (7 or 8 cows/pen, 10 x 20 m) equipped with individual feeders for forage (200-L fiberglass boxes in front of self-locking feeding places) and the daily concentrate ration was automatically distributed by feeding stations (ALPRO Herd Management 7.0, DeLaval) for the concentrate. Calves were stocked in straw-bedded cubicles adjacent to their dams' pens and had access to suckle their dams for 30 min at 06:00h and 14:00h.

The experiment started with a pre-challenge period (-4 to -1 d), followed by 3 sequential of 4 d restriction and 3 d refeeding periods (Figure. 1). The cows were fed a diet composed of different quantities of hay [dry matter (DM): 919 g/kg; crude protein (CP): 85 g/kg DM; neutral detergent fiber (NDF): 607 g/kg DM; acid detergent fiber (ADF): 332 g/kg DM; net energy: 5.4 MJ/kg DM; metabolizable protein: 59 g protein digestible in the intestine (PDI)/kg DM] and concentrate (DM: 915 g/kg; CP: 166 g/kg DM; NDF: 255 g/kg DM; ADF: 119g/kg DM; net energy: 7.6 MJ/kg DM; metabolizable protein:120 g PDI/kg DM). The INRA equations (INRA, 2007) were used to calculate the diets in order to meet either 100% (pre-challenge and refeeding periods) or 55% (restriction periods) of the net energy and metabolizable protein requirements for maintenance and lactation of a standard cow (BW: 615 kg, milk yield: 8.5 kg/d).

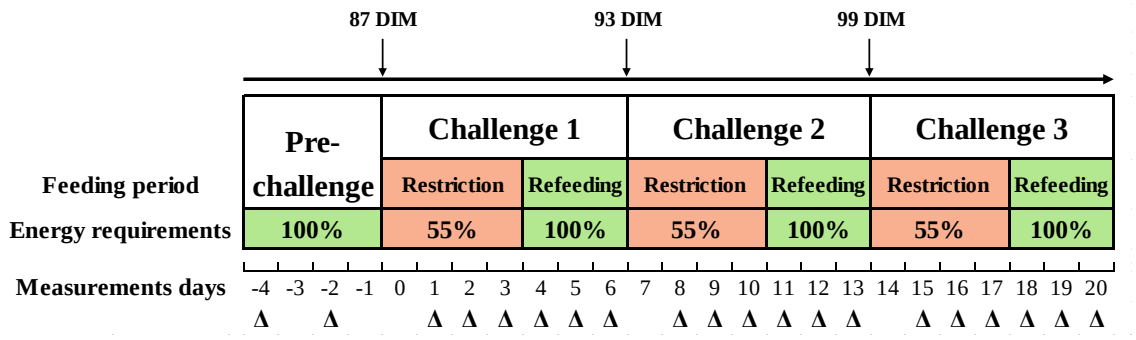


Figure 1. Schematic representation of the timeline of three repeated short nutritional challenges. Δ: sampling days for all traits.

All the cows were fed the same diet, which consisted of 7.4 kg DM of hay and 2.7 kg DM of concentrate during the pre-challenge and the refeeding periods and only 6.4 kg DM hay with no concentrate during the restriction periods. The hay was offered in a single meal in the individual feeders with the cows tied up for approximately 2 h until they finished their ration; the individual hay intake was registered daily. In the basal and refeeding periods, the ALPRO feeding stations were programmed to offer the concentrate to the cows and the individual concentrate intake was recorded daily. The cows had free access to water and minerals blocks during all the experiment.

2.2. Measurements, samplings and chemical analyses

All the measurements and samples of the feedstuffs, milk and blood were collected daily 24 h after the start of each feeding period and before the cows had access to the diet (Figure 1). The chemical composition of the feedstuffs was analyzed in duplicate following official methods as reported in Orquera-Arguero et al. (2022), and these data were used to calculate their nutritive value (INRA, 2007). The daily individual hay and concentrate intakes on DM basis were calculated.

The cows and calves were weighed on an electronic scale. Milk yield was estimated by the weigh-suckle-weigh technique of the calf (Le Neindre and Dubroeucq, 1973) as the sum of the milk consumed in both sucklings. After calf removal, cows were administered an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) 5 min before the manual extraction to facilitate the letdown of the residual milk. Composite milk samples collected from the four teats were preserved in 100-mL plastic tubes with sodium azide (PanReac, Barcelona, Spain) and refrigerated at 4 °C until the analysis of milk composition. The fat, protein, lactose and urea contents in milk were determined with an infrared scan (Milkoscan 7 RM, Foss Electric Ltd., Hillerød, Denmark).

Blood samples were collected from the coccygeal vein into heparinized tubes (BD Vacutainer Becton-Dickenson and Company, Plymouth, UK) to determine BHB and into K2 EDTA-containing tubes (BD Vacutainer Becton-Dickenson and Company, Plymouth, UK) to analyze NEFA and urea concentrations. Plasma, obtained after samples were centrifuged at 3500 rpm for 20 min at 4 °C, was frozen at -20 °C until further analysis. Randox kits (Randox Laboratories Ltd, Country Antrim, UK) were used to determine plasma concentrations of NEFA (colorimetric method, sensitivity: 0.072 mmol/L) and BHB (kinetic enzymatic method, sensitivity: 0.100 mmol/L). An automatic analyzer (Gernon, RAL S.A, Barcelona, Spain) was used to measure the plasma concentrations of urea (kinetic method, sensitivity: 0.056 mmol/L). The mean intra-and inter-assay coefficients were for NEFA: 4.3% and 4.7%, BHB: 6.6% and 7.4% and urea: 4.0% and 5.1%.

2.3. Calculations and statistical analyses

The energy balance (EB) was calculated using the INRA system (INRA, 2007). The difference between inputs, net energy (NE) intake (estimated from the individual intake and energy contents of the feedstuffs), and outputs, NE for maintenance (using the individual metabolic weight) and NE for lactation (using the milk yield and the contents of fat, and protein in milk). The magnitude of the effects of feed restriction and corresponding refeeding of each repeated challenge was evaluated by calculating the percentage of change relative to pre-challenge values (0%) for all the parameters analyzed in this study.

The statistical analyses were performed using SAS (version 9.4; SAS Institute Inc, Cary, NC, USA), and curve response variables and correlation heatmaps were produced using R (R Development Core Team, 2021). To explore the variability of milk yield, plasma NEFA and BHB, the distribution of data was represented by challenge and feeding period with violin plots, using ggplot2 package from R. The F-test was used to test whether variances from the different challenges and periods were equal. The individual responses of the cows to the nutritional challenges for milk yield, NEFA and BHB were selected for modelling analyses, because in previous studies they had shown the highest response to undernutrition and refeeding challenges (Orquera-Arguero et al., 2023b, 2023a). From the modelled curves, new response variables were obtained to cluster the cows as described in Orquera-Arguero et al. (2022). Briefly, the predicted curve for each trait (milk yield, NEFA, and BHB) was modelled using natural cubic splines with 8 knots with ns command with the library splines of R. New curve response variables summarizing animal response to challenge were calculated from the fitted curve.

Baseline: values in the absence of feed restriction, according to a linear interpolation from the basal to the refeeding period; peak: maximum difference between the actual daily value and the baseline value; days to peak: days from the start of restriction until the peak values were reached; days from the start of restriction until the contents reached the baseline again; area under the curve (AUC) during restriction (the estimated total loss/extra contents during restriction, as compared with baseline values); AUC during refeeding (the estimated total loss/extra contents during refeeding until baseline values were regained). These curve response variables were used to perform a principal component analysis (PCA function in FactoMineR package of R) to generate groups of cows according to their metabolic response (MR). Then a hierarchical clustering on these principal components (HCPC function in FactoMineR package of R) was carried out to group cows with a similar response pattern.

Daily data were averaged within animals and periods to perform comparisons among feeding periods. The curve response variables, the performance parameters, plasma metabolites and their percentage of change were analyzed with mixed models considering the MR cluster (High and Low), the time effects [i.e., day or feeding period (pre-challenge, Restriction 1, 2, 3 and Refeeding 1, 2, 3)] and their interaction as fixed effects, and the cow as the random effect. The degrees of freedom were adjusted with the Kenward-Roger correction to take into account-missing values. The variance components structure was selected on the basis of the lowest Akaike and Bayesian information criteria. The least square means and standard errors were obtained, and multiple comparisons adjusted with the Tukey correction. Associations among the performance parameters and plasma metabolites were explored by Pearson rank correlations (r) using the CORRPLOT procedure of R. We analyzed the overall daily database (n=620 per trait) and separately daily data collected when the cows received the diet formulated to meet 100% (pre-challenge and refeeding periods) and 55% (restriction periods) of the net energy and protein requirements for maintenance and lactation. For all statistical analyses, the significance level was predefined at $P < 0.05$, and trends were discussed when $0.05 \leq P < 0.10$.

3. Results

The individual variability in milk yield, NEFA, and BHB plasma concentrations according to the feeding period is shown in Figure 2.

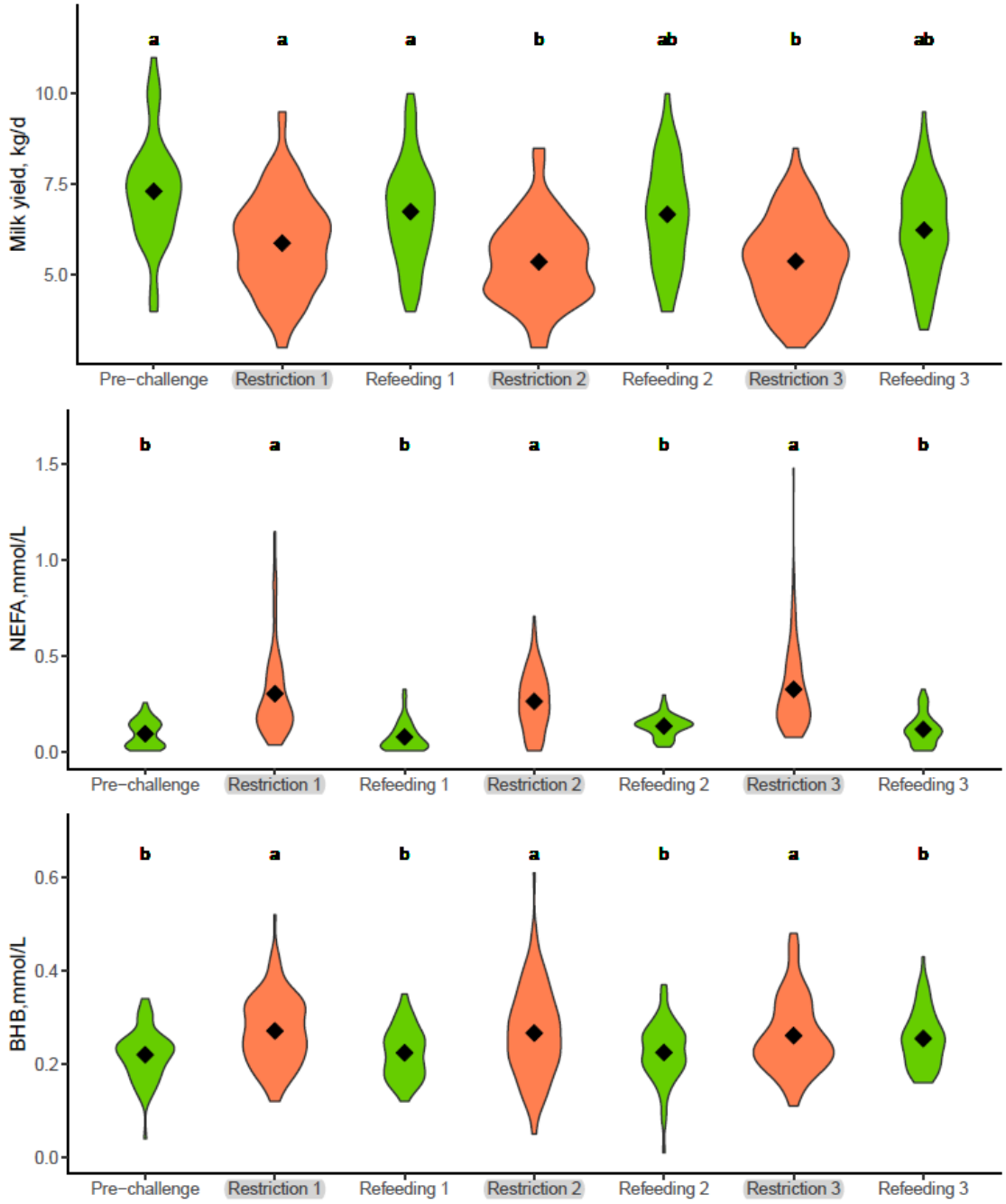


Figure 2. Frequency distribution (colored regions) of milk yield, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) by feeding period.

In orange, periods with a diet formulated to meet 55% of requirements (restriction); in green, periods with a diet formulated to meet 100 % of requirements (pre-challenge + refeeding). The black point indicates the mean value, significant differences ($P < 0.05$) between means are indicated with [a,b].

In the three parameters, the main differences were observed during the restriction periods, with a less variable milk yield response and more variable NEFA and BHB responses. Regarding milk yield, the pre-challenge variance was greater than those observed during the restrictions at challenges 2 and 3 ($P < 0.05$), whereas in the case of NEFA and BHB the pre-challenge variance was lower than those observed during restriction in the three challenges ($P \leq 0.02$). The variability in milk yield, NEFA and BHB was similar in the pre-challenge period and refeeding periods of the three challenges.

The PCA analysis performed on the curve response variables for milk yield, NEFA and BHB explained the 48% of the total variance with the first three principal components (Dim) (25%, 14%, and 9% in Dim 1, Dim 2, and Dim 3, respectively). The first Dim was related to milk yield curve variables, the second Dim was related to NEFA curve variables, and the third Dim was related to milk yield and BHB curve variables in the recovery phase. The clustering analysis generated two groups of cows that differed in their MR, hereafter denoted as High MR (n=15) and Low MR (n=16) cows. The mean values for the curve response variables are available in the Supplemental material. All the curve response variables for milk yield differed between MR clusters ($P \leq 0.02$; Table S1), the cows of the High MR cluster having highest baseline, greatest peak and AUC, and being fastest in reaching the peak and slowest in regaining the baseline. Regarding the metabolites curve response variables, cows of the High MR cluster had greater NEFA baseline values, greater NEFA and BHB peaks and AUC during restriction than their counterparts ($P \leq 0.004$; Table S2 and S3).

3.1. Performance parameters

Daily data and means by feeding period regarding cows' BW and milk yield throughout the experiment according to the MR cluster are plotted in Figure 3. Cow BW was affected by the interaction between the MR cluster and the feeding period ($P = 0.04$). Restriction reduced the cows' BW in both MR clusters in the three challenges to slightly different extents. The BW after refeeding recovered pre-challenge values only during challenge 1 in High MR cows ($P > 0.82$).

Milk yield was affected by the MR cluster and the feeding period ($P < 0.001$; Table 1). The High MR cows had greater milk yield than the Low MR cows ($P < 0.001$). Milk yield decreased with restriction and increased with refeeding in the three challenges ($P < 0.001$) but to different extents. The percentage loss of milk yield when compared to pre-challenge values was smaller during challenge 1 (-19%, $P < 0.001$) than during challenges 2 and 3 (-27% and -26% respectively, $P \leq 0.008$). Daily analyses showed that milk yield decreased on the first day of restriction of three challenges in both MR clusters

except for challenge 1, when it decreased on the second day in Low MR cows ($P < 0.001$, Figure 3). In the three challenges, pre-challenge milk yield was recovered on the first day of refeeding in the High MR cows but on the second day in the Low MR cows ($P < 0.004$, Figure 3).

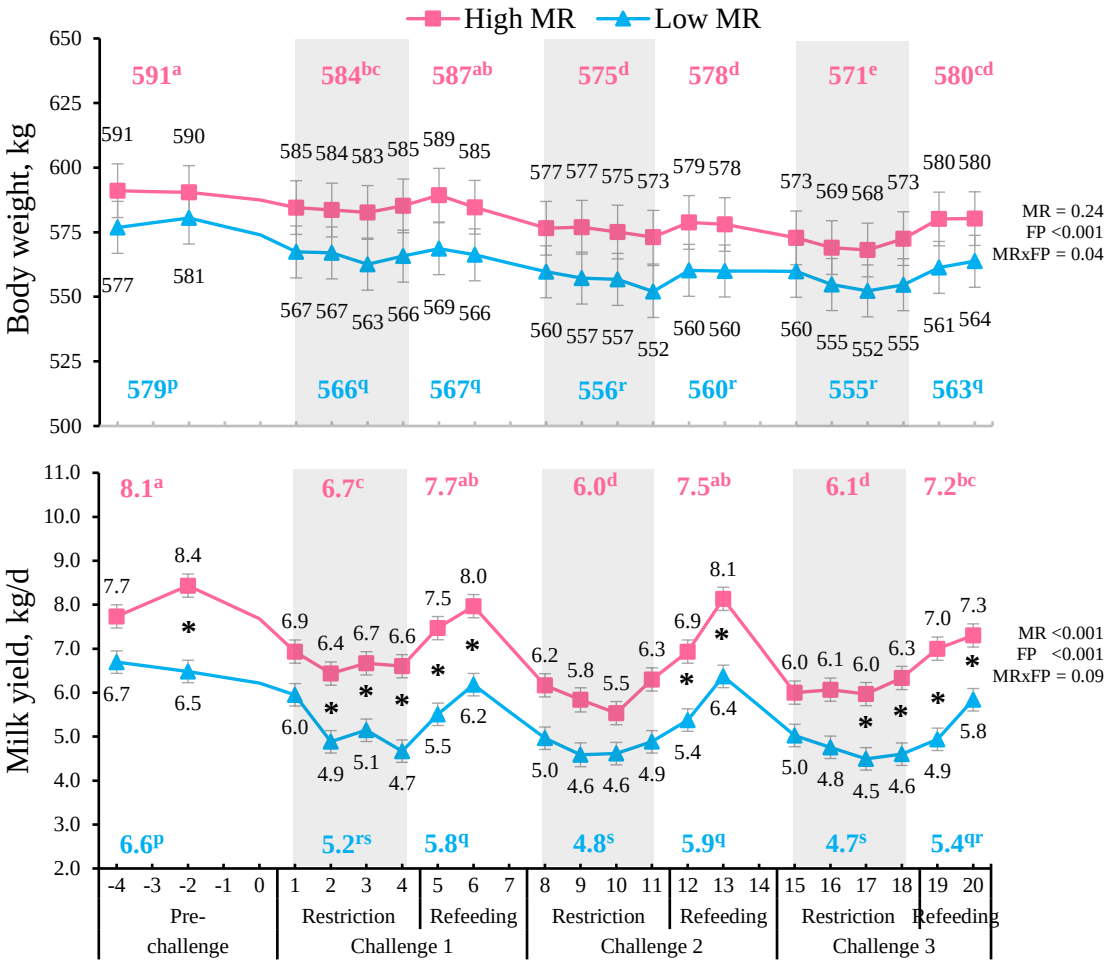


Figure 3. Daily weight and milk yield according to the metabolic response (MR) cluster throughout the experimental period.

The grey area represents the nutritional restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error.
Within each parameter, least square means for each feeding period (FP) are given in pink for High MR cows and in blue for Low MR cluster.
Differences between feeding periods are indicated with ^{a,b,c,d,e} in the High MR cluster and ^{p,q,r,s} in the Low MR cluster ($P < 0.05$).
* Denotes difference between clusters within a day at $P < 0.05$.

Milk composition according to the MR cluster and the feeding period is presented in Table 1. Milk protein content was affected by the interaction between the MR cluster and the feeding period ($P < 0.001$) whereas milk fat, lactose and urea contents were only affected by the feeding period ($P < 0.001$). Milk fat contents during challenge 1 were greater than during challenge 3 ($P = 0.03$). Milk protein contents were similar between High and Low MR clusters except for a tendency to differ during restriction in challenge 1 (2.82 vs. 3.04 g/100 g, $P = 0.06$, respectively) and a significant difference during restriction in challenge 3 (2.83 vs. 3.08 g/100g, $P = 0.02$, respectively). Lactose decreased with restriction and increased to pre-challenge contents during refeeding in challenges 1 and 2 ($P < 0.001$). Restriction increased milk urea content in all challenges (+38%, +10% and +3% in challenges 1, 2 and 3 respectively, $P < 0.05$), and decreased during refeeding to pre-challenge values in challenge 1 and even below in challenges 2 and 3 ($P < 0.05$).

Table 1. Effect of the metabolic response (MR) cluster and feeding period (FP) on milk composition of beef cows to a repeated 4 d restriction and 3 d refeeding challenge.

	Milk, kg/d	Fat, g/100g	Protein, g/100g	Lactose, g/100g	Urea, mg/dL
MR cluster					
High MR	7.0^{y}	4.26	2.85^{y}	4.71	22.6
Low MR	5.4^{x}	4.22	3.03^{x}	4.73	22.7
FP					
Pre-challenge	7.3^{a}	4.38^{ab}	2.96	4.70^{ab}	22.1^{c}
Challenge 1					
Restriction	5.9^{d}	4.43^{a}	2.93	4.64^{c}	30.3^{a}
Refeeding	6.8^{b}	4.55^{a}	2.91	4.73^{ab}	22.4^{c}
Challenge 2					
Restriction	5.4^{e}	4.31^{ab}	2.92	4.69^{b}	24.1^{b}
Refeeding	6.7^{b}	4.38^{ab}	2.96	4.75^{a}	19.4^{d}
Challenge 3					
Restriction	5.4^{e}	4.03^{bc}	2.96	4.74^{a}	22.7^{bc}
Refeeding	6.3^{c}	3.82^{c}	2.92	4.74^{a}	20.1^{d}
RSD[1]	0.79	0.981	0.112	0.108	2.53
P-values					
MR cluster	<0.001	0.91	0.009	0.63	0.91
FP	<0.001	<0.001	0.006	<0.001	<0.001
MR cluster x FP	0.09	0.62	<0.001	0.50	0.71

[1]Residual standard deviation.

^{x,y} Different superscripts indicate differences between MR clusters.

^{a,b,c,d} Different superscripts indicate differences among feeding periods ($P < 0.05$).

3.2. Plasma metabolites

Daily concentrations of plasma metabolites and their means by feeding period according to the MR cluster are plotted in Figure 4. The concentrations of NEFA were affected by the interaction between the MR cluster and the feeding period ($P < 0.001$). Cows on the High MR cluster had greater NEFA concentration than those on the Low MR cluster during the restriction period of challenges 1 ($P < 0.001$) and 2 ($P = 0.002$) but not in challenge 3 ($P = 0.17$). Restriction increased NEFA by threefold, up to similar mean concentrations in the three challenges within the High and Low MR cows ($P < 0.001$), although High MR cows reached a higher peak value in challenge 1 ($P < 0.05$).

Accordingly, percent change did not differ among challenges (+277%, +333% and +342% in challenges 1, 2 and 3 respectively, $P > 0.05$). Daily data showed that NEFA plasma concentration increased with restriction differently as the experiment progressed. During the restriction, NEFA concentrations in High MR cows were higher than their pre-challenge values during the four days in challenge 1, but only on days 2 and 3 in the subsequent challenges. In the Low MR cows, they only exceeded pre-challenge concentrations on day 2 in challenge 1 and on day 3 in the subsequent challenges ($P < 0.05$). Pre-challenge values were recovered on the first day of refeeding in the three challenges regardless of the MR cluster ($P > 0.05$, Figure 4).

Plasma BHB concentrations were affected by the interaction between the MR cluster and feeding period ($P = 0.005$), because they changed significantly in the High MR cows but remained stable in the Low MR cows (Figure 4). Consequently, they were higher in the High MR than the Low MR cows during restriction in challenges 1 and 2 ($P = 0.099$ and $P < 0.001$, respectively) but similar in challenge 3 ($P = 0.27$). With respect to pre-challenge values, BHB concentrations of High MR cows increased due to restriction similarly in the three challenges (+40%, +45%, +35% respectively, $P > 0.05$). Daily analyses showed that in the High MR cows they increased on the second day of restriction in all challenges ($P \leq 0.01$) and recovered pre-challenge concentrations on the first day of refeeding ($P > 0.05$, Figure 4).

Plasma urea concentrations were only affected by the feeding period ($P < 0.001$, Figure 4). They increased more due to restriction in challenge 1 (+20%, $P < 0.01$) than in the subsequent challenges (+12% and +11% respectively, $P > 0.05$) and decreased below pre-challenge concentrations during refeeding to a greater extent in challenge 2 ($P < 0.001$). Regarding the daily data, they responded immediately to the changes due to restriction and refeeding, irrespectively of the MR cluster.

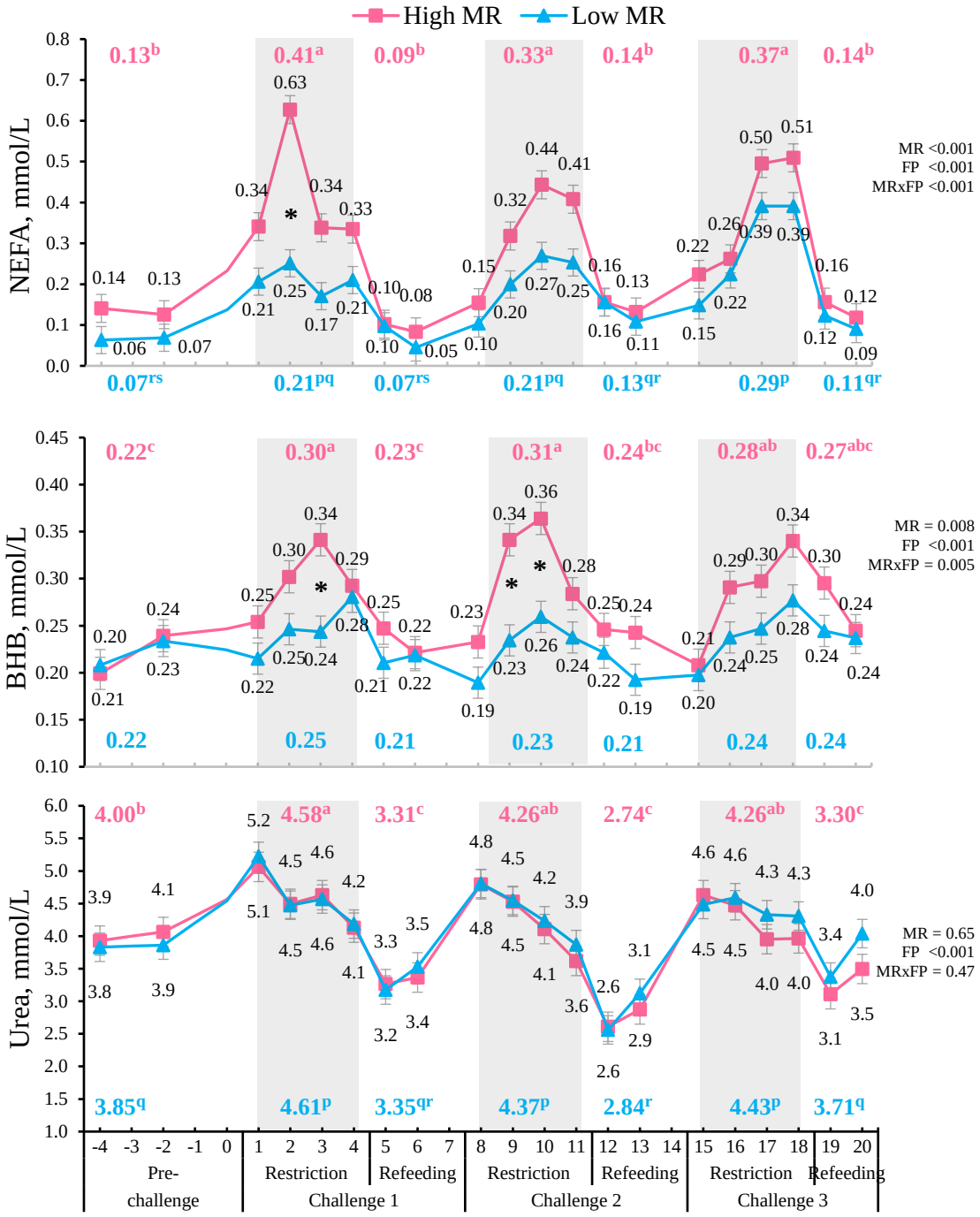


Figure 4. Daily plasma concentrations of non-esterified fatty acids (NEFA), and β-hydroxybutyrate (BHB) and urea according to the metabolic response (MR) cluster throughout the experimental period.

The grey area represents the nutritional restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error.

Within each parameter, least square means for each feeding period (FP) are given in pink for High MR cows and in blue for Low MR cluster.

Differences between feeding periods are indicated with ^{a,b,c,d,e} in the High MR cluster and ^{p,q,r,s} in the Low MR cluster ($P < 0.05$).

* Denotes difference between clusters within a day at $P < 0.05$.

The correlations among daily performance parameters and plasma metabolites are shown in Figure 5, which depicts both global correlations and those observed when the cows received the diet formulated to meet either 100% or 55% of their requirements. When correlations were studied separately for both diets, the BW and milk yield correlated strong and negatively with EB ($P < 0.001$) whereas milk urea correlated positively with plasma urea ($P < 0.001$). With the 100% diet there were weak negative correlations between the EB and plasma urea ($P < 0.001$), and milk urea and plasma NEFA ($P < 0.01$), and positive correlations between BW and milk urea ($P < 0.05$). When the cows were fed the 55% diet, plasma BHB correlated weak and positively with milk yield and plasma NEFA ($P < 0.001$).

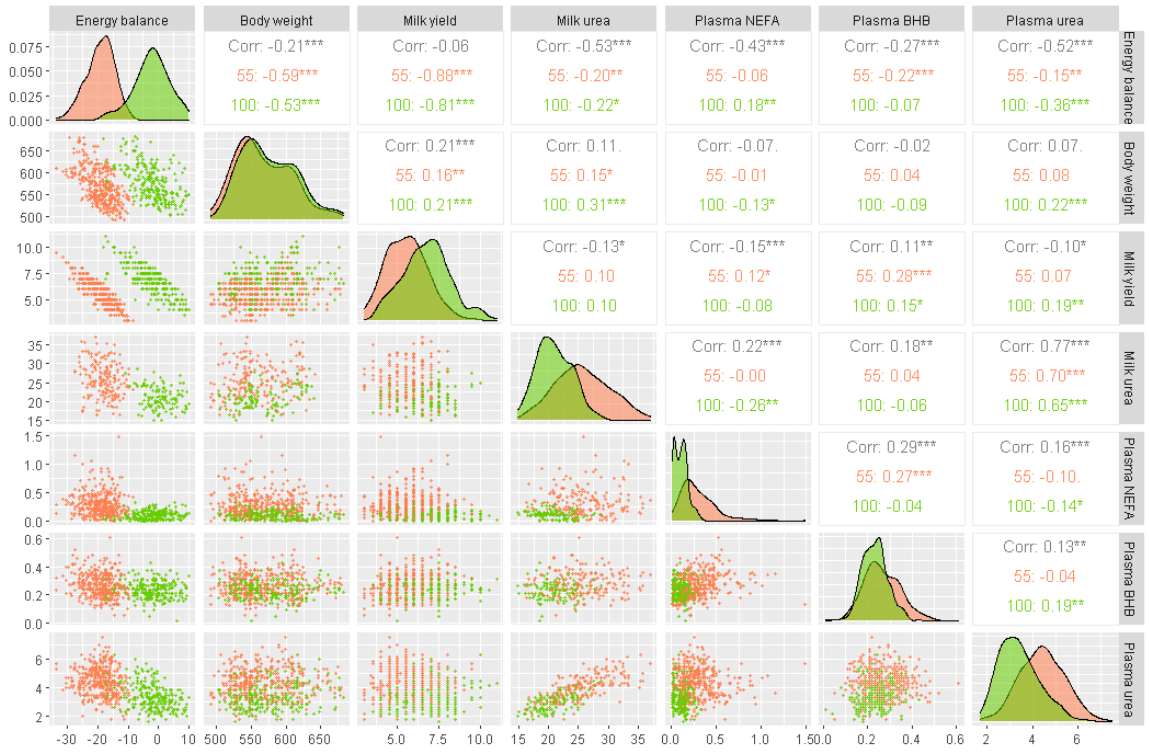


Figure 5. Pearson's rank correlations among performance parameters and plasma metabolites.

In black, global correlations independent of the diet.
In orange, correlations of the diet formulated to meet 55% of requirements (restriction).
In green, correlations of the diet formulated to meet 100 % of requirements (pre-challenge + refeeding).
Only significant correlations are presented, where ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.
NEFA: non-esterified fatty acids; BHB: β-hydroxybutyrate.

4. Discussion

Although some studies have previously evaluated the adaptation of cattle to repeated stressors of different nature, to the best of our knowledge this is the first report on the adaptive response of lactating beef cows to short repeated nutritional challenges. Negative stimuli of different origins trigger coping responses by the animals, which may depend on the nature, frequency, duration and intensity of the stressor (Chen et al., 2016). Dohme et al. (2008) assessed the degree of subacute ruminal acidosis with repeated challenges and found that its severity worsened with each successive challenge. On the opposite, Nagata et al. (2018) found an adaptation of ruminal bacterial populations during repeated acidosis challenges, which alleviated the adverse changes in ruminal pH. Rauch et al. (2021) reported that cow performance was not affected by repeated dietary protein oscillations, because despite they elicited transient effects, effective compensatory mechanisms were able to mitigate their potential negative consequences.

The study of the individual variability in the milk yield, plasma NEFA and BHB in response to the different diets showed that during the restriction periods the variance of milk production lowered while those of NEFA and BHB concentrations increased. This indicates that cows reacted to the restriction with large differences in their fat reserves mobilization capacity, which allowed to alleviate the negative impact of the reduced nutrient supply on their milk loss, which was less variable among cows (Agenäs et al., 2003; Berghof et al., 2019). This individual variability in the response and recovery from a challenge can be used to identify animal types (Friggens et al., 2016). For that purpose, we modelled the response curves of milk yield NEFA and BHB under the repeated feed challenges, by quantifying the gap between the potential and perturbed curve as an indicator of the animal's resilience (Poppe et al., 2020; Ben Abdelkrim et al., 2021; Barreto-Mendes et al., 2022). The response variables allowed to discriminate two distinct groups of cows with similar response profiles to the repeated challenges. This clustering analysis has proved useful to provide a basis for decision-making at the herd level (Tremblay et al., 2018; de Koster et al., 2019), because it identifies different patterns of aggregated response and provides more relevant information than differentiating cows only by a single trait. Furthermore, in a previous study the same cows had been clustered according to their response to short nutritional perturbations in different months of lactation (Orquera-Arguero et al., 2022), and most of them (27 out of 31) were classified into the same groups as in the current experiment according to their MR. This suggests that, regardless of the time and frequency of the feed challenges, there is a genetic

component that controls the partitioning of nutrients towards the different biological functions (Friggens and Newbold, 2007).

4.1 Performance parameters

Lactating cows are highly dependent on the supply of nutrients to the udder to support milk synthesis (Agenäs et al., 2003). Therefore, despite the array of physiological mechanisms which come into play to maintain homeostasis (Bauman and Currie, 1980; Baumgard et al., 2017), a rapid decline in milk yield can be expected during a feed restriction period, as observed in cows in different basal energy balance (Orquera-Arguero et al., 2023a). Herein, nutrient restriction reduced milk yield to a lesser extent in the first than in the subsequent challenges, which implies that the severity of the impact increased up to the second challenge but not thereafter. In general, the High MR cows had greater milk yield and showed a faster response to diet changes than the Low MR cows, which needed an additional day to recover pre-challenge yields. This could be related to differences in the nutrient partitioning, as observed by Baumgard et al. (2017) between high- and low-yielding dairy cows, where the former would show a higher priority for diverting absorbed nutrients to the mammary gland to ensure milk synthesis. Nevertheless, despite this difference both MR groups were able to regain their pre-challenge yield by the second day of refeeding, as observed previously in beef cows (De La Torre et al., 2022; Orquera-Arguero et al., 2022), regardless the repetition of challenges which indicates that beef cows were resilient under these conditions. Therefore, in beef cows milk yield could be considered a trait with elastic properties, as the deformation is reversible and can return to its original state (Blanc et al., 2010).

The repeated nutritional challenges produced minor changes in the milk components. Milk fat content only decreased during refeeding of challenge 3, suggesting it was only affected by the cumulative effect of the 3 challenges. The milk fat content in beef cows does not seem to be largely affected by short feed restrictions, but Orquera-Arguero et al. (2023a) reported a significant effect of diet changes on the fine fatty acid composition of milk fat. Milk urea changes were more evident because they reflect the balance of dietary protein and energy supply for ruminal microbial metabolism (Kessler et al., 2020), as is confirmed by the negative correlation with EB observed here. Conflicting results have been observed response to a feed restriction in dairy cows, from an increase in milk urea content (Carlson et al., 2006) attributed to amino acid catabolism, to a decrease (Abdelatty et al., 2017; Kvidera et al., 2017) associated with the decreased supply of amino acids from intestine absorption (Billa et al., 2020). Our results support the first hypothesis and suggest a greater protein catabolism in the first

challenge, reflected in greater milk urea content, which would decrease in challenges 2 and 3, as can be corroborated with the strongly correlated plasma urea concentrations. Overall, these results regarding milk yield and composition suggest that the severity of the restriction only increased with the second challenge but a further exposure to a third challenge did not trigger a more acute response.

4.2. Plasma metabolites

The effect of short term dietary restrictions and subsequent refeeding on plasma indicators of metabolic status has been documented in dairy (Billa et al., 2020; Bjerre-Harpøth et al., 2012; Leduc et al., 2021) and beef cattle (De La Torre et al., 2022; Orquera-Arguero et al., 2023b). Literature reports an increased release of NEFA from the adipose tissue to be used for milk fat synthesis by the mammary gland or oxidized into the liver in ketone bodies such as BHB, acetoacetate or acetone, and these can be used as energy fuel to support milk production (Bell, 1995; Puppel and Kuczyńska, 2016). Here, the rise of NEFA concentrations in response to feed restriction in all challenges shows that cows of both MR groups underwent lipid mobilization; reaching higher peak values in the High MR cows. Concentrations were lower than those observed in beef cows at earlier stages of lactation, when the metabolic demand and priority for milk production is greater (Orquera-Arguero et al., 2023b). Changes in NEFA contents were concomitant with an increase in the BHB plasma concentration only in the High MR cows, where a greater metabolic demand provoked a greater lipolysis to support the greater milk yield. Apparently, in the Low MR cows, the lower NEFA increases were insufficient to trigger ketogenesis, resulting in no change in BHB plasma concentration (McArt et al., 2013). The similar values of both metabolites among challenges imply that cows did not react differently in response to the repeated bouts of underfeeding.

The daily analysis provided further insight on the effects of restriction and refeeding on the dynamic response patterns according to the MR clusters. Both groups showed a rise in NEFA for at least one day of the 4-day restriction period. This increment was faster and reached higher values in the High MR cluster, with peak contents being close to and even exceeding (cf. challenge 1) the threshold of 0.60 mmol/L proposed as an indicator of risk of metabolic disease (Ospina et al., 2010). Furthermore, it appears that NEFA plasma concentrations responded faster to restriction than BHB in both groups, in agreement with Puppel and Kuczyńska (2016), whereas the BHB threshold for risk of ketosis (1.2 mmol/L, McArt et al., 2013) was never reached in either group. Despite the differences between MR clusters during the restrictions, during the refeeding

phases both groups had similar NEFA and BHB plasma concentrations as in the pre-challenge period. Accordingly, Ferraretto et al. (2014) reported that, after dairy cows received 25% or 50% feed restriction, circulating NEFA returned to basal concentrations one day after dairy cows returned to normal intake.

The plasma concentrations of urea are influenced by the dietary protein intake but also by the muscle tissue catabolism under limited energy intake, when glucogenic amino acids are mobilized to supply glucose, generating urea in the process (Agenäs et al., 2003; Bell, 1995; Burgos et al., 2001). Some studies in dairy cows have reported plasma urea to decrease (Bjerre-Harpøth et al., 2012) or remain unchanged under feed restriction (Carlson et al., 2006), whereas Horn et al. (2014) described increased urea in underfed cows. Here we observed significant increments associated to the mobilization of body protein, with full recovery during the refeeding phase. As in the previous metabolites, the similar values among challenges indicate that cows showed neither metabolic habituation nor sensitization to repeated underfeeding (Blumstein, 2016). In the conditions of this experiment, our results show the plasticity of cows of both groups to respond to and recover after repeated underfeeding, which can be associated with their ability to mobilize and reconstitute body reserves (Blanc et al., 2010).

5. Conclusions

Repeated challenges of short-term feed restriction and refeeding caused effects of different magnitude on the productive and metabolic traits of lactating beef cows. Cows with different metabolic response profiles reacted differently in terms of milk yield and plasma NEFA and BHB concentrations, all of which recovered basal values after a short refeeding. The milk loss in response to restriction worsened after the first challenge, but the indicators of lipid and protein mobilization responded similarly across repetitions, showing no under- or over-reaction to repeated underfeeding. It remains to be determined whether additional challenges would trigger a different response.

Supplemental material

Supplemental Table 1. Milk yield curve response variables according to the metabolic response (MR) cluster and challenge.

	Baseline, mmol/L	Peak, mmol/L	days to peak	days to regain	AUC_{rest}, mmol x d/L	AUC_{refeed}, mmol x d/L
MR cluster						
High MR	7.7^{x}	-1.8^{y}	2.6^{y}	5.6^{x}	-5.7^{y}	-1.1^{y}
Low MR	5.9^{y}	-1.3^{x}	3.1^{x}	5.3^{y}	-4.0^{x}	-0.7^{x}
Challenge						
1	6.9^{a}	-1.3^{b}	3.2^{a}	5.6	-3.7^{a}	-0.8
2	6.9^{a}	-1.9^{a}	2.7^{b}	5.5	-5.9^{b}	-1.1
3	6.6^{b}	-1.5^{b}	2.7^{b}	5.4	-4.9^{b}	-0.8
RSD	0.4	0.6	0.9	0.5	1.9	0.6
P-values						
MR cluster	<0.001	0.003	0.02	0.004	0.006	0.002
Challenge	0.002	<0.001	0.03	0.46	<0.001	0.099
MR cluster x Challenge	0.50	0.54	0.20	0.20	0.82	0.25

AUC: area under the curve.

Supplemental Table 2. NEFA curve response variables according to the metabolic response (MR) cluster and challenge.

	Baseline, mmol/L	Peak, mmol/L	days to peak	days to regain	AUC_{rest}, mmol x d/L	AUC_{refeed}, mmol x d/L
MR cluster						
High MR	0.12^{x}	0.35^{x}	3.1	5.3	0.77^{x}	0.11
Low MR	0.09^{y}	0.20^{y}	3.1	5.5	0.47^{y}	0.09
Challenge						
1	0.08^{b}	0.27^{ab}	2.4^{b}	5.5	0.80^{a}	0.06^{b}
2	0.12^{a}	0.21^{b}	3.3^{a}	5.4	0.41^{b}	0.08^{b}
3	0.11^{a}	0.34^{a}	3.6^{a}	5.2	0.64^{ab}	0.15^{a}
Cluster x Challenge						
High MR 1	0.10	0.39	2.4	5.4	1.12^{a}	0.06
High MR 2	0.13	0.29	3.3	5.3	0.53^{b}	0.12
High MR 3	0.13	0.38	3.6	5.1	0.66^{b}	0.16
Low MR 1	0.06	0.15	2.5	5.6	0.49^{b}	0.07
Low MR 2	0.11	0.14	3.3	5.4	0.29^{b}	0.05
Low MR 3	0.09	0.29	3.6	5.4	0.61^{b}	0.15
RSD	0.04	0.15	0.6	0.7	0.41	0.08
P-values						
MR cluster	0.001	<0.001	0.97	0.19	<0.001	0.38
Challenge	0.002	0.006	<0.001	0.26	0.002	<0.001
MR cluster x Challenge	0.78	0.15	0.92	0.92	0.02	0.16

AUC: area under the curve.

Supplemental Table 3. BHB curve response variables according to the metabolic response (MR) cluster and challenge.

	Baseline, mmol/L	Peak, mmol/L	days to peak	days to regain	AUC_{rest}, mmol x d/L	AUC_{refeed}, mmol x d/L
MR cluster						
High MR	0.25	0.09^{x}	3.1	5.5	0.13^{x}	0.03
Low MR	0.23	0.04^{y}	2.9	5.4	0.02^{y}	0.02
Challenge						
1	0.23^{b}	0.07	3.1^{ab}	5.4	0.13^{a}	0.03
2	0.23^{b}	0.08	2.6^{b}	5.4	0.11^{a}	0.02
3	0.25^{a}	0.05	3.3^{a}	5.4	0.002^{b}	0.03
RSD	0.03	0.04	0.9	0.9	0.13	0.05
P-values						
MR cluster	0.16	0.001	0.45	0.67	0.004	0.67
Challenge	0.005	0.07	0.03	0.99	0.001	0.77
MR cluster x Challenge	0.70	0.46	0.10	0.69	0.248	0.68

AUC: area under the curve.

Capítulo IV

“Rendimiento y perfil de ácidos grasos de la leche de vacas de carne con diferente estatus energético durante una restricción nutricional corta y realimentación”

Correspondiente al artículo:

Performance and milk fatty acid profile of beef cows with a different energy status with short nutrient restriction and refeeding.

Karina G. Orquera-Arguero; Mireia Blanco; Juan R. Bertolín; Javier Ferrer; Isabel Casasús. 2023.

Journal of Animal Science 101: 1-11. https://doi.org/10.1093/jas/skad053

1. Introduction

Wide seasonal variations in the availability and quality of feeding resources in extensive ruminant systems imply that animals are often subjected to underfeeding-refeeding cycles (Bocquier and González-García, 2010). When undernutrition occurs in lactating cows, both homeostatic and homeorhetic controls bring about adaptations to help to maintain balance and to supply nutrients to the mammary gland (Bauman and Currie, 1980) to support the high metabolic priority of milk production. Strategies to cope with the physiological imbalance caused by feed restriction depend, among other factors, on: restriction duration and its severity (Leduc et al., 2021); lactation stage (Orquera-Arguero et al., 2022); individual variability (Bjerre-Harpøth et al., 2012; Gross et al., 2011a). In beef cows, the impacts of restriction and refeeding on cow metabolism have been well assessed in the long term (Fiems et al., 2015), and only recently with short-term restrictions (De La Torre et al., 2022; Orquera-Arguero et al., 2022). Furthermore, *ad libitum* or individual feeding strategies are commonly used in dairy cattle, where individual concentrate allocation based on milk yield can improve the energy balance (EB) and cow performance (Lawrence et al., 2016), while other studies report no milk yield differences (Henriksen et al., 2019). On extensive beef cow farms, feeding management is often simplified by adopting a flat-rate regime (Manninen et al., 2004), which involves all cows receiving the same diet irrespectively of their individual requirements. This common feeding can cause disruptive situations under an eventual restriction in nutrient intake, with the most sensitive individuals, those with greater requirements, being the most affected (Bocquier and González-García, 2010). Clustering analyses have been used to group dairy cows according to their performance, plasma metabolites, hormones, and milk traits to identify animals with different strategies to face metabolic challenges (De Koster et al., 2019; Xu et al., 2019; Orquera-Arguero et al., 2022), which could facilitate herd management decisions.

Major changes occur in adipose tissue in response to a negative EB, which results in the mobilization of body reserves and an increase in circulating nonesterified fatty acids (NEFA) and ketones to provide energy and precursors for milk synthesis (Baumgard et al., 2017). Plasma concentrations of these and other metabolites, such as malondialdehyde (MDA), associated with oxidative status (Castillo et al., 2006) or urea as an indicator of protein metabolism (Bittante, 2022), have been used as biomarkers of cow metabolic load. In the last few years, milk composition traits have been examined as non-invasive indicators of dairy cows' nutritional status (Billa et al., 2020; Gross and Bruckmaier, 2019a) because they can be cost-efficiently and routinely measured from test-day milk samples (Mäntysaari et al., 2019). Of them, milk fatty acid (FA) contents

are promising indicators of energy status in dairy cows (Khiaosa-ard et al., 2020) given that FA C4:0 to C14:0 are synthesized *de novo* in the mammary gland, whereas those longer than C18:0 and around 50% of C16:0 originate from diet and lipid mobilization (Chilliard et al., 2000b; Palmquist, 2009). In fact C16:0, C18:0, and 18:1 cis-9 are the most abundant FA in plasma and body fat stores (Hostens et al., 2012), and their concentrations and ratios are closely related to the EB in dairy cows (Dórea et al., 2017), but no information on this is available in beef cows. We hypothesized that the response to restriction and refeeding would be driven by each cow's weight, milk yield, and nutritional status before the challenge. Therefore, the main objectives of this study were to: i) evaluate the effects of a negative EB induced by a short feed restriction on the performance, metabolites, and milk FA profile in two groups of beef cows classified according to their previous performance; ii) confirm the potential use of milk FA composition as a biomarker of metabolic status in beef cows.

2. Materials and methods

The Animal Ethics Committee of the Research Centre approved the experimental procedures (protocol no. CEEA-03-2018-01), which followed the guidelines of EU Directive 2010/63 on the protection of animals used for experimental and other specific purposes (EU, 2010). The experiment was conducted in the Pyrenees Mountain area at the CITA La Garcipollera Research Station (Spain, 42°37' N, 0°30' W, 945 m a.s.l.).

2.1. Animal management, diets, and experimental design

The study was conducted with 32 multiparous Parda de Montaña beef cows [at calving: body weight (BW): 626 ± 47.7 kg; body condition score (BCS, on a 5-point scale): 2.8 ± 0.22; age: 7.5 ± 2.91 years)]. One cow was removed from the study due to physical injury. After calving, cows were randomly allocated in pens (8 cows/pen, 10x20 m) equipped with individual feeders for forage (200-l fiberglass boxes in front of self-locking feeding places) and automatic feeding stations (ALPRO Herd Management 7.0, DeLaval) for concentrate. Calves were penned in straw-bedded cubicles adjacent to their dams. They were allowed to suckle their dams twice daily for 30 min at 06:00h and 14:00h.

Cows were fed a flat-rate regime during lactation. They all received the same amount of feed. Diets were calculated by considering the net energy (NE) and metabolisable protein requirements for the maintenance and lactation of a standard cow (615 kg BW; milk yield: 8.5 kg/d) using INRA equations (INRA, 2007). From calving to the start of the experiment 2 months later, cows were fed a formulated diet to meet 100% standard cow energy requirements (Table 1).

Table 1. Chemical composition, fatty acids (FA) composition and nutrition value (mean ± SD) of the feedstuffs offered to beef cows.

Parameter	Hay	Concentrate
Chemical composition		
Dry matter (DM), g/kg	922 ± 11.7	906 ± 4.0
Ash, g/kg DM	86.4 ± 24.4	68.3 ± 1.4
Crude protein, g/kg DM	109 ± 18.3	167 ± 4.7
Neutral detergent fibre, g/kg DM	570 ± 52.4	256 ± 23.2
Acid detergent fibre, g/kg DM	324 ± 32.9	114 ± 11.1
Lignin, g/kg DM	35.2 ± 12.8	29.4 ± 8.8
FA composition		
C16:0, g/100 g ID FAME[1]	32.2 ± 2.37	19.2 ± 0.60
C18:0, g/100 g ID FAME[1]	14.1 ± 2.02	5.3 ± 0.02
C18:1 cis-9, g/100 g ID FAME[1]	4.5 ± 1.15	23.6 ± 0.32
C18:2 n-6, g/100 g ID FAME[1]	15.7 ± 3.30	44.4 ± 1.78
C18:3 n-3, g/100 g ID FAME[1]	26.6 ± 10.17	1.8 ± 0.31
Total, mg ID FAME[1]/g DM	18.5 ± 2.99	65.7 ± 2.15
Nutritive value		
Net energy, MJ/kg DM	5.5 ± 0.15	7.3 ± 0.41
Metabolisable protein, g PDI[2]/kg DM	81 ± 17.9	123 ± 2.4

[1] identified fatty acid methyl esters.

[2] true protein digestible in the small intestine.

The experiment was conducted at the end of the second lactation month and involved three consecutive periods, where d 0 was taken as the first day of restriction [days in milk (DIM): 58 ± 6.3]. Cows were first fed a diet that met 100% of their energy and metabolisable protein requirements (d -2 to -1, basal period), then 55% of those requirements for 4 d (d 0 to 3, restriction period) and, finally, 100% again on the following 4 d (d 4 to 7, refeeding period). Diets consisted of 8.0 kg hay and 3.0 kg of concentrate (as-fed basis) during the basal and refeeding periods, and 7.0 kg hay during the restriction period. Animals had free access to water and mineral blocks throughout the experiment.

2.2. Measurements

Samples of feedstuffs were collected daily (d -2 to 8) and lyophilized in a Genesis Freeze Dryer 25 (Hucoa Erlöss, SA/Thermo Fisher Scientific) to determine their chemical composition and FA profile. Hay was offered daily at 08:00h as a single meal in individual troughs, where cows were tied up until they finished their ration, during approximately 2 h. ALPRO feeding stations were programmed to offer 3 kg of concentrate daily (as-fed

basis) to all the cows during the basal and refeeding periods. Individual concentrate intake was recorded daily.

The BCS was recorded upon calving, 30 DIM, and on experimental period d -2 and 8. It was determined by a trained person on a 1-5 scale, based on estimating the fat covering ribs, loin, and tailhead (Lowman et al., 1976). Cows were weighed on an electronic scale upon calving and then at 07:00h on 30 and 31 DIM and on experiment d -2, 1, 3, 5, 6, and 8. Milk yield was estimated on the same days by the weight-suckle-weight technique (Le Neindre and Dubroeucq, 1973). Calves were weighed before and after the two daily 30-min periods in which they had access to suckle their dams. The daily milk yield was estimated as the sum of the milk consumed by the calf in these two suckling periods. Milk samples were manually taken from each dam after the morning suckling. Five min before the manual extraction, all cows received an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) to accelerate the letdown of the residual milk. A 100-ml sample was collected to determine milk composition, added with sodium azide (PanReac) as a preservative and refrigerated at 4 °C until the analysis. To determine FA composition, a second 40-ml sample was collected, lyophilized, and stored at -20 °C until analyzed.

Cows were bled on the same experiment days described above to assess their metabolic profile. Blood samples were collected from the coccygeal vein at 07:00h after suckling and before offering hay. Heparinized tubes (BD Vacutainer Becton-Dickenson and Company) were used for the β- hydroxybutyrate (BHB) and MDA determinations, and the tubes that contained K2 EDTA (BD Vacutainer Becton-Dickenson and Company) were used to analyze glucose, NEFA, and urea concentrations. Immediately after collection, blood samples were centrifuged at 3500 rpm for 20 min at 4 °C. Plasma was collected and frozen at -20 °C until further analyses.

2.3. Analyses

2.3.1. Feedstuffs and milk

The chemical composition of feedstuffs was analyzed in duplicate as described in Orquera-Arguero et al. (2022). Briefly, dry matter (DM) and ash content were determined according to AOAC methods (AOAC, 2000). Nitrogen content was determined following the Dumas Procedure (index no. 968.06) with a nitrogen analyzer (Model NA 2100, CE Instruments, Thermoquest SA., Barcelona, Spain). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were analyzed following the sequential procedure of Van Soest et al. (1991) with an Ankom 200/220 fiber analyzer (Ankom Technology Corporation, Fairport, NY,

USA). In milk samples, fat, protein, and urea contents were analyzed by an infrared scan (Milkoscan 7 RM, Foss Electric Ltd., Hillerød, Denmark). The FA of the freeze-dried feedstuffs were extracted and methylated as proposed by Sukhija and Palmquist (1988). The fatty acid methyl esters (FAME) of the freeze-dried milk samples were obtained as described by Kramer et al. (1997). Determination was done by gas chromatography with a flame ionization detector and Bruker Scion 436-GC (Bruker, Billerica, USA) equipped with a CP-8400 Autosampler (Bruker, Billerica, USA), a cyanopropyl capillary column SP-2560 (100 m x 0.25 mm ID x 0.20 µm thickness for feedstuffs and 200 m x 0.25 mm ID x 0.20 µm thickness for milk) (Sigma-Aldrich, Sant Louis, USA) and the Compass CDS software. FAME was ID using the GLC-532, GLC-401, GLC-643, GLC-642, GLC-463 C18:1 t11, C19:0, C23:0 (Nu-Chek-Prep Inc.), mixture BR1, mixture BR4 (Larodan Research Grade Lipids) standard references, and the relative retention times observed in the bibliography (Kramer et al., 1997; Shingfield et al., 2003; De La Fuente et al., 2015). Fatty acid quantification was performed as described in UNE-EN ISO 12966-4:2015 and expressed as a percentage of the total amount of identified FAME. The chemical composition and FA profile of the feedstuffs are presented in Table 1.

2.3.2. Blood metabolites

Glucose (enzymatic-colorimetric method, sensitivity: 0.06 mmol/L) and urea (kinetic method, sensitivity: 0.056 mmol/L) concentrations were determined in plasma with an automatic analyzer (Gernon, RAL S.A, Barcelona, Spain). The mean intra- and interassay CV were 1.5% and 1.9% for glucose and 3.2% and 4.8% for urea, respectively. Plasma BHB (kinetic enzymatic method, sensitivity: 0.100 mmol/L) and NEFA (colorimetric method, sensitivity: 0.072 mmol/L) were determined using Randox kits (Randox Laboratories Ltd., Country Antrim, UK). The mean intra- and interassay CV were respectively 3.3% and 3.7% for NEFA and 6.2% in both cases for BHB. Oxidative status was determined using MDA as a biomarker of lipid peroxidation. This indicator was determined by liquid chromatography using an Acquity UPLC H-Class liquid chromatograph (Waters, Milford, Massachusetts, USA) equipped with a silica-based bonded phase column (Acquity UPLC HSS PFP, 100 mm × 2.1 mm × 1.8 µm, Waters), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ detector, Waters) and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters). The quantification of MDA was done by fluorescence detection at $\lambda_{excitation}$ = 530 nm and $\lambda_{emission}$ = 550 nm following the chromatographic conditions described in Bertolín et al. (2019). The mean intra- and interassay CV were 4.6% and 7.3%, respectively.

2.4 Calculations

The chemical composition of feedstuffs was employed to calculate their NE content using INRA equations (INRA, 2007). Individual EB was estimated by calculating the difference between inputs (NE intake) and outputs (NE for maintenance and NE for lactation) (INRA, 2007). Net energy intake was estimated from the individual intake and energy contents of feedstuffs. Net energy for maintenance was calculated from the individual metabolic weight. Net energy for production was obtained using the milk yield, fat, and protein contents in milk.

In milk, FA were grouped according to their degree of saturation as saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) according to their origin from *de novo* synthesis (C4:0 - C15:1), of mixed origin (C16:0 - C16:1), and from mobilization (≥ C17:0) (Palmquist, 2009). The C18:1 cis-9 to C15:0 FA ratio was calculated to assess its relation with the EB and metabolic profile.

2.5. Statistical analyses

All the data were analyzed using the SAS statistical package v 9.4 (SAS Institute Inc., Cary, NC, USA). Cows were assigned to clusters according to their resemblance in terms of Euclidean distance calculated using data from BW and BCS at calving and BW, BCS, milk yield, and EB at 30 and 31 DIM. A non-hierarchical clustering was performed using the k-means method (FASTCLUS procedure). The selection of the optimum number of clusters was based on cubic conglomerating criteria. Two clusters (hereafter referred to as status clusters) were obtained, namely Balanced and Imbalanced. An analysis of variance was performed on the classifying variables using a general linear model (GLM procedure) and taking the cluster as a fixed effect.

Cows' metabolic and production data were studied in two sets of analyses, which considered different time effects during the experiment: feeding period (basal, restriction, refeeding) and day (d -2 to 8). In both cases, mixed models for repeated measures (MIXED procedure) were used by considering the status cluster (Balanced and Imbalanced), time (feeding period or d), their interaction as fixed effects and cow as the random effect. The model used was $Y_{ijk} = \mu + S_j + T_k + S_j \times T_k + C_i + e_{ijk}$, where Y_{ijk} was the dependent variable at each time point for the i^{th} cow; μ, the overall mean; S_j, the effect of the status cluster; T_k, the effect of time (either feeding period or day); C_i, the random effect of cow i and e_{ijk} was the experimental error. Degrees of freedom were adjusted with the Kenward-Roger correction to take into account missing values. The variance components structure was selected on the basis of the lowest Akaike and Bayesian information criteria. Least square means and associated standard errors were obtained,

and multiple comparisons were adjusted with Tukey correction. Pearson's relations (r) between variables were obtained and presented on heatmaps for cow performance, plasma metabolites, and milk FA composition variables using the CORRPLOT package of R (R Core Team, 2021). The data set used for the correlation analyses corresponds to all traits and samples collected per cow at d -2, 1, 3, 5, 6, and 8 of experiment (n=186 values per trait). The *P*-value for significance was set at $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$.

3. Results

The results of the status cluster and feeding period effects appear in the tables. The results of the status cluster and day effects are plotted in the figures. The clustering analysis resulted in two cow clusters, which differed in terms of their pre-experimental BW, milk yield, and EB (Table 2). Cows in the first cluster were classified as Balanced and those in the second cluster as Imbalanced. Balanced cows were lighter, had a lower milk yield and a less negative EB than Imbalanced cows in the second cluster ($P \leq 0.03$).

Table 2. Initial cow characteristics (30-31 days in milk) according to the status cluster[1].

Item	Balanced	Imbalanced	SEM	*P*-value
n	15	16	-	-
Body weight, kg	563	633	4.12	< 0.001
Body condition score (scale 1 to 5)	2.8	2.9	0.04	0.18
Milk yield, kg/d	7.5	8.6	0.17	0.03
Energy balance, MJ NE[2]/d	-3.5	-10.0	0.77	< 0.001

[1] cows clustered according to the analysis based on pre-challenge cow traits and energy status.

[2] Net energy.

3.1. Cow performance

Dry matter intake (DMI) was only affected by feeding period ($P < 0.001$; Table 3). According to the experimental design, DMI was lower during the restriction than during the basal and refeeding periods ($P < 0.001$), and so were energy intakes (59.8, 34.9 and 59.8 MJ NE/d during the basal, restriction, and refeeding periods, respectively, $P < 0.001$) and metabolisable protein intakes (859, 471, and 859 g/d, respectively; $P < 0.001$). The BCS was affected by the status cluster (2.65 and 2.81 in Balanced and Imbalanced cows, respectively, $P < 0.001$), and tended to decrease between d -2 and d 8 (2.75 and 2.71, respectively, $P = 0.08$). Cow BW was affected by the interaction between status cluster and feeding period (Table 3) because restriction decreased BW in both groups ($P < 0.001$), but during refeeding BW decreased even more in Imbalanced

cows (P = 0.03), whereas it was maintained in Balanced cows ($P \geq 0.23$). In any case, Balanced cows were lighter than their Imbalanced counterparts throughout the experiment ($P < 0.001$). Regarding daily changes, BW of Imbalanced cows lowered from the start (d -2) to the end of the experiment (d 8) ($P < 0.05$), while that of Balanced cows decreased until d 6 ($P < 0.01$), but then regained basal values on d 8 (Figure 1).

Table 3. Effect of the status cluster[1] and feeding period (FP) on beef cows' performance.

Item	Status cluster		RSD[2]	*P*-value		
	Balanced	Imbalanced		Status	FP	Status × FP
Dry matter intake, kg/d			0.16	0.98	<0.001	0.51
Basal	10.0[a]	10.1[a]				
Restriction	6.4[b]	6.5[b]				
Refeeding	10.1[a]	10.0[a]				
Body weight, kg			6.55	<0.001	<0.001	0.01
Basal	553[a, y]	621[a, x]				
Restriction	542[b, y]	611[b, x]				
Refeeding	543[b, y]	606[c, x]				
Milk yield, kg/d			0.70	0.10	<0.001	0.001
Basal	7.7[a]	8.2[a]				
Restriction	6.3[c]	6.9[b]				
Refeeding	7.0[b]	8.3[a]				
EB[3], MJ NE[4]/d			2.46	<0.001	<0.001	<0.001
Basal	0.1[b, x]	-5.4[a, y]				
Restriction	-20.3[c, x]	-25.3[b, y]				
Refeeding	2.8[a, x]	-5.1[a, y]				

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] Residual standard deviation.

[3] Energy balance.

[4] Net energy.

[a,b,c] Different superscripts indicate differences between feeding periods ($P < 0.05$).

[x,y] Different superscripts indicate differences between status clusters ($P < 0.05$).

Milk yield was affected by the status cluster-feeding period interaction ($P < 0.001$, Table 3). Milk yield lowered similarly during the restriction in both status clusters (-18% and -17% for Balanced and Imbalanced cows, respectively). During refeeding, it increased again to the basal values for Imbalanced cows but did not fully recover for Balanced cows (-9%). Milk yield loss due to the restriction varied between -3% and -37% among cows. On average, Imbalanced cows had a numerically, but non significantly greater milk yield (7.0 vs. 7.8 kg/d in Balanced vs. Imbalanced cows, respectively, $P = 0.10$). In fact, when analyzed by day Imbalanced cows showed faster milk yield regain during the refeeding period (Figure 1).

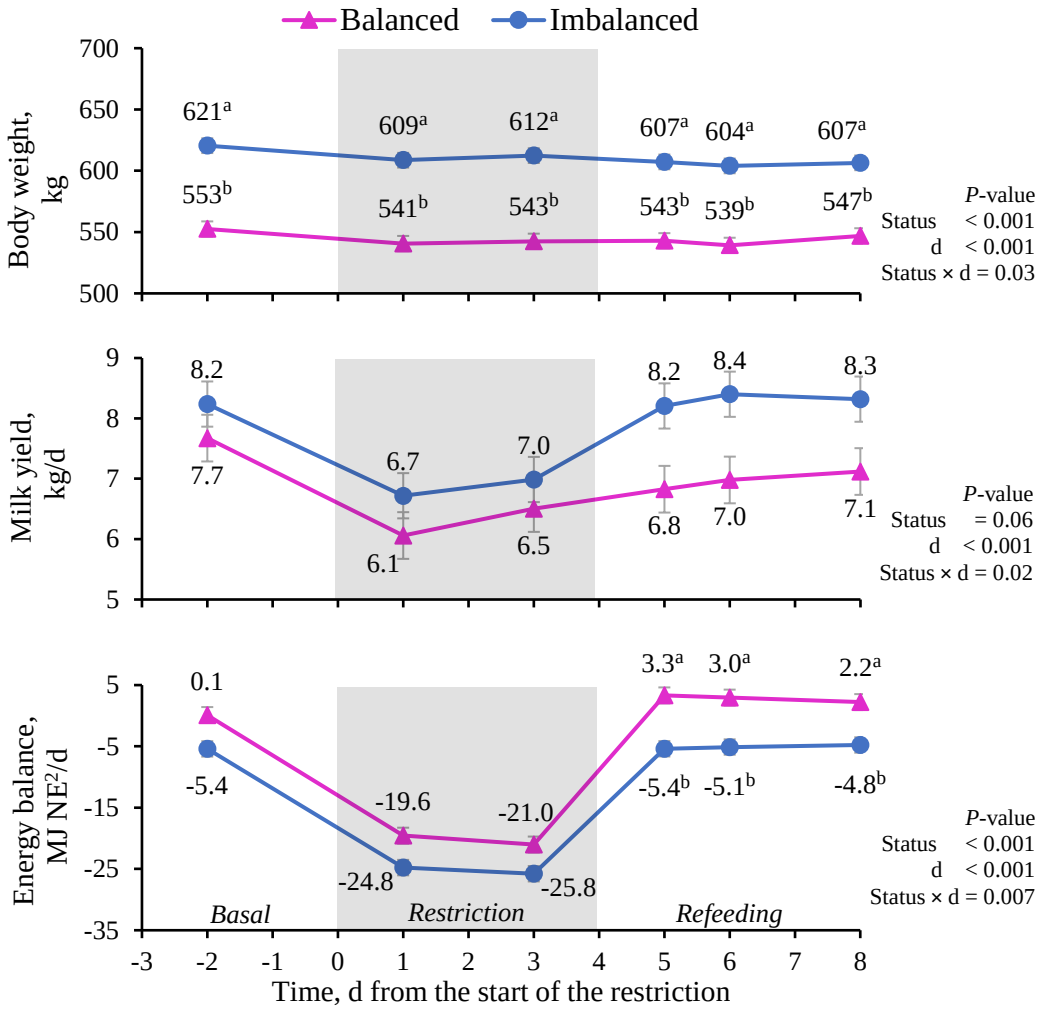


Figure 1. Effect of the status cluster1 and day (d) on beef cows' body weight, milk yield, and energy balance. The grey area represents the 4 d feed restriction at 55% of cows' energy and metabolisable protein requirements. Vertical bars indicate the standard error.

[1]according to the clustering analysis based on pre-challenge cow traits and energy status.[2] Net energy.[a, b] Within a day, different superscripts indicate differences between status clusters ($P < 0.05$).

Cow EB was affected by the status cluster and feeding period interaction (P < 0.001) because the difference between Balanced and Imbalanced cows was greater during the refeeding period than during the basal and restriction periods (Table 3). In both groups, EB was more negative during the restriction period than in the other periods (P < 0.001). This was confirmed when analyzed by day, where the differences between status clusters were only significant on d 5, 6, and 8 during the refeeding period (Figure. 1). Milk fat content only tended to be affected by the status cluster, with a lower content in Balanced than in Imbalanced cows (P = 0.09; Table 4). Milk protein and milk urea contents were affected only by feeding period (P < 0.001; Table 4). Milk protein content was lesser and milk urea content was greater during the restriction compared to the other periods (P < 0.001), which was corroborated by the negative correlation between milk urea and EB (Figure 2).

Table 4. Effect of the status cluster[1] and feeding period (FP) on beef cows' milk composition.

	Status cluster		FP				*P*-value[3]	
Item	Balanced	Imbalanced	Basal	Restriction	Refeeding	RSD[2]	Status	FP
Fat, g/100g	4.28	4.77	4.58	4.57	4.41	0.80	0.09	0.37
Protein, g/100g	2.91	2.91	2.93[a]	2.85[b]	2.95[a]	0.01	0.94	<0.001
Urea, mg/dL	22.8	24.5	22.7[b]	25.5[a]	22.8[b]	2.45	0.29	<0.001

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] Residual standard deviation.

[3] The interaction was never significant (P = 0.31 to 0.94).

[a,b] Different superscripts indicate differences among feeding periods (P < 0.05).

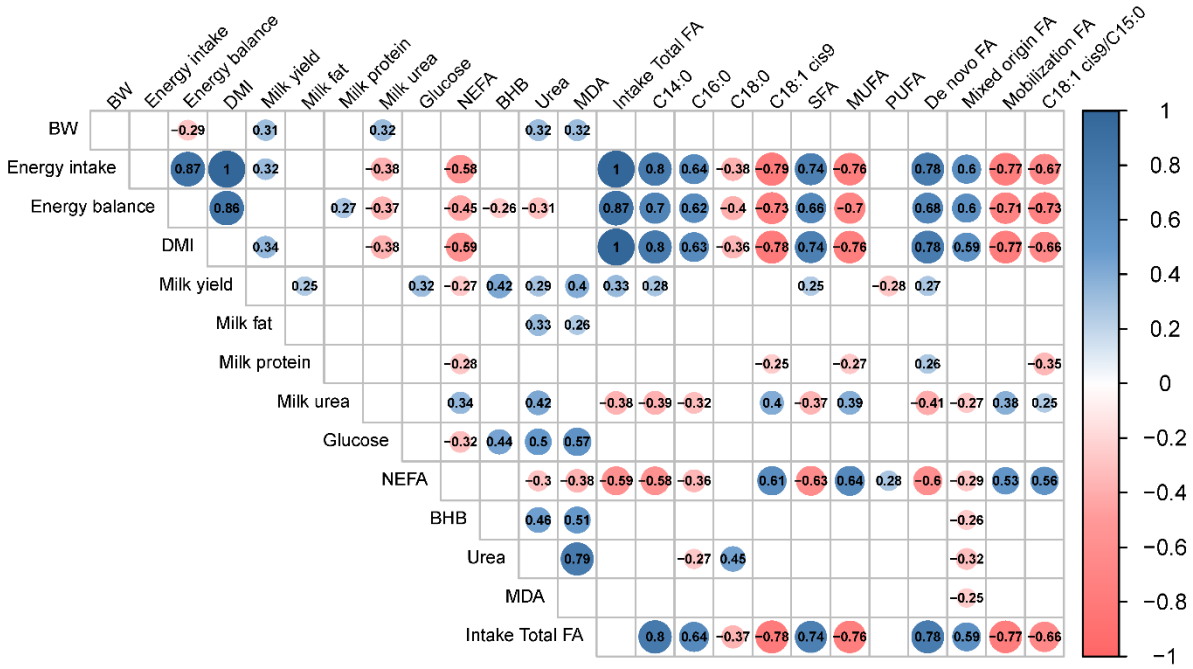


Figure 2. Significant Pearson's correlations ($P < 0.05$) among beef cow performance, metabolic profile variables and milk fatty acids (FA) composition.

BW: Body weight; DMI: dry matter intake; NEFA: nonesterified fatty acids; BHB: β-hydroxybutyrate; MDA: malondialdehyde; SFA: Saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA; *De novo* FA (C4:0 - C15:1), mixed origin FA (C16:0 - C16:1), and mobilization FA (≥ C17:0).

3.2. Blood metabolites

Plasma glucose concentration was affected only by feeding period ($P < 0.001$; Table 5). Glucose concentrations were similar during the basal and restriction periods, but rose during the refeeding period ($P < 0.001$). Plasma NEFA concentration was affected by feeding period ($P < 0.001$, Table 5), and increased during the restriction before decreasing during the refeeding period. When NEFA concentration was analyzed by day, an immediate response to diet changes was observed, with a rise after only 1 d on the restricted diet (d 1) and the basal values recovered after 1 d of refeeding (d 5) (Figure 3). Daily NEFA concentration in plasma correlated negatively with energy intake and EB ($P < 0.001$; Figure 2). Plasma BHB concentration was not affected by either the status cluster or the feeding period (Table 5). However, when analyzed by day, minor fluctuations in BHB concentrations occurred (Figure 3). Daily plasma BHB concentration weakly, but positively, correlated with both milk yield and glucose plasma concentration ($P < 0.001$; Figure 2).

Table 5. Effect of the status cluster[1] and feeding period (FP) on beef cows' plasma metabolite concentrations.

Item	Status cluster		FP			RSD[2]	*P*-value[3]	
	Balanced	Imbalance	Basal	Restriction	Refeeding		Status	FP
Glucose, mmol/L	2.18	2.31	2.10[b]	2.15[b]	2.48[a]	0.35	0.28	<0.001
NEFA[4], mmol/L	0.29	0.23	0.10[c]	0.49[a]	0.19[b]	0.17	0.33	<0.001
BHB[5], mmol/L	0.18	0.22	0.20	0.20	0.20	0.06	0.10	0.78
Urea, mmol/L	3.35	4.55	4.21[a]	4.08[a]	3.56[b]	0.84	0.03	<0.001
MDA[6], µmol/L	4.18	5.64	4.91	4.83	5.00	0.51	0.07	0.10

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] Residual standard deviation.

[3] The interaction was never significant (P = 0.08 to 0.92).

[4] Nonesterified fatty acids.

[5] β- hydroxybutyrate.

[6] Malondialdehyde.

[a,b,c] Different superscripts indicate differences between feeding periods (P < 0.05).

Plasma urea concentrations were affected by both the status cluster (P = 0.03), with lesser values in Balanced than in Imbalanced cows, and the feeding period (P < 0.001; Table 5), with lesser concentrations during refeeding than the other periods. When plasma urea was analyzed daily (Figure 3), it decreased from d 1 of the restriction to d 6 of refeeding, and then increased and reached the basal values by the end of the experiment (d 8). Plasma urea concentration positively correlated with milk urea and plasma glucose and BHB concentrations (P < 0.001; Figure 2).

Plasma MDA concentration tended to be affected by status cluster (P = 0.07; Table 5), and Balanced cows tended to have lesser concentrations than Imbalanced cows. Despite no clear differences being observed for feeding period, an increase in plasma MDA was observed by d 3 of the restriction as compared to previous basal values (P < 0.05) when analyzed by day (see Figure 3) and up to the start of the refeeding period (d 5 and 6). Basal values had recovered by the end of refeeding (d 8). Plasma MDA concentration positively correlated with glucose, BHB, and urea plasma concentrations (P < 0.001; Figure 2).

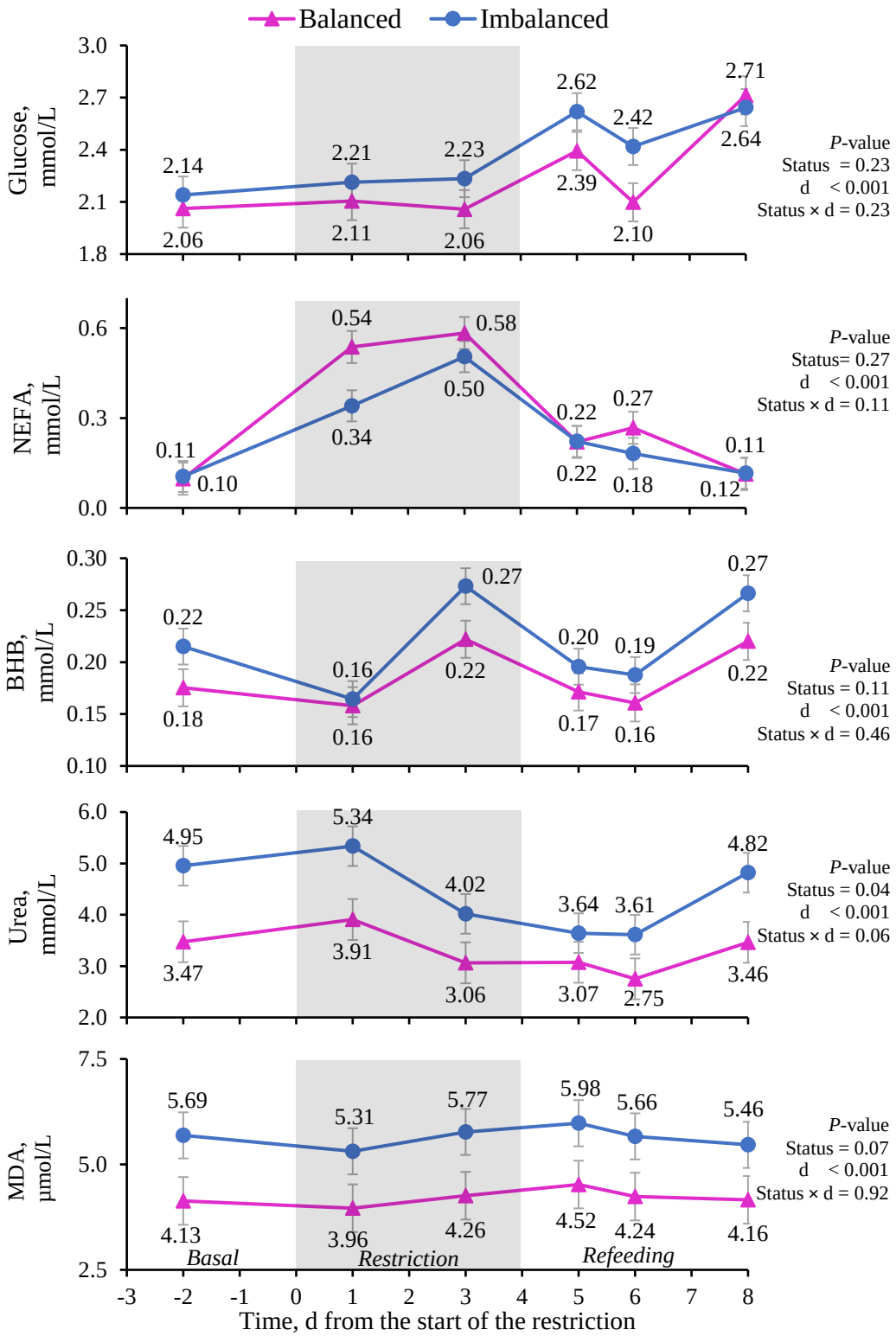


Figure 3. Effect of the status cluster1 and the day (d) on the plasma metabolites2 of the beef cows. The grey area represents the 4 d feed restriction at 55% of cows' energy and metabolisable protein requirements. Vertical bars indicate the standard error.

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.[2] NEFA: non-esterified fatty acids; BHB: β- hydroxybutyrate (BHB); MDA: malondialdehyde.

3.3. Diet fatty acids intake and milk fatty acids content

Diet FA intake were affected only by feeding period ($P < 0.001$), decreased during the restriction and increased to the basal intakes during refeeding (Table 6). Regarding the individual FA in milk, the status cluster tended to affect C16:0 ($P = 0.09$) and C18:1 cis-9 ($P = 0.002$), with greater concentrations in Imbalanced than in Balanced cows. All the major milk FA were affected by feeding period ($P < 0.001$). Restriction lowered the milk contents of C14:0 and C16:0 and increased those of C18:1 cis-9. During refeeding, C14:0 and C16:0 increased, while C18:0 and C18:1 cis-9 decreased. The time effect was confirmed when analyzing C14:0 and C16:0 on a daily basis. Feed restriction elicited an immediate response with nadir values on d 1 and 3, and then increased during refeeding. With C14:0, a status cluster and day interaction ($P = 0.01$) took place because of the slightly different recovery pattern noted during refeeding (Figure 4). The C18:1 cis-9 content increased steadily on d 1 and 3 of the restriction, and then decreased on the first day of refeeding (Figure 4). Milk contents of C14:0 and C16:0 positively correlated, whereas C18:1 cis-9 correlated negatively with EB ($P < 0.001$; Figure 2). Milk C14:0 correlated negatively and C18:1 cis-9 positively with NEFA plasma content ($P < 0.001$, Figure 2).

When FA were analyzed according to their degree of saturation, both SFA and MUFA were affected by the status cluster ($P < 0.05$) and the feeding period ($P < 0.001$), and PUFA only by feeding period ($P < 0.01$) (Table 6). The milk FA profile of Balanced cows had greater SFA and lesser MUFA contents than that Imbalanced cows, whereas PUFA contents were similar in both status clusters. During the restriction, SFA content lowered, while MUFA and PUFA rose ($P < 0.001$). During refeeding, SFA increased but did not reach the basal values, MUFA decreased to the basal values and PUFA remained unchanged. When analyzed by day, the SFA basal values had recovered by d 6 and after 2 d on the refeeding diet (Figure 5). For PUFA, a status cluster and day interaction was observed ($P = 0.01$, Figure 5) because Balanced cows had not regained the basal values by d 8, whereas Imbalanced cows had. Altogether, milk SFA contents correlated highly and positively with total diet FA intake and cow EB ($P < 0.001$; Figure 2), while negative correlations were observed between milk MUFA content and both parameters ($P < 0.001$). SFA negatively and MUFA positively correlated with NEFA plasma contents ($P < 0.001$).

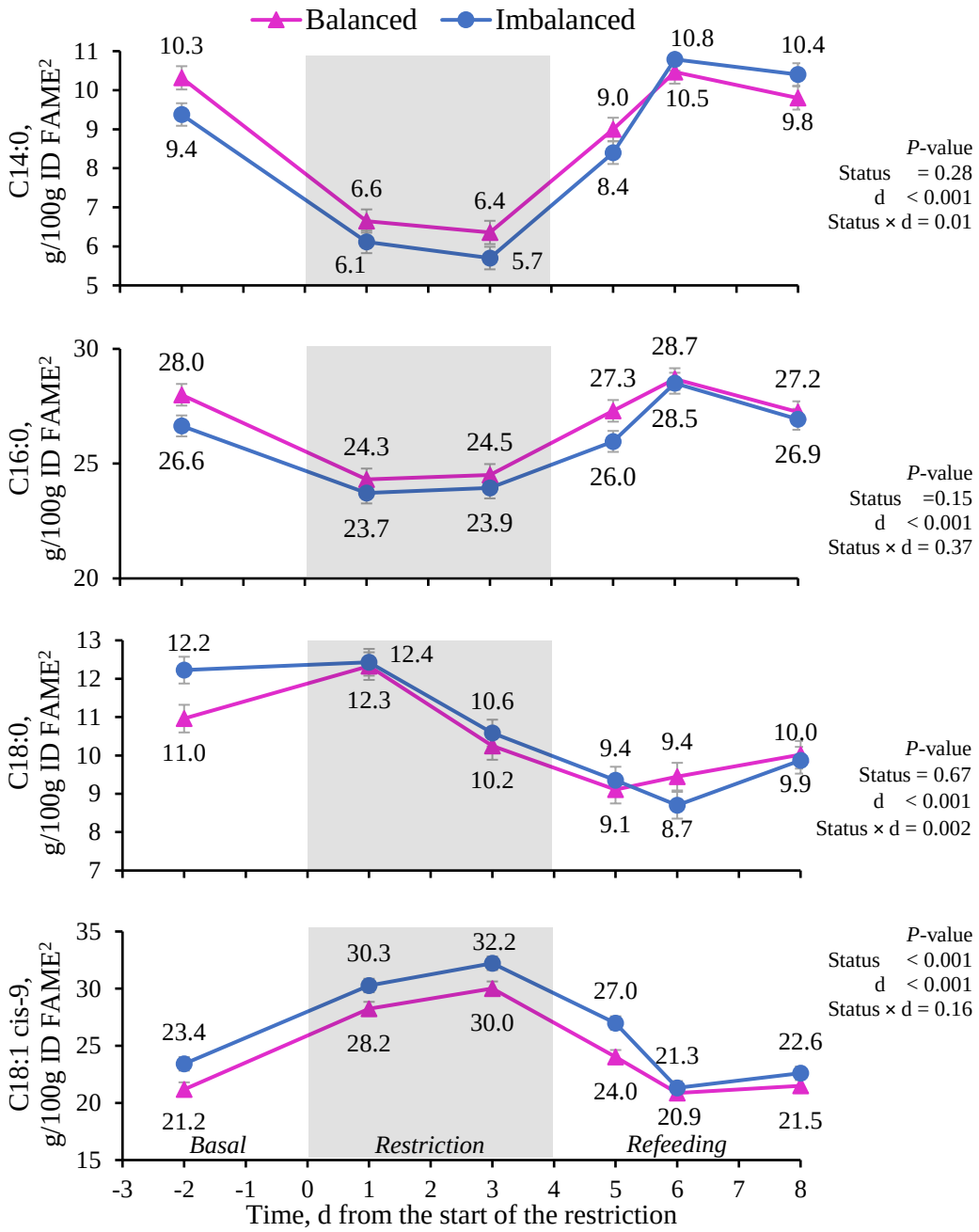


Figure 4. Effect of the status cluster1 and day (d) on beef cows' milk concentrations of individual milk fatty acids: C14:0, C16:0, C18:0, and C18:1 cis-9. The grey area represents the 4 d feed restriction at 55% of cows' energy and metabolisable protein requirements. Vertical bars indicate the standard error.

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] identified fatty acid methyl esters.

Table 6. Effect of the status cluster[1] and feeding period (FP) on beef cows' dietary intake of fatty acids (FA) and on the major FA in milk, FA according to their saturation and origin, and the C18:1 cis-9 to C15:0 ratio.

	Status cluster		FP				*P*-value[3]	
Item	Balanced	Imbalanced	Basal	Restriction	Refeeding	RSD[2]	Status	FP
Intake of dietary FA, g/d								
C16:0	64.3	64.1	77.2[a]	38.1[b]	77.2[a]	2.01	0.55	<0.001
C18:0	24.4	24.4	28.3[a]	16.6[b]	28.3[a]	0.56	0.74	<0.001
C18:1 cis-9	33.5	33.1	47.4[a]	5.2[b]	47.3[a]	2.47	0.34	<0.001
C18:2 n-6	72.4	71.6	98.9[a]	18.3[b]	98.8[a]	4.65	0.36	<0.001
C18:3 n-3	38.2	38.3	40.9[a]	33.0[b]	40.9[a]	0.19	0.12	<0.001
Total	248	247	312[a]	119[b]	312[a]	10.48	0.45	<0.001
Milk FA, g/100 g ID FAME [4]								
C14:0	8.9	8.4	9.8[a]	6.2[b]	9.8[a]	1.16	0.10	<0.001
C16:0	26.7	25.9	27.3[a]	24.1[b]	27.4[a]	1.49	0.09	<0.001
C18:0	10.6	11	11.6[a]	11.4[a]	9.4[b]	1.14	0.31	<0.001
C18:1 cis-9	24.1	26.1	22.3[b]	30.2[a]	22.9[b]	2.55	0.002	<0.001
FA according to saturation								
Saturated FA	61.9	60.3	64.7[a]	55.6[c]	63.0[b]	2.95	0.04	<0.001
Monounsaturated FA	32.9	34.6	30.8[b]	38.8[a]	31.7[b]	2.6	0.01	<0.001
Polyunsaturated FA	5.2	5.1	4.5[b]	5.6[a]	5.4[a]	0.66	0.46	<0.001
FA according to origin								
De novo (C4:0 to C15:1)	22.1	20.8	23.4[a]	16.8[b]	24.1[a]	2.41	0.04	<0.001
Mixed origin (C16:0 + C16:1)	29.1	28.2	29.5[a]	26.7[b]	29.8[a]	1.48	0.09	<0.001
Mobilization (≥ C17:0)	48.8	51.0	47.2[b]	56.5[a]	46.1[b]	3.52	0.02	<0.001
C18:1 cis-9 to C15:0 ratio	16.6	19.2	15.5[b]	21.7[a]	16.5[b]	2.18	0.001	<0.001

[1] according to the clustering analysis based on pre-challenge cow traits and energy status. [2] Residual standard deviation. [3] The interactions were not significant (P = 0.06 to 0.70). [4] identified fatty acid methyl esters. [a,b,c] Different superscripts indicate differences among feeding periods (P < 0.05).

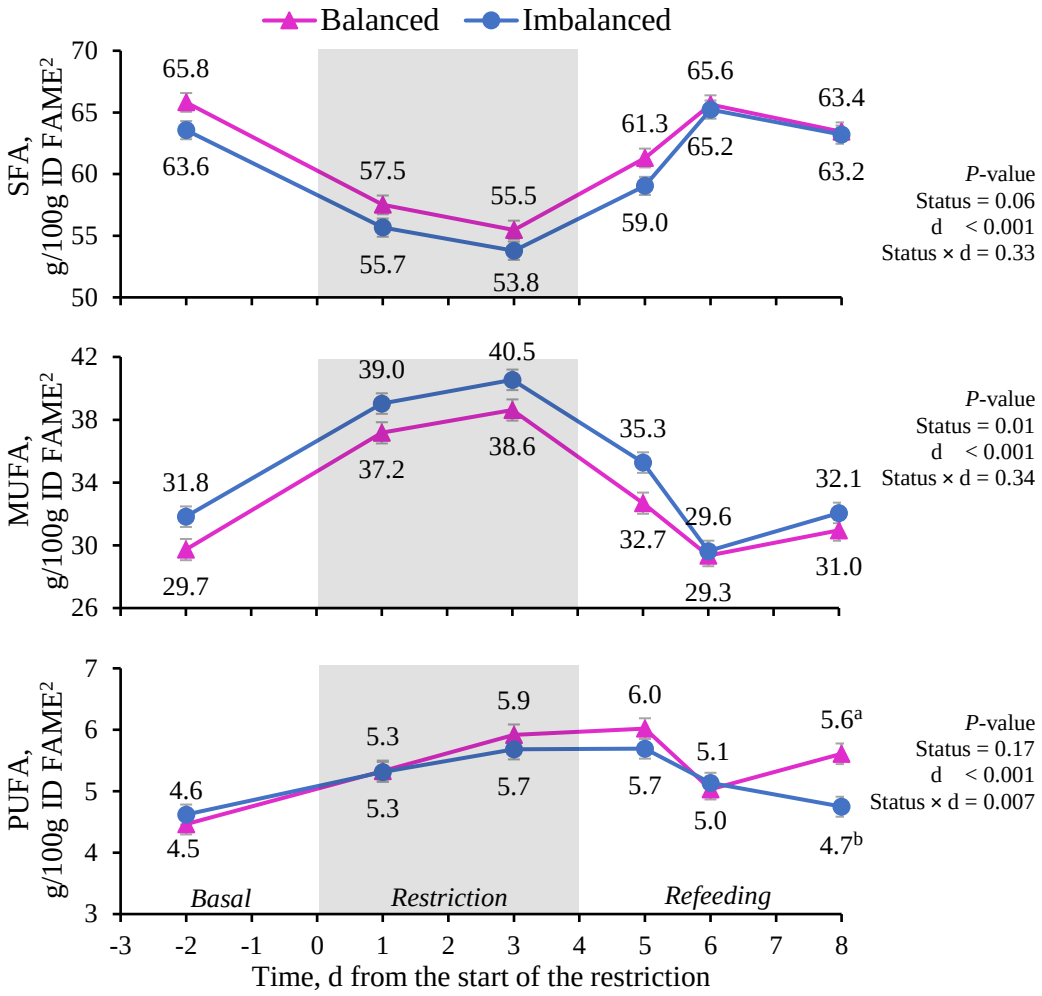


Figure 5. Effect of the status cluster1 and day (d) on beef cows' milk concentrations of grouped fatty acids (FA) according to their saturation: saturated FA (SFA), monounsaturated FA (MUFA), and polyunsaturated FA (PUFA). The grey area represents the 4 d feed restriction at 55% of cows' energy and metabolisable protein requirements. Vertical bars indicate the standard error.

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] identified fatty acid methyl esters.

[a, b] Within a day, different superscripts indicate differences between status clusters ($P < 0.05$).

Regarding the effect on the FA grouped according to their origin, the status cluster affected *de novo* (C4:0 - C15:1) and mobilization FA ($P < 0.05$), and tended to affect mixed origin FA (C16:0 - C16:1) ($P = 0.09$) with Balanced cows having greater *de novo* FA contents, slightly greater mixed origin FA and lesser mobilization FA than Imbalanced cows (Table 6). Feeding period affected the three FA groups ($P < 0.001$). *De novo* and mixed origin FA decreased, while mobilization FA increased during the restriction before returning to the basal values during refeeding. When analyzed by day,

an immediate effect was noted on *de novo* FA during the restriction in both status clusters, with low and constant values on d 1 and 3 (Figure 6). They thereafter increased during refeeding to the basal values on d 5 in both status clusters, but continued to rise even beyond the basal values on d 6 and 8 in Imbalanced cows. Similarly, the daily values of mixed origin FA lowered immediately with the restriction and increased from the start of refeeding irrespectively of the status cluster (Figure 6).

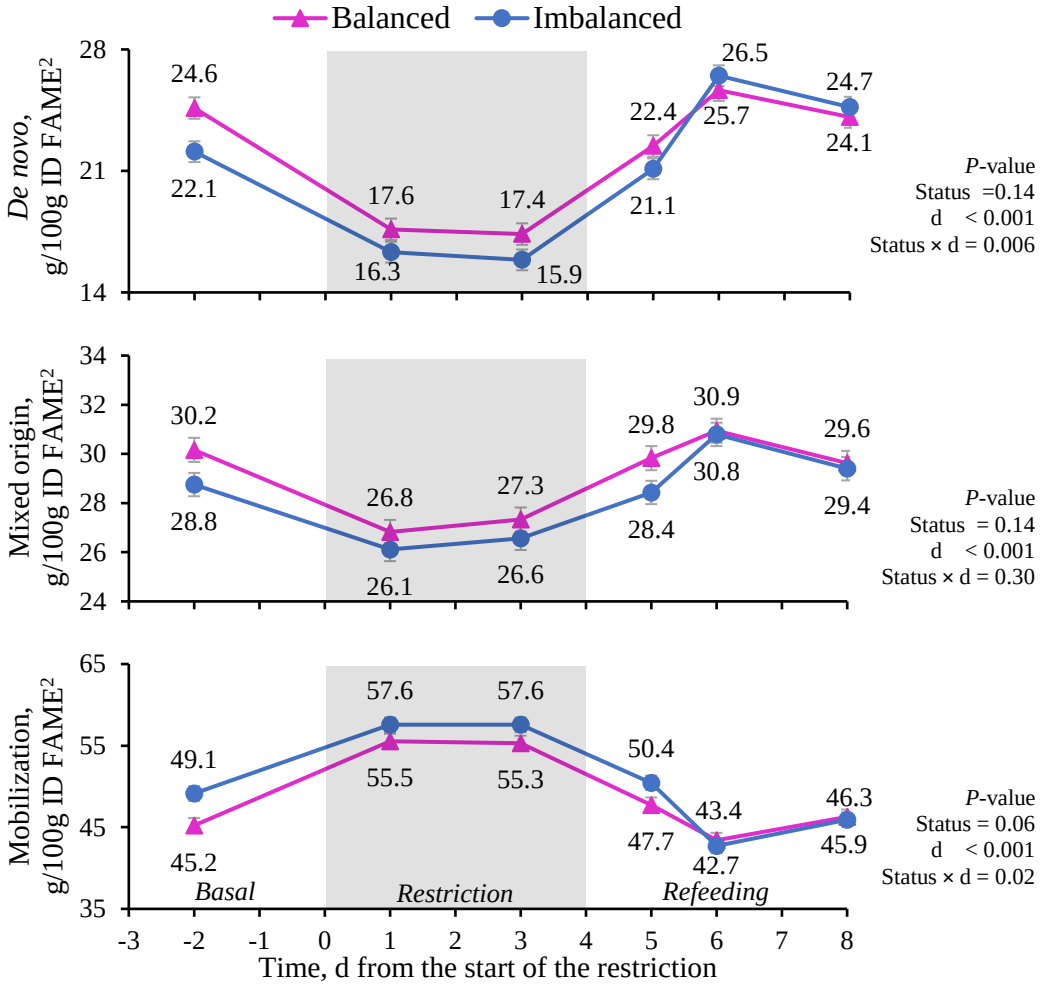


Figure 6. Effect of the status cluster1 and day (d) on beef cows' milk concentrations of grouped fatty acids (FA) according to their origin: De novo FA (C4:0 - C15:1), mixed origin FA (C16:0 - C16:1), and mobilization FA (≥ C17:0). The grey area represents the 4 d feed restriction at 55% of cows' energy and metabolisable protein requirements. Vertical bars indicate the standard error.

[1] according to the clustering analysis based on pre-challenge cow traits and energy status.

[2] identified fatty acid methyl esters.

Mobilization FA of both Balanced and Imbalanced cows sharply rose on the first day of restriction (d 1), decreased with refeeding below the basal values on d 6 and

returned to the baseline values on d 8 (Figure 6). Daily individual EB correlated highly and positively with milk contents of *de novo* and mixed origin FA ($P < 0.001$) (Figure 2), but negatively with mobilization FA ($P < 0.001$). *De novo* and mobilization FA obtained correlations of a different sign with NEFA plasma concentrations ($P < 0.001$).

The C18:1 cis-9 to C15:0 ratio was affected by the status cluster ($P = 0.001$), with greater values in Imbalanced cows than in their Balanced counterparts, and also by the feeding period ($P < 0.001$) with an increment during the restriction and a return to the basal values during the refeeding period (Table 6). This ratio correlated negatively with EB ($P < 0.001$) and positively with plasma NEFA concentrations ($P < 0.001$), but not with the other plasma metabolites (Figure 2).

4. Discussion

This study investigated the pattern of beef cows' adaptive responses in different energy statuses to a short, but intense, feed restriction, and subsequent refeeding. Their pre-challenge performance and energy status were established by retrospective cow classification according to their previous BW, milk yield, and EB. We obtained two distinct status clusters: Imbalanced cows were heavier, tended to have greater milk yields and a more negative EB, whereas Balanced cows fed the same diets were lighter, had lesser milk yields and a neutral EB. When subjected to nutrient restriction, and despite wide between-cow variability, most of the parameters that describe cows' performance, plasma metabolites, and milk composition were affected by time (feeding period or day). A less marked effect was observed for the status cluster (Balanced vs. Imbalanced cows).

4.1. Cow performance

According to the experimental design, DMI (64%) and both energy (55%) and protein (53%) intake lowered during the restriction period, which resulted in lighter BW (-2%), lower milk yield (-17%), and less milk protein content (-3%) compared to the basal values. Milk fat content did not change, and milk urea content increased (+13%). The BW loss could be a consequence of the reduced DMI and the concomitant loss of gut fill, together with the mobilization of body reserves in response to the restriction (Gross et al., 2011a). This mobilization was probably larger for Imbalanced cows, which were heavier and had a lower EB throughout the study, which allowed them to cope with the metabolic challenge, but resulted in net BW loss at the end of the refeeding period.

The diminished milk yield during the restriction was associated with reduced energy supply, as observed in other studies. The -17% reduction herein observed for

beef cows after a 4 d restriction at 55% of their requirements was similar to the -19% to -20% reduction after a 4-5 d restriction at 50-60% of previous intake for dairy cows (Abdelatty et al., 2017; Carlson et al., 2006). A greater (-30%) reduction was observed when dairy cows were restricted more intensely (48% of their requirements) for 4 d (Bjerre-Harpøth et al., 2012). In beef cattle, Charolais cows had -12% milk loss under a similar restriction condition, which was probably related to a less negative physiological imbalance (De La Torre et al., 2022). As observed here, all the aforementioned studies report a wide variation in cows' individual adaptive ability to counterbalance the feed restriction, which Orquera-Arguero et al. (2022) associated to the cows milk yield potential and capacity of mobilization of fat reserves.

Despite the fact that the basal milk yield did not differ between status clusters, it was not only numerically greater in Imbalanced cows, as observed by De Koster et al. (2019) in two groups of cows clustered according to their metabolic profiles, but also recovered more quickly when refeeding started. According to Baumgard et al. (2017), milk yield would be a major driver of the different partition of nutrients toward milk production or fat reserves in cows and would, therefore, condition their response to feed restriction. The slower recovery observed in Balanced cows resulted in their EB being even better during refeeding than during the basal period because energy intake exceeded their requirements for a numerically lesser milk yield. When analyzed by day, the basal values had recovered in both status clusters by the end of the refeeding period. This finding agrees with other studies in beef (De La Torre et al., 2022) and dairy (Bjerre-Harpøth et al., 2012; Gross et al., 2011a) cows, which reflects the plasticity of the cow response to a short nutritional challenge.

Several studies report greater milk fat content associated with a negative EB and body fat mobilization (Agenäs et al., 2003; Kessel et al., 2008), whereas others report no difference between cows with different fat mobilization intensities (Schuh et al., 2019). In the present study, no changes were observed in response to a short feed restriction, which agrees with the results of Carlson et al. (2006), who worked with dairy cows under similar conditions, although they also found increased plasma indicators of lipolysis (NEFA and BHB). As pointed out by Schuh et al. (2019), the fact that milk fat did not mirror the increase in circulating NEFA could be explained by them being partly diverted to other tissues to be used as an energy substrate rather than to the mammary gland to be converted into milk FA. Milk fat content tended to be greater in Imbalanced cows, which agrees with the observations made by Stoop et al. (2009) when comparing cows with different EB, which could reflect a longer term difference in the nutritional status of cows with different BW and milk yields fed at a flat rate since lactation onset.

The immediate milk protein content reduction during the restriction period observed in similar studies with dairy cows (Billa et al., 2020; Gross et al., 2011a) can be ascribed to reduced dietary energy and protein intake, which compromise both microbial protein synthesis and by-pass protein flux to the intestine. Similarly, Bjerre-Harpøth et al. (2012) confirmed that milk protein content lowered during the restriction and returned to the prerestriction content during refeeding regardless of the lactation stage. The rise in milk urea contents during feed restriction agrees with the observations made by Broderick (2003), who described that when dietary energy lowers, milk yields, and milk protein contents decrease, while milk urea increases, in response to the lower amino acid requirements for lesser milk secretion (Bittante, 2022).

4.2. Blood metabolites

In the present experiment, the metabolites associated with energy metabolism and oxidative status were not affected by the status cluster, except for greater plasma urea concentration in Imbalanced cows. Glucose, NEFA, and urea immediately responded to diet changes, while a delayed response was noted for BHB and MDA. Plasma glucose concentration strongly depended on the current energy and protein intake at a given time, and also on diet composition. They were all similar for both status clusters and, thus, their glucose concentration did not differ. Plasma glucose did not change during the restriction, although it was expected to decrease as a consequence of lower feed and energy intake. This lack of response could be due to the lower gluconeogenesis associated with lower ruminal propionic acid production (Kessel et al., 2008) caused by the lower proportion of concentrate in the restriction diet. However, circulating glucose also depends on uptake by mammary glands for milk lactose production, as observed in other studies (Agenäs et al., 2003; Carlson et al., 2006). The increment that occurred in the refeeding phase agrees with the observations made by Bjerre-Harpøth et al. (2012), for whom glucose also peaked at the start of refeeding due to metabolic readjustment.

An increase in circulating NEFA concentration is an indicator of adipose tissue catabolism in response to a negative EB to supply FA, which can be converted into milk triglycerides in the mammary gland or oxidized in the liver as an energy substrate (Bell, 1995). In the current study, NEFA did not differ among cows in both status clusters, probably because the actual difference in EB between them was too narrow to elicit a response. However, they responded immediately to the large differences in energy intake among feeding periods, with which they correlated. A critical threshold of 0.57 mmol NEFA/L was set by Ospina et al. (2010) as an early postpartum indicator of

increased risk of clinical ketosis in dairy cows, which was only just reached by Balanced cows on d 3 in our experiment.

Excessive NEFA mobilization can impair the liver's metabolic capacity to completely oxidize them, which results in the production of ketone bodies, such as BHB, acetoacetate, and acetone (Jorjong et al., 2015; Mann et al., 2016). In our experiment, the tendency of a greater BHB concentration for Imbalanced than Balanced cows, plus the positive correlation between BHB and milk yield, suggest increased NEFA oxidation to provide energy substrates for milk production (Wathes et al., 2007). The BHB concentrations did not differ among feeding periods, as observed in dairy and beef cows at mid-lactation with a similar feed restriction period lasting 4 d (Bjerre-Harpøth et al., 2012; Carlson et al., 2006; De La Torre et al., 2022). These results imply that NEFA mobilization did not exceed the liver's metabolizing capacity and provided sufficient energy supply for nutrient-restricted cows. However, a peak occurred at the end of the restriction phase, with a delayed response to energy intake compared to NEFA, as observed by Gross et al. (2011a) in dairy cows at mid-lactation. The extent of this delay can be influenced by the lactation stage and restriction duration (Carlson et al., 2006; Orquera-Arguero et al., 2022). Apparently, feed restriction length did not suffice here to have a prolonged effect on BHB. Plasma BHB can be used as an indirect marker of a negative EB in dairy cows, but has been shown to be a poor indicator in beef cattle (De La Torre et al., 2022; Orquera-Arguero et al., 2022), as observed here. Hyperketonemia, defined when BHB exceeds a critical threshold of 1.2 mmol/L, is associated with increased risk of disease, milk yield losses, and impaired reproductive performance in dairy cows (Jorjong et al., 2015). In our study, both NEFA and BHB concentrations were below the above-mentioned thresholds because our beef cows had a less severe negative EB due to their lower milk yields.

Lack of differences in these metabolites between status clusters was not expected. De Koster et al. (2019) observed that plasma glucose was greater and NEFA and BHB were lesser in balanced than in imbalanced dairy cows. Vossebeld et al. (2022)clustered cows according to their postpartum EB profile. They found that those with a more negative EB had greater plasma NEFA and BHB concentrations. However, differences in EB between the dairy cow groups in both studies, and associated with their different DMI, BW, and milk yield, were much larger than those herein recorded. Our similar results for both cow groups in different EB could be partly ascribed to wide individual variation in cows' metabolic adaptive capacity, as pointed out by Kessel et al. (2008), or to the lower milk yield and associated metabolic load in beef cows.

Circulating urea in lactating ruminants originates from either dietary protein intake or the catabolism of body protein reserves when energy intake is restricted and the AA stored in skeletal muscle are mobilized (Bell, 1995). Given their similar protein intake, the greater plasma urea concentrations in Imbalanced cows indicate greater body protein turnover to support gluconeogenesis and to cope with their more negative EB. These differences observed in plasma were probably not large enough to be reflected in milk urea contents, despite them being significantly correlated, as observed by Kessler et al. (2020). The minor differences among d, which decreased at the end of the restriction and had risen by the end of the refeeding period, showed a delayed response to diet changes, which falls in line with Bjerre-Harpøth et al. (2012).

Oxidative stress occurs during periods of high metabolic demand, when the production of free oxidant radicals cannot be counteracted by the natural anti-oxidant system. Castillo et al. (2006) found increased lipid peroxidation only at very early postpartum, with wide individual variation. Bernabucci et al. (2005) reported that dairy cows with greater BCS loss, and greater BHB and NEFA concentrations, also had greater concentration of reactive oxygen metabolites, which agrees with Schuh et al. (2019), plus lesser concentrations of antioxidants. In our study, Imbalanced cows tended to have greater MDA concentrations, which mirrored the trend observed for BHB concentrations. This finding also reflects fat mobilization and oxidation, and is associated with hepatic stress. This positive correlation between MDA and BHB agrees with those observed by Li et al. (2016) in dairy cows, who also report a positive association with NEFA, but it was not observed in our experiment. This supports the lack of differences in oxidative status among feeding periods, where the increased NEFA and the decreased milk yield allowed cows to cope with metabolic stress without further lipid oxidation. In line with our results, Urh et al. (2019) found that diets that included different amounts of concentrate affected NEFA concentrations, but neither BHB nor the oxidative status of dairy cows, which they associated with relatively small differences in cows' energy intake, as we observed here with a flat-rate feeding regime.

4.3. Diet fatty acids intake and milk fatty acids content

The total FA intake decreased by -62% due to the restriction, whereas the extent of the decrease in individual FA intake varied, with a greater reduction (-81% to -89%) for those that were more abundant in the concentrate (C18:2 n-6 and C18:1 cis-9) than for those that were predominant in hay (C16:0 and C18:0). These differences in relative individual FA intake reflected both the reduction in DMI and the change in diet among periods. Diet composition affects the milk FA profile because short- and medium-chain

milk FA derive from *de novo* synthesis from acetate and the transformations of butyrate that occur during the ruminal fermentation of carbohydrates (Bauman and Griinari, 2003), both of which increase when the forage proportion in diet increases. However, the milk FA profile does not exactly mirror the relative intake of the different FA because they can be modified by ruminal biohydrogenation and mammary lipogenic and Δ-9 desaturation pathways (Chilliard et al., 2007).

Research into the relation between energy intake and EB with the milk FA profile is extensive in dairy cows, but literature on milk FA composition of beef cows is scarce. To the best of our knowledge, this is the first study to report changes in beef cows' milk FA contents in response to feed restriction. As in the case of milk yield and circulating metabolites, the response patterns of milk FA in beef cows follow the trends observed in dairy cows although the changes are of a lesser magnitude. Here we observed that energy status had a marked effect in both the long (differences between status clusters, e.g. C14:0 and C16:0 tended to be greater and C18:1 cis-9 lesser in Balanced vs. Imbalanced cows) and short terms (differences among feeding periods, e.g., lowest C14:0 and C16:0 and highest C18:1 cis-9 during the restriction) on milk contents of major FA and different FA proportions according to both their degree of saturation and origin. When a negative EB induces body fat mobilization, the major FA in subcutaneous and abdominal depots (C16:0, C18:0, and C18:1 cis-9) are released to plasma, where they constitute a high proportion of circulating NEFA, and where C18:1 cis-9 is the most abundant FA in both dairy (Hostens et al., 2012) and beef (Lake et al., 2007) cows. These NEFA are taken up by the mammary gland and directly used for milk fat synthesis (Bauman and Griinari, 2003). Consequently, their relative proportions in milk fat should reflect this lipid mobilization in response to EB. Furthermore, when these long-chain FA are released into plasma, *de novo* synthesis of short-chain FA by the mammary gland is inhibited (Chilliard et al., 2007). Gross et al. (2011b) described how the milk FA profile responds quickly to dietary energy changes, with significant reductions in most FA of ≤ C16:0 and increments of preformed FA of > C16:0 within 1 week of feed restriction, and the basal values recover within 1 week of refeeding. This pattern was confirmed in our experiment, even on the first day after diet change. As we noted, C14:0 milk contents were positively associated with EB, and increased with improved energy status with advancing dairy cows' lactation (Craninx et al., 2008). On C16:0, literature offers conflicting results, which are explained by its mixed origin (Chilliard et al., 2000b). C16:0 contents increased with either a negative EB (Stoop et al., 2009) or feed restriction (Abdelatty et al., 2017), but the decrease herein observed during the restriction period agrees with the patterns reported by Gross et al. (2011b) and Billa et al. (2020), which

suggests that despite its mixed origin, here it reflects the reduced *de novo* FA synthesis. Regarding long-chain FA, milk C18:0 did not increase during the restriction, unlike previous reports (Billa et al., 2020; Gross et al., 2011b), but decreased with refeeding as a result of less fat mobilization, which agrees with the aforementioned studies. Finally, milk oleic acid contents (C18:1 cis-9) have been associated with a negative EB and high plasma NEFA concentrations (Dórea et al., 2017; Jorjong et al., 2014; Stoop et al., 2009), which agrees with our results. It has even been proposed as an early predictor of subclinical ketosis in dairy cows (Van Haelst et al., 2008), and as a better indicator of a negative EB than actual plasma NEFA and BHB concentrations (Churakov et al., 2021), which can vary diurnally depending on the time that elapses between feeding and blood sampling (Mäntysaari et al., 2019). This was confirmed herein by the stronger correlation of EB with milk C18:1 cis-9 contents than with these plasma metabolites. This relation also explains the greater milk contents of C18:1 cis-9 in Imbalanced cows, and the rise that occurred during the restriction period in association with a more negative EB in both cases.

According to their degree of saturation, the differences between status clusters and feeding periods followed the differences in major FA and in other less abundant ones. During the feed restriction, SFA decreased by -14% whereas MUFA and PUFA increased by +26% and +24%, respectively. This agrees with the results of Gross et al. (2011b) except for their stable PUFA concentrations, but contrasts with those of Stoop et al. (2009), who found greater proportions of SFA, mainly C16:0 and C18:0 from body fat, in those cows with a greater energy imbalance. The reduction in SFA during the restriction and the lesser concentration in SFA in Balanced cows in our study seemed to be driven by the predominant behavior of C16:0 as a *de novo* synthesized FA, and also by the minimal response of C18:0 to EB, as observed by Abdelatty et al. (2017). Regarding the origin of milk FA, Grummer (1991) suggests that almost all the C4:0 to C14:0, and about half the C16:0 in milk, are synthesized *de novo* in the mammary gland, whereas the rest of the C16:0 and all long-chain FA derive from mammary uptake of circulating triacylglycerol and NEFA. Unless diet composition significantly varies (Khiaosa-ard et al., 2020), the relative proportions of *de novo* synthesized and preformed FA mainly reflect changes in the EB (Gross et al., 2011b). Accordingly in our study, milk *de novo* FA content was significantly greater and that of mobilization FA was lesser in Balanced vs. Imbalanced cows. In dairy cows that underwent a 6 d 50% energy restriction, Billa et al. (2020) reported that milk contents C10:0 to C15:0 decreased by -37%, and those of C16:0 by -23%, while FA > C16:0 rose by almost +60%, and basal contents were recovered within a week of refeeding. Here with a similar but shorter feed

restriction in beef cows, the relative changes were less intense, i.e. both *de novo* and mixed origin FA decreased (by -28% and -10%), while mobilization FA increased by +20%, and the basal values were also regained during the refeeding period in response to the improved EB. These changes are consistent with the strong correlations of the FA of different origins with EB and NEFA contents, as also described by Khiaosa-ard et al. (2020), who also found correlations with BHB contents that were not herein observed.

Several ratios between milk FA of different origins (mostly long-chain vs. short- and medium-chain FA or linear and branched FA) have been proposed as indicators related to cow diet or energy status (Craninx et al., 2008; Dórea et al., 2017). Of them, Jorjong et al. (2015) established that the C18:1 cis-9 to C15:0 ratio was the most discriminating factor for early hyperketonemia diagnosis (BHB ≥ 1.2 mmol/L), for which they proposed a threshold of between 34 and 45. Dórea et al. (2017) indicated that it could also be used to accurately predict plasma NEFA and that when this ratio exceeded 62, the cows would be at risk of developing metabolic disorders. In our experiment, the C18:1 cis-9 to C15:0 ratio differed between the status clusters and feeding periods by following the differences observed in EB and plasma NEFA contents, with which it correlated, and could therefore be used as a biomarker of the energy status of cows. However, our values were far from the above-mentioned thresholds described for dairy cows.

5. Conclusions

A short-term feed restriction and refeeding induced a transient negative EB in beef cows, to which they responded with lower milk yield and changes in plasma metabolites and milk composition, which are associated with the mobilization of body reserves. Despite some of these traits differing between Balanced and Imbalanced cows, with different BW, milk yields and EB before the challenge, they responded similarly to dietary changes by showing a consistent pattern across several individual nutritional statuses. The milk FA profile, which has been rarely studied in beef cows for practical purposes, also differed between Balanced and Imbalanced cows. In particular, the milk C18:1 cis-9 to C15:0 ratio proved to be an accurate indicator of metabolic status, which supports its use in experimental models.

V. Consideraciones Finales

Las vacas manejadas en sistemas extensivos se ven sometidas durante su ciclo productivo a periodos de restricción-realimentación, cuya frecuencia se puede ver incrementada según las previsiones relacionadas con el cambio climático. En este contexto, la presente tesis doctoral ha abordado los efectos de una restricción nutricional de 4 días de duración, al 55% de los requerimientos de energía y proteína, en diferentes momentos de la lactación y también de forma consecutiva en vacas de raza Parda de Montaña. Se han estudiado los mecanismos de respuesta que se desencadenan, para profundizar en el conocimiento sobre la resiliencia y la habituación ante las perturbaciones.

5.1 Modelizado de curvas de respuesta y análisis de agrupamiento

El estudio de la resiliencia de las vacas se realizó mediante la modelización de las **curvas de respuesta** para cuantificar la diferencia entre la curva potencial y la observada durante los retos alimenticios en diferentes momentos de la lactación. El modelizado de curvas se ha utilizado para estudiar la producción lechera especialmente en el vacuno de leche (Adriaens et al., 2021; Wood, 1967) y en menor medida en vacuno de carne (Espasandin et al., 2016; Iewdiukow et al., 2020). La respuesta a una perturbación se expresa a través de múltiples parámetros, por lo que utilizar un enfoque multivariante resulta interesante para caracterizar mejor la resiliencia (Ben Abdelkrim et al., 2023). Por ello, realizamos un análisis multivariante (análisis de componentes principales), que permitió identificar aquellas variables de carácter productivo y metabólico que explicaban la mayor parte de la variabilidad de respuesta, para luego aplicar un **análisis de agrupamiento** de animales con patrones similares de respuesta ante la restricción. En nuestro estudio, dichas variables fueron la producción de leche y la concentración plasmática de NEFA y BHB. En otros estudios realizados con vacuno de leche se seleccionaron para diferenciar los grupos: la concentración plasmática de glucosa, NEFA, BHB, insulina e IGF-1 (de Koster et al., 2019; Heirbaut et al., 2022) o de NEFA, BHB y la relación grasa:proteína de la leche (Tremblay et al. (2018). Nuestro enfoque de realizar el agrupamiento en función de la producción de leche y metabolitos sanguíneos es más viable en vacuno de carne ya que no es tan sencillo obtener muestras de leche como en vacuno de leche.

Respecto al agrupamiento de las vacas a partir de las variables de las curvas de respuesta, las diferencias entre grupos se dieron principalmente en las variables de magnitud, siendo las variables de tiempo más similares. Las diferencias se asociaron principalmente al periodo de restricción (línea base, pico y área bajo la curva durante la restricción), tanto en la producción de leche como en la concentración plasmática de

NEFA, pero de manera menos evidente en la de BHB. Además, cabe recalcar que las vacas asignadas a cada clúster en el capítulo I y capítulo III son coincidentes en la mayoría de los casos. Solamente 4 animales de los 31 utilizados en el estudio cambiaron de grupo; el 87% de las vacas se clasificaron en el mismo grupo de acuerdo con su respuesta metabólica (alta vs. baja). Esto indica que de forma general los animales responden de manera similar independiente de si se trata de un solo reto en distintos meses de lactación o si se enfrentan a tres retos consecutivos. Estos hallazgos mostrarían que, al igual que en vacuno de leche, en vacuno de carne también existe un componente genético en la respuesta adaptativa, que conlleva una partición más o menos similar de los nutrientes hacia las diferentes funciones (Friggens y Newbold, 2007). Por otro lado, en el capítulo IV se utilizó un enfoque diferente, ya que las vacas se agruparon en función de semejanza en su estado nutricional previo al inicio del ensayo (en equilibrio vs. en desequilibrio). Para este capítulo se usaron datos solamente del mes 3 (58 días postparto), y su clasificación se realizó a partir de los datos de peso vivo y CC al parto y de peso vivo, CC, producción lechera y BE a los 30 y 31 días postparto. Dado que los parámetros para clasificar a las vacas fueron diferentes de los usados en el análisis de agrupamiento anterior, esta clasificación de vacas no coincidió con la anterior habiendo 6 vacas en equilibrio y 9 en desequilibrio en el grupo de vacas de alta respuesta metabólica y 9 vacas en equilibrio y 6 vacas en desequilibrio en el de baja respuesta metabólica.

Agrupar animales que presentan una respuesta metabólica similar podría ser interesante para tomar ciertas decisiones de manejo a nivel de explotación. Las vacas clasificadas como de alta respuesta metabólica podrían considerarse más resilientes ya que, a pesar de las variaciones observadas, fueron capaces de recuperarse de la perturbación en menor tiempo cuando el suministro de la dieta se restauró y volvió a sus niveles habituales. Para Elgersma et al. (2018) las vacas lecheras con menos fluctuaciones en el rendimiento lechero bajo perturbaciones naturales son más resilientes, porque la menor varianza en el rendimiento se correlaciona genéticamente con una mejor salud y longevidad.

5.2 Efecto de una restricción-realimentación sobre los parámetros productivos

Los periodos de **restricción-realimentación** aplicados en este experimento y el **momento de la lactación** afectaron en diferente magnitud a los parámetros de producción analizados. El **peso vivo** de las vacas sufrió una ligera una disminución a causa de la restricción, independientemente de si se trató de retos en diferentes puntos de la lactación o retos consecutivos, asociada a la pérdida de contenido digestivo. La

medición de la CC como indicador de la movilización de reservas grasas no resultó ser un buen indicador a corto plazo, ya que no mostró cambios significativos durante los periodos de estudio. Tampoco lo fue el espesor de grasa subcutánea estimada mediante ultrasonidos al inicio de cada periodo en los distintos retos (Orquera et al., 2020), aunque ambos métodos permitieron detectar diferencias en el largo plazo, entre los meses de lactación.

La **producción de leche** fue uno de los parámetros más afectados por la restricción nutricional, independientemente de que el reto ocurriera en diferentes meses de la lactación o fueran retos consecutivos. Sin embargo, hubo un amplio rango de variación en los porcentajes de caída de producción de leche durante los periodos de restricción (desde -1% hasta -66%). Esta amplia la variabilidad individual en la respuesta a la restricción se asocia a diferencias en la partición de nutrientes (Baumgard et al., 2017), condicionadas por un componente genético (Friggens y Newbold, 2007). Adicionalmente, sería interesante medir la repetibilidad individual de la respuesta entre los meses, es decir, si con el avance de la lactación los animales presentan la misma respuesta. En caso de ser así, la resiliencia ante las perturbaciones sería un carácter con una marcada base genética, por lo que la intensidad y repetibilidad de la respuesta podría utilizarse como base para la selección de los animales más resilientes.

Los estudios de los capítulos I y III mostraron las diferencias en la producción de leche de las vacas clasificadas como de alta y baja respuesta metabólica, así como el efecto de los diversos retos. En ambos estudios, el análisis de curvas de respuesta en la leche mostró que las vacas de alta respuesta metabólica tuvieron mayor producción inicial y una caída durante la restricción fue más abrupta. Este efecto se observó tanto en los retos en diferentes meses de la lactación como en los retos consecutivos. La caída en la producción de leche se vio afectada por el mes de lactación, con mayores pérdidas porcentuales (-20%) en el mes 3 y 4 de la lactación (58 y 87 días postparto) que en el mes 2. Por otro lado, en los retos consecutivos la leche disminuyó en mayor proporción en el segundo y tercer reto consecutivo (-26%) que en el primero. Bjerre-Harpøth et al. (2012) indicaron en vacuno de leche que la producción previa a la restricción, y no tanto la fase de la lactación, sería un factor predictivo importante de la caída en el rendimiento lechero por causa de la restricción. Es decir, que los animales con mayor producción inicial activarían mecanismos adaptativos más intensos para mantener su producción. Nuestros resultados demostraron que en vacuno de carne tanto el estado de lactación como la producción de leche previa al reto fueron determinantes para determinar el ajuste en la partición de nutrientes y tratar de mantener la producción de leche.

Dado que la resiliencia se define como la capacidad de recuperarse tras una perturbación, en este trabajo se evaluaron los rendimientos cuando los animales volvieron a su dieta normal tras la restricción. En los retos en diferentes meses de lactación, el tiempo propuesto de recuperación (4 días) fue suficiente para que los animales pudieran regresar a su producción de leche previa. En los retos consecutivos, las vacas de alta respuesta metabólica tuvieron una respuesta más rápida a los cambios en la dieta que las vacas de baja repuesta, que necesitaron un día adicional para recuperar los rendimientos. En vacuno de carne De La Torre et al. (2022) observaron que tras 4 días de restricción al 50%, la producción de leche inicial se recuperaba en dos días, pero indicaban que el tiempo de recuperación podría ser mayor con restricciones más largas.

La respuesta de la producción de leche al reto presentó características elásticas, ya que la deformación debido a la restricción fue reversible y se recuperó la producción inicial tanto en los retos en diferentes meses de la lactación como en los retos consecutivos. Adicionalmente, observamos que no hubo una habituación en este parámetro a los retos consecutivos, ya que provocaron una disminución de la producción de leche que empeoró tras el primer reto y se mantuvo posteriormente.

En cuanto a los principales **componentes de la leche,** el contenido de grasa no se vio afectado por los retos alimenticios en diferentes meses o en retos consecutivos, contrariamente a lo descrito en vacuno de leche (Bjerre-Harpøth et al., 2012; Rico y Razzaghi, 2023). Sin embargo, sí observamos un efecto de la subnutrición sobre la composición de dicha grasa, aspecto muy novedoso en vacuno de carne ya que no se había estudiado hasta el momento. La restricción provocó cambios inmediatos, reflejados en una disminución de los ácidos grasos saturados, los ácidos grasos *de novo* (C4:0 al C14:0) y los de origen mixto (C16:0 y C16:1), y un aumento de los ácidos grasos mono- y poli-insaturados, y los ácidos grasos de movilización (> C16:0). Al mismo tiempo se evaluaron distintos ratios de ácidos grasos empleados en vacas lecheras para evaluar el estado energético de los animales (Dórea et al., 2017; Jorjong et al., 2015). El ratio C18:1 cis-9/C15:0 se correlacionó con el BE y la concentración plasmática de NEFA, por lo que se podría utilizar como indicador del estado nutricional de las vacas de carne. En vacuno de leche otros indicadores se han propuesto como candidatos atractivos para fenotipar la resiliencia, entre ellos algunos metabolitos de la leche como BHB, lactato-deshidrogenasa o glucosa (Ben Abdelkrim et al., 2023), que podría ser interesante analizar en vacuno de carne.

El contenido de **proteína** y **urea** en leche se vieron influenciados por la restricción nutricional, reflejando la reducción en el contenido proteico de la dieta. Así el contenido de proteína en leche disminuyó durante la restricción en el mes 2 y 3, pero fue constante durante los retos consecutivos, lo que indica que estaría más bien condicionado por el mes de lactación. En cambio, la restricción incrementó el contenido de urea en leche en todos los retos nutricionales independientemente del mes de lactación o de retos consecutivos, siendo especialmente llamativo el gran incremento en el mes 4. El contenido de urea en leche no solo depende de la ingestión de proteína de la dieta, sino también está influenciada por la transferencia de urea del plasma sanguíneo a la leche. Durante este periodo final de la lactación, a pesar de la movilización de las reservas corporales en forma de grasa, sorprende observar un proceso de catabolismo proteico como parece haber sido el caso (Getahun et al., 2019; Spek et al., 2016).

En este estudio ni la lactosa ni el conteo de células somáticas mostraron ser buenos indicadores del efecto de una restricción nutricional. La lactosa presentó pequeñas variaciones durante la restricción y se recuperó con la realimentación. Este metabolito es un importante agente osmótico de la leche el cual influye en el volumen producido, pero su contenido generalmente permanece constante durante la lactación (Guinard-Flament et al., 2006). Por otro lado el conteo de células somáticas, aunque presentó ciertas variaciones, siempre estuvo por debajo del umbral de mastitis subclínica (200 × 103 células/mL, Dervishi et al., 2017).

Además de los parámetros productivos analizados en este estudio, una perturbación alimentaria desencadena cambios metabólicos, fisiológicos y de comportamiento que pueden repercutir en otros aspectos. De forma complementaria a los ensayos descritos en esta tesis, se evaluó el comportamiento de los animales mediante acelerómetros, observando que durante los periodos de restricción se reducía notablemente el tiempo de ingestión y rumia e incrementaba el descanso, en distinta medida según el mes de lactación (Orquera et al., 2021a). Dado que la nutrición es también determinante de los rendimientos reproductivos en vacuno de carne (Noya et al., 2020), de forma paralela se analizó el efecto de estos cortos periodos de restricción sobre la dinámica folicular de las vacas en el mes 3 de lactación (Orquera et al., 2021b). Aparentemente, para observarse diferencias a este nivel sería necesaria una restricción más prolongada, de al menos dos a tres semanas (Mackey et al., 1999).

5.3 Efecto de una restricción-realimentación sobre los indicadores metabólicos

Los metabolitos plasmáticos utilizados en este trabajo han sido ampliamente utilizados como indicadores del estado energético, proteico y oxidativo del ganado vacuno. En este trabajo, algunos metabolitos tuvieron una mayor respuesta que otros durante los periodos de restricción y algunos no siguieron pautas claras.

Así, pudimos observar que la **glucosa en plasma** en vacuno de carne, al igual que se ha descrito en vacuno de leche, no resulta ser un buen indicador del estado energético del animal ya que no se observaron pautas claras. Su concentración está principalmente condicionada por la ingestión de la dieta, pero puede verse influenciada por el proceso de la gluconeogénesis. Los rumiantes dependen en gran medida de la gluconeogénesis hepática para mantener el metabolismo de la glucosa en todo el cuerpo y para el suministro de glucosa para la síntesis de la lactosa. La tasa de gluconeogénesis responde al nivel de producción, a la disponibilidad de sustrato y las concentraciones relativas de los precursores gluconeogénicos (Velez y Donkin, 2005).

Las concentraciones de **NEFA y BHB en plasma** han resultado indicadores inmediatos de la movilización de grasa de las reservas corporales, aunque el primero ha presentado mayores cambios. Por la inmediatez en la respuesta, la concentración de NEFA sería el indicador de elección para evaluar la lipólisis a corto plazo, a diferencia de caracteres como la CC o el espesor de grasa subcutánea cuya respuesta es detectable a largo plazo (Orquera et al., 2020). La variabilidad individual se evidenció en la respuesta a la restricción, en NEFA con incrementos entre +11% y +1138% y en BHB con incrementos entre el 2% y +160%. La concentración de NEFA ha reflejado diferencias entre grupos de vacas de alta y baja respuesta metabólica, presentando las primeras una concentración de NEFA superior, y también se vio fuertemente influenciada por el mes de lactación, con mayor aumento durante la restricción de los meses 2 y 3 que en lactación más avanzada. Por otro lado, observamos una respuesta menos acusada en la concentración de BHB por el efecto de la restricción, y más evidente en las vacas de alta respuesta metabólica. De hecho, en los retos consecutivos en lactación avanzada (mes 4), el BHB solo se vio modificado por periodos de restricción-realimentación en el grupo de alta respuesta metabólica. En cualquier caso, al finalizar los retos ambos metabolitos regresaron a sus concentraciones previas a la restricción, observándose de nuevo una respuesta elástica. Además, el hecho de que la intensidad de la respuesta de ambos metabolitos ante el cambio de dieta no cambiase durante los retos consecutivos indica que, en estas condiciones, las vacas nodrizas ni aumentaron (sensibilización) ni disminuyeron su respuesta (habituación) ante una

exposición repetida. Sería interesante analizar si esto es así en caso de aumentar el número de repeticiones, o de producirse en otra fase con mayor demanda metabólica, como al inicio de la lactación.

La concentración de **urea en plasma** se incrementó durante los periodos de restricción, independientemente del grupo de respuesta metabólica, en todos los meses de lactación pasando del +11% al 20%. Este parámetro es indicador del catabolismo de las reservas proteicas corporales, que en este estudio tendría especialmente a partir del mes 4, cuando se observaron los mayores incrementos. Aunque en el vacuno de leche no se ha descrito tal efecto más que al inicio de la lactación, nuestros resultados apuntan a que la restricción nutricional en esta fase más tardía desencadenaría un catabolismo proteico (Chilliard et al., 1998), incrementando la urea en plasma y en leche.

No se observó un efecto claro a corto plazo de la subnutrición sobre la concentración de **MDA en plasma,** que habitualmente se utiliza como indicador del estado oxidativo. Sin embargo, sí podría ser un buen indicador a largo plazo, ya que sus concentraciones fueron mayores durante el mes 2 de lactación que en el resto, asociadas al mayor contenido de NEFA.

Como hemos descrito, una restricción nutricional a corto plazo desencadena respuestas de diferente magnitud en las vacas de carne según la etapa de la lactación, las características individuales de los animales, o la exposición repetida a la subnutrición. Se han identificado diversos parámetros asociados a los rendimientos productivos y al metabolismo energético y proteico que responden en distinta medida a las variaciones en el aporte nutricional, lo que permite recomendarlos o descartarlos como indicadores del estado metabólico. Además, se ha detectado una variabilidad individual en la respuesta que puede ser la base para la selección eficiente de los animales con mayor capacidad de afrontar estos retos y recuperarse de ellos, es decir, más resilientes.

VI. Conclusiones

En las condiciones que se desarrolló el experimento de la presente tesis doctoral, se pueden determinar las siguientes conclusiones generales:

1. El modelizado de curvas de respuesta de tipo “spline” resultó un método adecuado para evaluar la resiliencia en vacas de carne que se enfrentaron a retos nutricionales en distintos meses de lactación o repetidos de manera consecutiva en un mes. La dinámica de reacción permitió identificar nuevas variables, tanto de magnitud como de tiempo de respuesta de los animales.

2. El uso conjunto de los datos de producción de leche y metabolitos plasmáticos NEFA y BHB para agrupar las vacas en función de su estado metabólico es más recomendable que la utilización de su información individual. Este enfoque nos permitió caracterizar mejor su resiliencia ante desafíos nutricionales.

3. Las vacas presentaron una amplia variabilidad en la respuesta a los desafíos nutricionales. La repetibilidad en el agrupamiento de vacas enfrentadas a los retos de alimentación en tres meses diferentes o de manera consecutiva indicó una componente genética importante para la resiliencia. Agrupar animales con perfiles similares de respuesta puede ser una herramienta para facilitar las decisiones de manejo en la explotación ganadera, así como para la selección de animales más resilientes.

4. Las restricciones alimenticias de 4 días de duración al 55% de los requerimientos de energía y proteína durante el segundo, tercer y cuarto mes de la lactación provocaron una caída en la producción de leche (-17,5%), e incrementos en las concentraciones plasmáticas de NEFA (+212%), BHB (+18%) y urea (+18%, en el cuarto mes). La producción de leche mostró ser un factor determinante para desencadenar la activación de ciertos mecanismos fisiológicos de adaptación a la subnutrición. La proporción en la que se redujo su producción estuvo condicionada por el rendimiento lechero previo al reto y por la fase de la lactación.

5. La mayoría de los parámetros evaluados se recuperaron rápidamente con la realimentación, algunos casi inmediatamente como las concentraciones plasmáticas de NEFA y BHB, mientras que otros necesitaron algunos días más, como sería el caso de la producción de leche.

6. Las estrategias de adaptación de las vacas de carne ante una restricción nutricional cambiaron a medida que avanzaba la lactación, al disminuir la demanda de nutrientes para la producción de leche. Durante las primeras etapas de la lactación se produjo mayoritariamente una movilización de reservas

corporales en forma de grasa, mientras que en estados más avanzados de la lactación pudo tener lugar un proceso de movilización de reservas de origen proteico.

7. Al aplicar una serie de tres retos repetidos de manera consecutiva, la producción de leche cayó más a partir del segundo y tercer reto, pero el resto de parámetros no mostraron habituación ni sensibilización ante una exposición repetida, desencadenándose una respuesta similar en cada uno de los retos. Los intervalos de realimentación de 3 días entre retos fueron suficientes para que las vacas recuperasen los valores previos al reto de todos los parámetros evaluados, tanto productivos como metabólicos.

8. A nivel plasmático la concentración de NEFA fue el metabolito que reflejó de forma más clara la movilización de reservas corporales a corto plazo. Tanto en los retos en diferentes meses de lactación como en los retos consecutivos, este proceso de lipólisis fue fundamental para que los animales pudieran amortiguar el impacto de la subnutrición sobre su producción de leche. La duración y/o la severidad de la restricción no fueron suficientes para elevar en la misma proporción la concentración de BHB.

9. El perfil de ácidos grasos de la leche respondió rápidamente a los cambios energéticos de la dieta, y el ratio C18:1 cis-9/ C15:0 fue un indicador preciso del estado metabólico de los animales. A pesar de que la leche no es una muestra de fácil obtención en vacuno de carne en condiciones de granja, estos análisis podrían utilizarse con fines experimentales por su relación con el BE y la movilización de reservas corporales en forma de grasa.

VII. Referencias

Abdelatty, A.M., Iwaniuk, M.E., Garcia, M., Moyes, K.M., Teter, B.B., Delmonte, P., Kadegowda, A.K.G., Tony, M.A., Mohamad, F.F., Erdman, R.A., 2017. Effect of short-term feed restriction on temporal changes in milk components and mammary lipogenic gene expression in mid-lactation Holstein dairy cows. J. Dairy Sci. 100, 4000–4013. https://doi.org/10.3168/jds.2016-11130

Abuelo, A., Hernández, J., Benedito, J.L., Castillo, C., 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 99, 1003–1016. https://doi.org/10.1111/jpn.12273

Adriaens, I., van den Brulle, I., D'Anvers, L., Statham, J.M.E., Geerinckx, K., De Vliegher, S., Piepers, S., Aernouts, B., 2021. Milk losses and dynamics during perturbations in dairy cows differ with parity and lactation stage. J. Dairy Sci. 104, 405–418. https://doi.org/10.3168/jds.2020-19195

Agenäs, S., Dahlborn, K., Holtenius, K., 2003. Changes in metabolism and milk production during and after feed deprivation in primiparous cows selected for different milk fat content. Livest. Prod. Sci. 83, 153–164. https://doi.org/10.1016/S0301-6226(03)00096-4

Agenäs, S., Heath, M.F., Nixon, R., Wilkinson, J.M., Phillips, C., 2006. Indicators of undernutrition in cattle. Anim. Welf. 15, 149-160. https://doi.org/10.1017/S0962728600030232

Alvarenga, A.B., Oliveira, H.R., Turner, S.P., Garcia, A., Retallick, K.J., Miller, S.P., Brito, L.F., 2023. Unraveling the phenotypic and genomic background of behavioral plasticity and temperament in North American Angus cattle. Genet. Sel. Evol. 55, 3. https://doi.org/10.1186/s12711-023-00777-3

Alvarez-Rodríguez, J., Palacio, J., Casasús, I., Revilla, R., Sanz, A., 2009. Performance and nursing behaviour of beef cows with different types of calf management. Animal 3, 871–878. https://doi.org/10.1017/S175173110900408X

Alvarez-Rodríguez, J., Palacio, J., Sanz, A., 2010. Effects of nursing frequency and parity on the productive, metabolic and reproductive parameters of beef cows. Livest. Sci. 129, 111–121. https://doi.org/10.1016/j.livsci.2010.01.013

AOAC, 2000. Official Methods of Analysis, in: Association of Official Analytical Chemist. Arlington, VA, USA.

Barreto-Mendes, L., De La Torre, A., Ortigues-Marty, I., Cassar-Malek, I., Pires, J., Blanc, F., 2022. How to approach the resilience of livestock exposed to environmental challenges? Quantification of individual response and recovery by means of differential calculus. Anim. - Open Sp. 1, 100008. https://doi.org/10.1016/J.ANOPES.2022.100008

Bastin, C., Laloux, L., Gillon, A., Miglior, F., Soyeurt, H., Hammami, H., Bertozzi, C., Gengler, N., 2009. Modeling milk urea of Walloon dairy cows in management perspectives. J. Dairy Sci. 92, 3529–3540. https://doi.org/10.3168/jds.2008-1904

Bauman, D., Currie, B., 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63,

1514–1529. https://doi.org/10.3168/jds.S0022-0302(80)83111-0

Bauman, D.E., 2000. Regulation of nutrient partitioning during lactation: homeostasis and homeorhesis revisited., in: Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. CABI Wallingford UK. 311–328. https://doi.org/10.1079/9780851994635.0311

Bauman, D.E., Griinari, J.M., 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23, 203–227. https://doi.org/10.1146/annurev.nutr.23.011702.073408

Baumgard, L.H., Collier, R.J., Bauman, D.E., 2017. A 100-Year Review: Regulation of nutrient partitioning to support lactation. J. Dairy Sci. 100, 10353–10366. https://doi.org/10.3168/jds.2017-13242

Bell, A., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73, 2804–2819. https://doi.org/10.2527/1995.7392804x

Bell, A., Bauman, D., 1997. Adaptations of glucose metabolism during pregnancy and lactation. J. Mammary Gland Biol. Neoplasia 2, 265–278. https://doi.org/10.1023/A:1026336505343

Ben Abdelkrim, A., Ithurbide, M., Larsen, T., Schmidely, P., Friggens, N.C., 2023. Milk metabolites can characterise individual differences in animal resilience to a nutritional challenge in lactating dairy goats. animal 17, 100727. https://doi.org/10.1016/J.ANIMAL.2023.100727

Ben Abdelkrim, A., Puillet, L., Gomes, P., Martin, O., 2021a. Lactation curve model with explicit representation of perturbations as a phenotyping tool for dairy livestock precision farming. Animal 15, 100074. https://doi.org/10.1016/j.animal.2020.100074

Ben Abdelkrim, A., Tribout, T., Martin, O., Boichard, D., Ducrocq, V., Friggens, N.C., 2021b. Exploring simultaneous perturbation profiles in milk yield and body weight reveals a diversity of animal responses and new opportunities to identify resilience proxies. J. Dairy Sci. 104, 459–470. https://doi.org/10.3168/jds.2020-18537

Benedet, A., Manuelian, C.L., Zidi, A., Penasa, M., De Marchi, M., 2019. β-hydroxybutyrate concentration in blood and milk and its associations with cow performance. Animal 13, 1676–1689. https://doi.org/10.1017/S175173111900034X

Benoit, M., Mottet, A., 2023. Energy scarcity and rising cost: Towards a paradigm shift for livestock. Agric. Syst. 205, 103585. https://doi.org/10.1016/J.AGSY.2022.103585

Berghof, T.V.L., Poppe, M., Mulder, H., 2019. Opportunities to improve resilience in animal breeding programs. Front. Genet. 9, 1–15. https://doi.org/ttps://doi.org/10.3389/fgene.2018.00692

Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88, 2017–2026. https://doi.org/10.3168/jds.S0022-0302(05)72878-2

Bertolín, J.R., Joy, M., Blanco, M., 2019. Malondialdehyde determination in raw and processed meat products by UPLC-DAD and UPLC-FLD. Food Chem. 298, 125009. https://doi.org/10.1016/j.foodchem.2019.125009

Billa, P.A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J.A.A., 2020. Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. J. Dairy Sci. 103, 3133–3146. https://doi.org/10.3168/jds.2019-17466

Bittante, G., 2022. Effects of breed, farm intensiveness, and cow productivity on infrared predicted milk urea. J. Dairy Sci. 105, 5084–5096. https://doi.org/10.3168/JDS.2021-21105

Bjerre-Harpøth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., Moyes, K.M., 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. J. Dairy Sci. 95, 2362–2380. https://doi.org/10.3168/jds.2011-4419

Blanc, F., Dumont, B., G., B., Francois, B., Agabriel, J., 2010. Robustesse, flexibilité, plasticité : des processus adaptatifs révélés dans les systèmes d'élevage extensifs de ruminants. Prod. Anim. -Paris- Inst. Natl. la Rech. Agron. 23, 65–80. https://doi.org/10.20870/productions-animales.2010.23.1.3288

Blanc, F., Francois, B., Agabriel, J., D'hour, P., Chilliard, Y., 2006. Adaptive abilities of the females and sustainability of ruminant livestock systems. A review. http://dx.doi.org/10.1051/animres:2006040 55. https://doi.org/10.1051/animres:2006040

Blanco, M., Villalba, D., Ripoll, G., Sauerwein, H., Casasús, I., 2009. Effects of early weaning and breed on calf performance and carcass and meat quality in autumn-born bull calves. Livest. Sci. https://doi.org/10.1016/j.livsci.2008.05.003

Blanco, M., Villalba, D., Ripoll, G., Sauerwein, H., Casasús, I., 2008. Effects of pre-weaning concentrate feeding on calf performance, carcass and meat quality of autumn-born bull calves weaned at 90 or 150 days of age. Animal. https://doi.org/10.1017/S1751731108001808

Blumstein, D.T., 2016. Habituation and sensitization: new thoughts about old ideas. Anim. Behav. 120, 255–262. https://doi.org/10.1016/J.ANBEHAV.2016.05.012

Bocquier, F., González-García, E., 2010. Sustainability of ruminant agriculture in the new context: feeding strategies and features of animal adaptability into the necessary holistic approach. Animal 4, 1258–1273. https://doi.org/10.1017/S1751731110001023

Boutinaud, M., Herve, L., Quesnel, H., Lollivier, V., Finot, L., Dessauge, F., Chanat, E., Lacasse, P., Charton, C., Guinard-Flament, J., 2019. The cellular mechanisms underlying mammary tissue plasticity during lactation in ruminants. Animal 13, s52–s64. https://doi.org/10.1017/S1751731119000624

Bradford, B.J., Yuan, K., Farney, J.K., Mamedova, L.K., Carpenter, A.J., 2015. Inflammation during the transition to lactation: New adventures with an old flame. J.

Dairy Sci. 98, 6631–6650. https://doi.org/10.3168/jds.2015-9683

Broderick, G.A., 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. J. Dairy Sci. 86, 1370–1381. https://doi.org/10.3168/jds.S0022-0302(03)73721-7

Bruckmaier, R.M., Gross, J.J., 2017. Lactational challenges in transition dairy cows. Anim. Prod. Sci. 57, 1471–1481.

Burgos, M., Senn, M., Sutter, F., Kreuzer, M., Langhans, W., 2001. Effect of water restriction on feeding and metabolism in dairy cows. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, R418-27. https://doi.org/10.1152/ajpregu.2001.280.2.R418

Caldeira, R.M., Belo, A.T., Santos, C.C., Vazques, M.I., Portugal, A. V, 2007. The effect of long-term feed restriction and over-nutrition on body condition score, blood metabolites and hormonal profiles in ewes. Small Rumin. Res. 68, 242–255. https://doi.org/10.1016/j.smallrumres.2005.08.026

Carlson, D.B., Litherland, N.B., Dann, H.M., Woodworth, J.C., Drackley, J.K., 2006. Metabolic effects of abomasal L-carnitine infusion and feed restriction in lactating holstein cows. J. Dairy Sci. 89, 4819–4834. https://doi.org/10.3168/jds.S0022-0302(06)72531-0

Casasús, I., Sanz, A., Villalba, D., Ferrer, R., Revilla, R., 2004. Intake capacity of two breeds of suckler cattle of different milk yield potential and validation of prediction models. Livest. Prod. Sci. 89, 195–207. https://doi.org/10.1016/j.livprodsci.2004.02.003

Casasús, I., Sanz, A., Villalba, D., Ferrer, R., Revilla, R., 2002. Factors affecting animal performance during the grazing season in a mountain cattle production system. J. Anim. Sci. 80, 1638–1651. https://doi.org/10.2527/2002.8061638x

Castillo, C., Hernández, J., Valverde, I., Pereira, V., Sotillo, J., Löpez Alonso, M., Benedito, J.L., 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. Res. Vet. Sci. 80, 133–139. https://doi.org/10.1016/j.rvsc.2005.06.003

Chen, Y., Stookey, J., Arsenault, R., Scruten, E., Griebel, P., Napper, S., 2016. Investigation of the physiological, behavioral, and biochemical responses of cattle to restraint stress1. J. Anim. Sci. 94, 3240–3254. https://doi.org/10.2527/jas.2016-0549

Chilliard, Y., 1999. Metabolic adaptations and nutrient partitioning in the lactating animal. Chill. Y., 1999. Metab. Adapt. Nutr. partitioning Lact. Anim. Biol. Lact. 503–552.Biology Lact. 503–552.

Chilliard, Y., Bocquier, F., Doreau, M., 1998. Digestive and metabolic adaptations of ruminants to undernutrition, and consequences on reproduction. Reprod. Nutr. Dev. 38, 131–152. https://doi.org/10.1051/rnd:19980201

Chilliard, Y., Ferlay, A., Faulconnier, Y., Bonnet, M., Rouel, J., Bocquier, F., 2000a. Adipose tissue metabolism and its role in adaptations to undernutrition in ruminants. Proc. Nutr. Soc. 59, 127–134. https://doi.org/10.1017/S002966510000015X

Chilliard, Y., Ferlay, A., Mansbridge, R.M., Doreau, M., 2000b. Ruminant milk fat plasticity: Nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Anim. Res. 49, 181–205. https://doi.org/10.1051/animres:2000117

Chilliard, Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J., Doreau, M., 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. Eur. J. Lipid Sci. Technol. 109, 828–855. https://doi.org/10.1002/ejlt.200700080

Churakov, M., Karlsson, J., Edvardsson Rasmussen, A., Holtenius, K., 2021. Milk fatty acids as indicators of negative energy balance of dairy cows in early lactation. Animal 15, 100253. https://doi.org/10.1016/j.animal.2021.100253

Cinar, M., Serbester, U., Ceyhan, A., Gorgulu, M., 2015. Effect of somatic cell count on milk yield and composition of first and second lactation dairy cows. Ital. J. Anim. Sci. 14, 105–108. https://doi.org/10.4081/ijas.2015.3646

Claffey, A., Delaby, L., Boland, T.M., Egan, M., 2020. Implications of adapting autumn grazing management on spring herbage production–The effect on late lactation milk production and the subsequent response in early lactation animal performance. Livest. Sci. 231, 103870. https://doi.org/10.1016/J.LIVSCI.2019.103870

Codrea, M., Højsgaard, S., Friggens, N.C., 2011. Differential smoothing of time-series measurements to identify disturbances in performance and quantify animal response characteristics: An example using milk yield profiles in dairy cows. J. Anim. Sci. 89, 3089–3098. https://doi.org/10.2527/jas.2010-3753

Colditz, I.G., Hine, B.C., 2016. Resilience in farm animals: Biology, management, breeding and implications for animal welfare. Anim. Prod. Sci. 56, 1961–1983. https://doi.org/10.1071/AN15297

Cortés-Lacruz, X., Casasús, I., Revilla, R., Sanz, A., Blanco, M., Villalba, D., 2017. The milk yield of dams and its relation to direct and maternal genetic components of weaning weight in beef cattle. Livest. Sci. 202, 143–149. https://doi.org/10.1016/j.livsci.2017.05.025

Craninx, M., Steen, A., Van Laar, H., Van Nespen, T., Martin-Tereso, J., De Baets, B., Fievez, V., 2008. Effect of lactation stage on the odd- and branched-chain milk fatty acids of dairy cattle under grazing and indoor conditions. J. Dairy Sci. 91, 2662–2677. https://doi.org/10.3168/jds.2007-0656

De Koster, J., Salavati, M., Grelet, C., Crowe, M.A., Matthews, E., O'Flaherty, R., Opsomer, G., Foldager, L., Hostens, M., 2019. Prediction of metabolic clusters in early-lactation dairy cows using models based on milk biomarkers. J. Dairy Sci. 102, 2631–2644. https://doi.org/10.3168/jds.2018-15533

De la Fuente, M.A., Rodríguez-Pino, V., Juárez, M., 2015. Use of an extremely polar 100-m column in combination with a cyanoalkyl polysiloxane column to complement the study of milk fats with different fatty acid profiles. Int. Dairy J. 47, 52–63. https://doi.org/10.1016/j.idairyj.2015.02.010

De La Torre, A., Barreto-Mendes, L., Pires, J.A.A., Cassar-Malek, I., Ortigues-Marty, I., Blanc, F., 2022. Exploration of robustness indicators using adaptive responses to short-term feed restriction in suckling primiparous beef cows. animal 16, 100556.

https://doi.org/10.1016/J.ANIMAL.2022.100556

De La Torre, A., Recoules, E., Blanc, F., Ortigues-Marty, I., D'Hour, P., Agabriel, J., 2015. Changes in calculated residual energy in variable nutritional environments: An indirect approach to apprehend suckling beef cows' robustness. Livest. Sci. 176, 75–84. https://doi.org/10.1016/j.livsci.2015.03.008

Dematawewa, C.M.B., Pearson, R.E., VanRaden, P.M., 2007. Modeling Extended Lactations of Holsteins. J. Dairy Sci. 90, 3924–3936. https://doi.org/10.3168/JDS.2006-790

DePeters, E.J., Ferguson, J.D., 1992. Nonprotein Nitrogen and Protein Distribution in the Milk of Cows. J. Dairy Sci. 75, 3192–3209. https://doi.org/10.3168/jds.S0022-0302(92)78085-0

Deroche, B., Pradel, P., Baumont, R., 2020. Long-term evolution and prediction of feed value for permanent mountain grassland hay: Analysis of a 32-year data set in relation to climate change. Grass Forage Sci. 75, 18–27. https://doi.org/10.1111/gfs.12465

Dervishi, E., Blanco, M., Rodríguez-Sánchez, J.A., Sanz, A., Calvo, J.H., Casasús, I., 2017. Milk yield and genomewide expression profiling in the mammary gland of beef primiparous cows in response to the dietary management during the pre- and postweaning periods. J. Anim. Sci. 95, 4274–4287. https://doi.org/10.2527/jas2017.1736

Diskin, M.G., Mackey, D.R., Roche, J.F., Sreenan, J.M., 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim. Reprod. Sci. 78, 345–370. https://doi.org/10.1016/S0378-4320(03)00099-X

Dohme, F., DeVries, T.J., Beauchemin, K.A., 2008. Repeated ruminal acidosis challenges in lactating dairy cows at high and low risk for developing acidosis: Ruminal pH. J. Dairy Sci. 91, 3554–3567. https://doi.org/10.3168/JDS.2008-1264

Dórea, J.R.R., French, E.A., Armentano, L.E., 2017. Use of milk fatty acids to estimate plasma nonesterified fatty acid concentrations as an indicator of animal energy balance. J. Dairy Sci. 100, 6164–6176. https://doi.org/10.3168/jds.2016-12466

Drackley, J.K., 1999. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci. 82, 2259–2273. https://doi.org/10.3168/jds.s0022-0302(99)75474-3

Drackley, J.K., Cardoso, F.C., 2014. Prepartum and postpartum nutritional management to optimize fertility in high-yielding dairy cows in confined TMR systems. Animal 8, 5–14. https://doi.org/10.1017/S1751731114000731

Drackley, J.K., Overton, T.R., Douglas, G.N., 2001. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. J. Dairy Sci. 84, E100–E112. https://doi.org/10.3168/jds.S0022-0302(01)70204-4

Dumont, B., Andueza, D., Niderkorn, V., Lüscher, A., Porqueddu, C., Picon-Cochard, C., 2015. A meta-analysis of climate change effects on forage quality in grasslands: specificities of mountain and Mediterranean areas. Grass Forage Sci.

70, 239–254. https://doi.org/10.1111/gfs.12169

Elgersma, G.G., de Jong, G., van der Linde, R., Mulder, H.A., 2018. Fluctuations in milk yield are heritable and can be used as a resilience indicator to breed healthy cows. J. Dairy Sci. 101, 1240–1250. https://doi.org/10.3168/jds.2017-13270

Espasandin, A., Gutierrez, V., Casal, A., Graña, A., Bentancur, O., Carriquiry, M., 2016. Modeling Lactation Curve in Primiparous Beef Cattle. J. Agric. Sci. 8, 116. https://doi.org/10.5539/jas.v8n4p116

EU, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [WWW Document].

Ferraretto, L.F., Gencoglu, H., Hackbart, K.S., Nascimento, A.B., Dalla Costa, F., Bender, R.., Guenther, J.N., Shaver, R.D., Wiltbank, M.C., 2014. Effect of feed restriction on reproductive and metabolic hormones in dairy cows. J. Dairy Sci. 97, 754–763. https://doi.org/10.3168/jds.2013-6925

Fiems, L.O., De Boever, J.L., Ampe, B., Vanacker, J.M., De Campeneere, S., Sonck, B., 2015. Effect of energy restriction and dietary protein level during the re-alimentation period on feed efficiency in double-muscled Belgian Blue cows. Livest. Sci. 174, 46–52. https://doi.org/10.1016/j.livsci.2015.01.017

Fiems, L.O., De Campeneere, S., De Boever, J.L., Van Caelenbergh, W., De Brabander, D.L., 2009. Effect of indoor energy restriction level and management on beef production in Belgian Blue double-muscled cow-calf pairs. J. Anim. Physiol. Anim. Nutr. (Berl). 93, 678–687. https://doi.org/10.1111/j.1439-0396.2008.00856.x

Fiems, L.O., Vanacker, J.M., De Boever, J.L., Van Caelenbergh, W., Aerts, J.M., De Brabander, D.L., 2007. Effect of energy restriction and re-alimentation in Belgian Blue double-muscled beef cows on digestibility and metabolites. J. Anim. Physiol. Anim. Nutr. (Berl). 91, 54–61. https://doi.org/10.1111/j.1439-0396.2006.00641.x

Friggens, N., Adriaens, I., Boré, R., Cozzi, G., Jurquet, J., Kamphuis, C., Leiber, F., Lora, I., Sakowski, T., Statham, J.M.E., Haas, Y., 2022. Resilience: reference measures based on longer-term consequences are needed to unlock the potential of precision livestock farming technologies for quantifying this trait. Peer Community J. 2, e38. https://doi.org/10.24072/pcjournal.136

Friggens, N.C., Brun-Lafleur, L., Faverdin, P., Sauvant, D., Martin, O., 2013. Advances in predicting nutrient partitioning in the dairy cow: recognizing the central role of genotype and its expression through time. animal 7, 89–101. https://doi.org/DOI: 10.1017/S1751731111001820

Friggens, N.C., Duvaux-Ponter, C., Etienne, M.P., Mary-Huard, T., Schmidely, P., 2016. Characterizing individual differences in animal responses to a nutritional challenge: Toward improved robustness measures. J. Dairy Sci. 99, 2704–2718. https://doi.org/10.3168/jds.2015-10162

Friggens, N.C., Newbold, J.R., 2007. Towards a biological basis for predicting nutrient partitioning: The dairy cow as an example. Animal 1, 87–97. https://doi.org/10.1017/S1751731107657772

Getahun, D., Alemneh, T., Akeberegn, D., Getabalew, M., Zewdie, D., 2019. Urea Metabolism and Recycling in Ruminants 20, 14790–14796. https://doi.org/10.26717/BJSTR.2019.20.003401

Gross, J.J., Bruckmaier, R.M., 2019a. Metabolic challenges in lactating dairy cows and their assessment via established and novel indicators in milk. Animal 13, s75–s81. https://doi.org/10.1017/S175173111800349X

Gross, J.J., Bruckmaier, R.M., 2019b. Metabolic challenges and adaptation during different functional stages of the mammary gland in dairy cows: Perspectives for sustainable milk production. J. Dairy Sci. 102, 2828–2843. https://doi.org/10.3168/jds.2018-15713

Gross, J.J., Bruckmaier, R.M., 2015. Repeatability of metabolic responses to a nutrient deficiency in early and mid lactation and implications for robustness of dairy cows. J. Dairy Sci. 98, 8634–8643. https://doi.org/10.3168/jds.2014-9246

Gross, J.J., van Dorland, H.A., Bruckmaier, R.M., Schwarz, F.J., 2011a. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. J. Dairy Sci. 94, 1820–1830. https://doi.org/10.3168/jds.2010-3707

Gross, J.J., Van Dorland, H.A., Bruckmaier, R.M., Schwarz, F.J., 2011b. Milk fatty acid profile related to energy balance in dairy cows. J. Dairy Res. 78, 479–488. https://doi.org/10.1017/S0022029911000550

Grossman, M., Koops, W.J., 2003. Modeling Extended Lactation Curves of Dairy Cattle: A Biological Basis for the Multiphasic Approach. J. Dairy Sci. 86, 988–998. https://doi.org/10.3168/JDS.S0022-0302(03)73682-0

Grummer, R.R., 1991. Effect of Feed on the Composition of Milk Fat. J. Dairy Sci. 74, 3244–3257. https://doi.org/10.3168/jds.S0022-0302(91)78510-X

Guinard-Flament, J., Delamaire, E., Lemosquet, S., Boutinaud, M., David, Y., 2006. Changes in mammary uptake and metabolic fate of glucose with once-daily milking and feed restriction in dairy cows. Reprod. Nutr. Dev. 46, 589–598. https://doi.org/10.1051/rnd:2006030

Harder, I., Stamer, E., Junge, W., Thaller, G., 2019. Lactation curves and model evaluation for feed intake and energy balance in dairy cows. J. Dairy Sci. 102, 7204–7216. https://doi.org/10.3168/jds.2018-15300

Heirbaut, S., Jing, X.P., Stefańska, B., Pruszyńska-Oszmałek, E., Buysse, L., Lutakome, P., Zhang, M.Q., Thys, M., Vandaele, L., Fievez, V., 2022. Diagnostic milk biomarkers for predicting the metabolic health status of dairy cattle during early lactation. J. Dairy Sci. https://doi.org/10.3168/JDS.2022-22217

Henriksen, J.C.S., Weisbjerg, M.R., Løvendahl, P., Kristensen, T., Munksgaard, L., 2019. Effects of an individual cow concentrate strategy on production and behavior. J. Dairy Sci. 102, 2155–2172. https://doi.org/10.3168/jds.2018-15477

Henry, B.K., Eckard, R.J., Beauchemin, K.A., 2018. Adaptation of ruminant livestock production systems to climate changes. Animal 12, s445–s456.

https://doi.org/10.1017/S1751731118001301

Herdt, T.H., 2000. Ruminant adaptation to negative energy balance: Influences on the etiology of ketosis and fatty liver. Vet. Clin. Food Anim. Pract. 16, 215–230. https://doi.org/10.1016/S0749-0720(15)30102-X

Hervé, L., Quesnel, H., Véron, M., Portanguen, J., Gross, J., Bruckmaier, R.M., Boutinaud, M., 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows. J. Dairy Sci. 102, 2670–2685. https://doi.org/10.3168/jds.2018-15398

Horn, M., Steinwidder, A., Pfister, R., Gasteiner, J., Vestergaard, M., Larsen, T., Zollitsch, W., 2014. Do different cow types respond differently to a reduction of concentrate supplementation in an Alpine low-input dairy system? Livest. Sci. 170, 72–83. https://doi.org/10.1016/J.LIVSCI.2014.10.006

Hostens, M., Fievez, V., Leroy, J.L.M.R., Van Ranst, J., Vlaeminck, B., Opsomer, G., 2012. The fatty acid profile of subcutaneous and abdominal fat in dairy cows with left displacement of the abomasum. J. Dairy Sci. 95, 3756–3765. https://doi.org/10.3168/jds.2011-5092

Iewdiukow, M., Lema, O.M., Velazco, J.I., Quintans, G., 2020. Is it possible to accurately estimate lactation curve parameters in extensive beef production systems? Appl. Anim. Sci. 36, 509–514. https://doi.org/10.15232/aas.2019-01965

Ingvartsen, K.L., 2006. Feeding- and management-related diseases in the transition cow: Physiological adaptations around calving and strategies to reduce feeding-related diseases. Anim. Feed Sci. Technol. 126, 175–213. https://doi.org/10.1016/j.anifeedsci.2005.08.003

Ingvartsen, K.L., Dewhurst, R.J., Friggens, N.C., 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest. Prod. Sci. 83, 277–308. https://doi.org/10.1016/S0301-6226(03)00110-6

INRA, 2007. Alimentation des bovins, ovins et caprins. Besoins des Animaux-Valeur des aliments., Quae Versailles. ed. Paris, France.

ISO, 2015. Animal and vegetable fats and oils. Gas chromatography of fatty acid methyl esters. Part 4: Determination by capillary gas chromatography.

Jorge-Smeding, E., Astigarraga, L., Loza, C., Carriquiry, M., Rico, D., Álvarez-Oxiley, A., 2022. Characterisation of productive performance, body reserve dynamics and energy metabolic parameters of Holstein Friesian and Normande dairy cows managed in a low-input pastoral dairy system. Anim. Prod. Sci. 62, 274–283. https://doi.org/10.1071/AN21250

Jorjong, S., van Knegsel, A.T.M., Verwaeren, J., Bruckmaier, R.M., De Baets, B., Kemp, B., Fievez, V., 2015. Milk fatty acids as possible biomarkers to diagnose hyperketonemia in early lactation. J. Dairy Sci. 98, 5211–5221. https://doi.org/10.3168/JDS.2014-8728

Jorjong, S., van Knegsel, A.T.M., Verwaeren, J., Lahoz, M.V., Bruckmaier, R.M., De

Baets, B., Kemp, B., Fievez, V., 2014. Milk fatty acids as possible biomarkers to early diagnose elevated concentrations of blood plasma nonesterified fatty acids in dairy cows. J. Dairy Sci. 97, 7054–7064. https://doi.org/10.3168/jds.2014-8039

Kessel, S., Stroehl, M., Meyer, H.H.D., Hiss-Pesch, S., Sauerwein, H., Schwarz, F., Bruckmaier, R.M., 2008. Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. J. Anim. Sci. 86, 2903–2912. https://doi.org/10.2527/jas.2008-1016

Kessler, E.C., Bruckmaier, R.M., Gross, J.J., 2020. Milk urea nitrogen concentration is higher in Brown Swiss than in Holstein dairy cows despite identical feeding. J. Anim. Physiol. Anim. Nutr. (Berl). 104, 1671–1677. https://doi.org/10.1111/jpn.13408

Khiaosa-ard, R., Kleefisch, M.T., Zebeli, Q., Klevenhusen, F., 2020. Milk fatty acid composition reflects metabolic adaptation of early lactation cows fed hay rich in water-soluble carbohydrates with or without concentrates. Anim. Feed Sci. Technol. 264, 1–14. https://doi.org/10.1016/j.anifeedsci.2020.114470

Knight, C., Alamer, M., Sorensen, A., Nevison, I., Flint, D., Vernon, R., 2004. Metabolic safety-margins do not differ between cows of high and low genetic merit for milk production. J. Dairy Res. 71, 141–153. https://doi.org/10.1017/S0022029904000044

Komaragiri, M., Erdman, R., 1997. Factors affecting body tissue mobilization in early lactation dairy cows. 1. Effect of dietary protein on mobilization of body fat and protein. J. Dairy Sci. 80, 929–937. https://doi.org/10.3168/jds.S0022-0302(97)76016-8

König, S., May, K., 2019. Phenotyping strategies and quantitative-genetic background of resistance, tolerance and resilience associated traits in dairy cattle. Animal, 897-908. https://doi.org/10.1017/S1751731118003208

Kramer, J.K.G., Fellner, V., Dugan, M.E.R., Sauer, F.D., Mossoba, M.M., Yurawecz, M.P., 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 32, 1219–1228. https://doi.org/10.1007/s11745-997-0156-3

Kuhla, B., Metges, C.C., Hammon, H.M., 2016. Endogenous and dietary lipids influencing feed intake and energy metabolism of periparturient dairy cows. Domest. Anim. Endocrinol. 56, S2–S10. https://doi.org/10.1016/j.domaniend.2015.12.002

Kvidera, S.K., Horst, E.A., Fernandez, M.V.S., Abuajamieh, M., Ganesan, S., Gorden, P.J., Green, H.B., Schoenberg, K.M., Trout, W.E., Keating, A.F., 2017. Characterizing effects of feed restriction and glucagon-like peptide 2 administration on biomarkers of inflammation and intestinal morphology. J. Dairy Sci. 100, 9402–9417. https://doi.org/10.3168/jds.2017-13229

Lacetera, N., 2019. Impact of climate change on animal health and welfare. Anim. Front. 9, 26–31. https://doi.org/10.1093/af/vfy030

Laeger, T., Görs, S., Metges, C.C., Kuhla, B., 2012. Effect of feed restriction on

metabolites in cerebrospinal fluid and plasma of dairy cows. J. Dairy Sci. 95, 1198–1208. https://doi.org/10.3168/jds.2011-4506

Lake, S.L., Weston, T.R., Scholljegerdes, E.J., Murrieta, C.M., Alexander, B.M., Rule, D.C., Moss, G.E., Hess, B.W., 2007. Effects of postpartum dietary fat and body condition score at parturition on plasma, adipose tissue, and milk fatty acid composition of lactating beef cows. J. Anim. Sci. 85, 717–730. https://doi.org/10.2527/jas.2006-353

Lawrence, D., O'Donovan, M., Boland, T.M., Lewis, E., Kennedy, E., 2016. An examination of two concentrate allocation strategies which are based on the early lactation milk yield of autumn calving Holstein Friesian cows. Animal 10, 796–804. https://doi.org/10.1017/S1751731115002724

Le Neindre, P., Dubroeucq, H., 1973. Observations sur l'estimation de la production laitière des vaches allaitantes per la peseè du veau avant et aprés la tétée. Ann. Zootech. 22, 413–422. https://doi.org/hal-00887309

Leduc, A., Souchet, S., Gelé, M., Le Provost, F., Boutinaud, M., 2021. Effect of feed restriction on dairy cow milk production: a review. J. Anim. Sci. 99, 1–12. https://doi.org/10.1093/jas/skab130

Li, P., Li, X.B., Fu, S.X., Wu, C.C., Wang, X.X., Yu, G.J., Long, M., Wang, Z., Liu, G.W., 2012. Alterations of fatty acid β-oxidation capability in the liver of ketotic cows. J. Dairy Sci. 95, 1759–1766. https://doi.org/10.3168/jds.2011-4580

Li, Y., Ding, H.Y., Wang, X.C., Feng, S.B., Li, X.B., Wang, Z., Liu, G.W., Li, X.W., 2016. An association between the level of oxidative stress and the concentrations of NEFA and BHBA in the plasma of ketotic dairy cows. J. Anim. Physiol. Anim. Nutr. (Berl). 100, 844–851. https://doi.org/10.1111/jpn.12454

Lowman, B.G., Scott, N.A., Somerville, S.H., East of Scotland College of Agriculture. Animal Production, A. and D.D., 1976. Condition Scoring of Cattle, Bulletin (East of Scotland College of Agriculture). Edinburgh School of Agriculture.

Mackey, D.R., Sreenan, J.M., Roche, J.F., Diskin, M.G., 1999. Effect of acute nutritional restriction on incidence of anovulation and periovulatory estradiol and gonadotropin concentrations in beef heifers. Biol. Reprod. 61, 1601–1607. https://doi.org/10.1095/biolreprod61.6.1601

Mann, S., Nydam, D. V, Lock, A.L., Overton, T.R., McArt, J.A.A., 2016. Short communication: Association of milk fatty acids with early lactation hyperketonemia and elevated concentration of nonesterified fatty acids. J. Dairy Sci. 99, 5851–5857. https://doi.org/10.3168/jds.2016-10920

Manninen, M., Saarijärvi, K., Huhta, H., Jauhiainen, L., Aspila, P., 2004. Effects of winter feeding strategies with alternative feeds on the performance of mature suckler cows and their progeny. Agric. Food Sci. 13, 348–362. https://doi.org/10.2137/1239099043633323

Mäntysaari, P., Mäntysaari, E.A., Kokkonen, T., Mehtiö, T., Kajava, S., Grelet, C., Lidauer, P., Lidauer, M.H., 2019. Body and milk traits as indicators of dairy cow energy status in early lactation. J. Dairy Sci. 102, 7904–7916.

https://doi.org/10.3168/jds.2018-15792

MAPA, 2022. Informe trimestral sobre el sector vacuno de carne. Subdirección Gen. Prod. Ganad. y Cinegéticas, D.G. Prod. y Mercados Agrar (accessed 3.26.23).

MAPA, 2021. Estudio del sector vacuno de carne español: segmento vacas nodrizas. Subdirección Gen. Prod. Ganad. y Cinegéticas Dir. Gen. Prod. y Mercados Agrar. (accessed 3.26.23).

Martin, P., Vinet, A., Allart, L., Launay, F., Dozias, D., Maupetit, D., Renand, G., 2022. Influence of feed restriction and subsequent recovery on lactating Charolais cows. Livest. Sci. 264, 105077. https://doi.org/10.1016/J.LIVSCI.2022.105077

McArt, J.A.A., Nydam, D. V., Oetzel, G., Overton, T.R., Ospina, P.A., 2013. Elevated non-esterified fatty acids and β-hydroxybutyrate and their association with transition dairy cow performance. Vet. J. 198, 560–570. https://doi.org/10.1016/J.TVJL.2013.08.011

Moyes, K.M., Drackley, J.K., Salak-Johnson, J.L., Morin, D.E., Hope, J.C., Loor, J.J., 2009. Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation. J. Dairy Sci. 92, 4301–4316. https://doi.org/10.3168/jds.2009-2170

Mulliniks, J., Beard, J., 2019. Sustainable and economically viable management options for cow/calf production through enhanced beef cow metabolic efficiency. J. Anim. Sci. 97, 1398–1406. https://doi.org/10.1093/jas/sky476

Murrieta, C.M., Hess, B.W., Lake, S.L., Scholljegerdes, E.J., Rule, D.C., 2010. Body condition score and day of lactation regulate fatty acid metabolism in milk somatic cells and adipose tissue of beef cows. Livest. Sci. 131, 65–72. https://doi.org/10.1016/J.LIVSCI.2010.02.028

Nagata, R., Kim, Y.-H., Ohkubo, A., Kushibiki, S., Ichijo, T., Sato, S., 2018. Effects of repeated subacute ruminal acidosis challenges on the adaptation of the rumen bacterial community in Holstein bulls. J. Dairy Sci. 101, 4424–4436. https://doi.org/10.3168/jds.2017-13859

Nardone, A., Ronchi, B., Lacetera, N., Ranieri, M.S., Bernabucci, U., 2010. Effects of climate changes on animal production and sustainability of livestock systems. Livest. Sci. 130, 57–69. https://doi.org/10.1016/j.livsci.2010.02.011

Nielsen, N.I., Ingvartsen, K.L., Larsen, T., 2003. Diurnal variation and the effect of feed restriction on plasma and milk metabolites in TMR-fed dairy cows. J. Vet. Med. Ser. A Physiol. Pathol. Clin. Med. 50, 88–97. https://doi.org/10.1046/j.1439-0442.2003.00496.x

Nousiainen, J., Shingfield, K.J., Huhtanen, P., 2004. Evaluation of milk urea nitrogen as a diagnostic of protein feeding. J. Dairy Sci. 87, 386–398. https://doi.org/10.3168/jds.S0022-0302(04)73178-1

Noya, A., Casasús, I., Achiaga, J., Sanz, A., 2019. Long-term effects of maternal subnutrition in early pregnancy on cow-calf performance, immunological and physiological profiles during the next lactation. Animals 9, 936.

https://doi.org/10.3390/ani9110936

Noya, A., Casasús, I., Rodríguez-Sánchez, J.A., Ferrer, J., Sanz, A., 2020. A negative energy balance during the peri-implantational period reduces dam IGF-1 but does not alter progesterone or pregnancy-specific protein B (PSPB) or fertility in suckled cows. Domest. Anim. Endocrinol. 72, 106418. https://doi.org/10.1016/j.domaniend.2019.106418

Ollion, E., Ingrand, S., Delaby, L., Trommenschlager, J.M., Colette-Leurent, S., Blanc, F., 2016. Assessing the diversity of trade-offs between life functions in early lactation dairy cows. Livest. Sci. 183, 98–107. https://doi.org/10.1016/j.livsci.2015.11.016

Orquera-Arguero, K.G., Blanco, M., Bertolín, J.R., Ferrer, J., Casasús, I., 2023a. Performance and milk fatty acid profile of beef cows with a different energy status with short nutrient restriction and refeeding. J. Anim. Sci. 101, 1–16. https://doi.org/10.1093/jas/skad053

Orquera-Arguero, K.G., Casasús, I., Ferrer, J., Blanco, M., 2023b. Beef cows' performance and metabolic response to short nutritional challenges in different months of lactation. Res. Vet. Sci. 159, 26–34. https://doi.org/10.1016/j.rvsc.2023.04.002

Orquera-Arguero, K.G., Villalba, D., Blanco, M., Ferrer, J., Casasús, I., 2022. Modelling beef cows' individual response to short nutrient restriction in different lactation stages. animal 16, 100619. https://doi.org/10.1016/j.animal.2022.100619

Orquera, K., Blanco, M., Ferrer, J., Casasús, I., 2021a. Daily activity budget of beef cows under occasional feed restriction periods throughout lactation, in: Book of Abstracts of the 72nd Annual Meeting of the European Federation of Animal Science, 615.

Orquera, K., Ripoll, G., Blanco, M., Ferrer, J., Bertolín, J.., Casasús, I., 2020. Indicators of body fat mobilisation of lactating beef cows under short nutritional challenges, Book of Abstracts of the 71st Annual Meeting of the European Federation of Animal Science, 368.

Orquera, K., Sanz, A., Ferrer, J., Blanco, M., Casasús, I., 2021b. Efecto del balance energético sobre la ciclicidad de vacas nodrizas en el segundo mes de lactación, XIX Jornadas sobre Producción Animal AIDA, 139.

Ospina, P.A., Nydam, D. V, Stokol, T., Overton, T.R., 2010. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. J. Dairy Sci. 93, 546–554. https://doi.org/10.3168/jds.2009-2277

Oteros-Rozas, E., Ontillera, R., Sanosa, P., Gómez-Baggethun, E., Reyes-García, V., González, J., 2013. Traditional ecological knowledge among transhumant pastoralists in Mediterranean Spain. Ecol. Soc. 18, 33. https://doi.org/10.5751/ES-05597-180333

Overton, T.R., Waldron, M.R., 2004. Nutritional management of transition dairy cows: strategies to optimize metabolic health. J. Dairy Sci. 87, E105–E119.

https://doi.org/10.3168/jds.S0022-0302(04)70066-1

Palmquist, D.L., 2009. Milk fat: Origin of fatty acids and influence of nutritional factors thereon, Advanced Dairy Chemistry 2, 43-92. https://doi.org/10.1007/0-387-28813-9_2

Pareek, N., Voigt, J., Bellmann, O., Schneider, F., Hammon, H.M., 2007. Energy and nitrogen metabolism and insulin response to glucose challenge in lactating German Holstein and Charolais heifers. Livest. Sci. 112, 115–122. https://doi.org/10.1016/j.livsci.2007.02.001

Pedernera, M., García, S.C., Horagadoga, A., Barchia, I., Fulkerson, W.J., 2008. Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. J. Dairy Sci. 91, 3896–3907. https://doi.org/10.3168/jds.2008-1098

Perperoglou, A., Sauerbrei, W., Abrahamowicz, M., Schmid, M., 2019. A review of spline function procedures in R. BMC Med. Res. Methodol. 19, 46. https://doi.org/10.1186/s12874-019-0666-3

Pires, J.A.A., Larsen, T., Leroux, C., 2022. Milk metabolites and fatty acids as noninvasive biomarkers of metabolic status and energy balance in early-lactation cows. J. Dairy Sci. 105, 201–220. https://doi.org/10.3168/jds.2021-20465

Pires, J.A.A., Pawlowski, K., Rouel, J., Delavaud, C., Foucras, G., Germon, P., Leroux, C., 2019. Undernutrition modified metabolic responses to intramammary lipopolysaccharide but had limited effects on selected inflammation indicators in early-lactation cows. J. Dairy Sci. 102, 5347–5360. https://doi.org/10.3168/jds.2018-15446

Poppe, M., Veerkamp, R.F., van Pelt, M.L., Mulder, H.A., 2020. Exploration of variance, autocorrelation, and skewness of deviations from lactation curves as resilience indicators for breeding. J. Dairy Sci. 103, 1667–1684. https://doi.org/10.3168/jds.2019-17290

Pullar, D., Rigby, I., 1993. A note on the comparison of barley-urea, maize gluten and rapeseed meal as supplements to ad libitum winter wheat straw for spring-calving suckler cows. Anim. Sci. 57, 169–173. https://doi.org/DOI: 10.1017/S0003356100006747

Puppel, K., Kuczyńska, B., 2016. Metabolic profiles of cow's blood; a review. J. Sci. Food Agric. 96, 4321–4328. https://doi.org/10.1002/jsfa.7779

R Development Core Team, 2021. R: A language and environment for statistical computing.

Rauch, R., Martín-Tereso, J., Daniel, J.B., Dijkstra, J., 2021. Dietary protein oscillation: Effects on feed intake, lactation performance, and milk nitrogen efficiency in lactating dairy cows. J. Dairy Sci. 104, 10714–10726. https://doi.org/10.3168/JDS.2021-20219

Rico, D.E., Razzaghi, A., 2023. Animal board invited review: The contribution of adipose stores to milk fat: implications on optimal nutritional strategies to increase milk fat

synthesis in dairy cows. animal 17, 100735. https://doi.org/10.1016/J.ANIMAL.2023.100735

Rodríguez-Sánchez, J.A., Sanz, A., Ferrer, J., Casasús, I., 2018. Influence of postweaning feeding management of beef heifers on performance and physiological profiles through rearing and first lactation. Domest. Anim. Endocrinol. 65, 24–37. https://doi.org/10.1016/j.domaniend.2018.05.001

Rodríguez-Sánchez, J.A., Sanz, A., Tamanini, C., Casasús, I., 2015. Metabolic, endocrine, and reproductive responses of beef heifers submitted to different growth strategies during the lactation and rearing periods1. J. Anim. Sci. 93, 3871–3885. https://doi.org/10.2527/jas.2015-8994

Rojas-Downing, M.M., Nejadhashemi, A.P., Harrigan, T., Woznicki, S.A., 2017. Climate change and livestock: Impacts, adaptation, and mitigation. Clim. Risk Manag. 16, 145–163. https://doi.org/10.1016/J.CRM.2017.02.001

Ruiz, M., Ruiz, J.P., 1986. Ecological history of transhumance in Spain. Biol. Conserv. 37, 73–86. https://doi.org/10.1016/0006-3207(86)90035-2

Rust, J.M., 2019. The impact of climate change on extensive and intensive livestock production systems. Anim. Front. 9, 20–25. https://doi.org/10.1093/af/vfy028

Sadri, H., Ghaffari, M.H., Sauerwein, H., 2023. Invited review: Muscle protein breakdown and its assessment in periparturient dairy cows. J. Dairy Sci. 106, 822–842. https://doi.org/10.3168/JDS.2022-22068

Sanz, A., Bernués, A., Villalba, D., Casasús, I., Revilla, R., 2004. Influence of management and nutrition on postpartum interval in Brown Swiss and Pirenaica cows. Livest. Prod. Sci. 86, 179–191. https://doi.org/10.1016/S0301-6226(03)00165-9

Sanz, A., Casasús, I., Bernués, A., Bergua, A., Revilla, R., Vijil, E., 2001. La raza Parda de Montaña en Aragón. Surcos de Aragón 73, 32–34.

Sapkota, D., Kelly, A.K., Crosson, P., White, R.R., McGee, M., 2020. Quantification of cow milk yield and pre-weaning calf growth response in temperate pasture-based beef suckler systems: A meta-analysis. Livest. Sci. 241, 104222. https://doi.org/10.1016/j.livsci.2020.104222

Sarikaya, H., Werner-Misof, C., Atzkern, M., Bruckmaier, R.M., 2005. Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. J. Dairy Res. 72, 486–492. https://doi.org/10.1017/S0022029905001317

Schröder, U.J., Staufenbiel, R., 2006. Invited Review: Methods to Determine Body Fat Reserves in the Dairy Cow with Special Regard to Ultrasonographic Measurement of Backfat Thickness. J. Dairy Sci. 89, 1–14. https://doi.org/10.3168/JDS.S0022-0302(06)72064-1

Schuh, K., Sadri, H., Häussler, S., Webb, L.A., Urh, C., Wagner, M., Koch, C., Frahm, J., Dänicke, S., Dusel, G., Sauerwein, H., 2019. Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated v.

normal body condition at dry-off. Animal 13, 1478–1488. https://doi.org/10.1017/S1751731118003385

Schütz, K.E., Cox, N.R., 2014. Effects of short-term repeated exposure to different flooring surfaces on the behavior and physiology of dairy cattle. J. Dairy Sci. 97, 2753–2762. https://doi.org/10.3168/JDS.2013-7310

Sepchat, B., D'hour, P., Agabriel, J., 2017. Production laitière des vaches allaitantes : caractérisation et étude des principaux facteurs de variation. INRAE Prod. Anim *30*(2), 139–152. https://doi.org/10.20870/productions-animales.2017.30.2.2240

Shi, X., Li, D., Deng, Q., Li, Y., Sun, G., Yuan, X., Song, Y., Wang, Z., Li, X., Li, X., Liu, G., 2015. NEFAs activate the oxidative stress-mediated NF-κB signaling pathway to induce inflammatory response in calf hepatocytes. J. Steroid Biochem. Mol. Biol. 145, 103–112. https://doi.org/10.1016/j.jsbmb.2014.10.014

Shingfield, K.J., Ahvenjärvi, S., Toivonen, V., Ärölä, A., Nurmela, K.V. V, Huhtanen, P., Griinari, J.M., 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Anim. Sci. 77, 165–179. https://doi.org/DOI: 10.1017/S1357729800053765

Sigl, T., Gellrich, K., Meyer, H.H.D., Kaske, M., Wiedemann, S., 2013. Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation – metabolism, productivity and effect of a short-term feed restriction. J. Anim. Physiol. Anim. Nutr. (Berl). 97, 278–296. https://doi.org/10.1111/j.1439-0396.2011.01268.x

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128, 104–109. https://doi.org/10.1016/J.VETIMM.2008.10.305

Spek, J.W., Dijkstra, J., Bannink, A., 2016. Influence of milk urea concentration on fractional urea disappearance rate from milk to blood plasma in dairy cows. J. Dairy Sci. 99, 3880–3888. https://doi.org/10.3168/jds.2015-9421

Spek, J.W., Dijkstra, J., Van Duinkerken, G., Bannink, A., 2013. A review of factors influencing milk urea concentration and its relationship with urinary urea excretion in lactating dairy cattle. J. Agric. Sci. 151, 407–423.

Stockman, C., Collins, T., Barnes, A., Miller, D., Wickham, S., Beatty, D., Blache, D., Wemelsfelder, F., Fleming, P., 2011. Qualitative Behavioural Assessment and quantitative physiological measurement of cattle naive and habituated to road transport. Anim. Prod. Sci. 51, 240–249. https://doi.org/10.1071/AN10122

Stoop, W.M., Bovenhuis, H., Heck, J.M.L., van Arendonk, J.A.M., 2009. Effect of lactation stage and energy status on milk fat composition of Holstein-Friesian cows. J. Dairy Sci. 92, 1469–1478. https://doi.org/10.3168/jds.2008-1468

Sukhija, P.S., Palmquist, D.L., 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36, 1202–1206. https://doi.org/10.1021/jf00084a019

Tremblay, M., Kammer, M., Lange, H., Plattner, S., Baumgartner, C., Stegeman,

J.A., Duda, J., Mansfeld, R., Döpfer, D., 2018. Identifying poor metabolic adaptation during early lactation in dairy cows using cluster analysis. J. Dairy Sci. 101, 7311–7321. https://doi.org/10.3168/JDS.2017-13582

Turk, R., Juretić, D., Gereš, D., Svetina, A., Turk, N., Flegar-Meštrić, Z., 2008. Influence of oxidative stress and metabolic adaptation on PON1 activity and MDA level in transition dairy cows. Anim. Reprod. Sci. 108, 98–106. https://doi.org/10.1016/j.anireprosci.2007.07.012

Urh, C., Denißen, J., Gerster, E., Kraus, N., Stamer, E., Heitkönig, B., Spiekers, H., Sauerwein, H., 2019. Pro- and antioxidative indicators in serum of dairy cows during late pregnancy and early lactation: Testing the effects of parity, different dietary energy levels, and farm. J. Dairy Sci. 102, 6672–6678. https://doi.org/10.3168/jds.2019-16248

van der Drift, S.G.A., Houweling, M., Schonewille, J.T., Tielens, A.G.M., Jorritsma, R., 2012. Protein and fat mobilization and associations with serum β-hydroxybutyrate concentrations in dairy cows. J. Dairy Sci. 95, 4911–4920. https://doi.org/10.3168/jds.2011-4771

Van Haelst, Y.N.T., Beeckman, A., Van Knegsel, A.T.M., Fievez, V., 2008. Short communication: Elevated concentrations of oleic acid and long-chain fatty acids in milk fat of multiparous subclinical ketotic cows. J. Dairy Sci. 91, 4683–4686. https://doi.org/10.3168/jds.2008-1375

van Knegsel, A.T.M., Hammon, H.M., Bernabucci, U., Bertoni, G., Bruckmaier, R.M., Goselink, R.M.A., Gross, J., Kuhla, B., Metges, C.C., Parmentier, H.K., 2014. Metabolic adaptation during early lactation: key to cow health, longevity and a sustainable dairy production chain. CAB Rev 9, 1–15.

van Knegsel, A.T.M., van den Brand, H., Dijkstra, J., Kemp, B., 2007. Effects of dietary energy source on energy balance, metabolites and reproduction variables in dairy cows in early lactation. Theriogenology 68, S274–S280. https://doi.org/10.1016/j.theriogenology.2007.04.043

van Knegsel, A.T.M., Van den Brand, H., Dijkstra, J., Tamminga, S., Kemp, B., 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod. Nutr. Dev. 45, 665–688. https://doi.org/10.1051/rnd:2005059

Van Saun, R., 2006. Metabolic profiles for evaluation of the transition period. Proc. Am. Assoc. Bov. Pr. 39, 130–138. https://doi.org/10.21423/aabppro20064693

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Vanbergue, E., Peyraud, J.L., Ferlay, A., Miranda, G., Martin, P., Hurtaud, C., 2018. Effects of feeding level, type of forage and milking time on milk lipolytic system in dairy cows. Livest. Sci. 217, 116–126. https://doi.org/10.1016/J.LIVSCI.2018.09.019

Veissier, I., Boissy, A., DePassillé, A.M., Rushen, J., van Reenen, C.G., Roussel, S.,

Andanson, S., Pradel, P., 2001. Calves' responses to repeated social regrouping and relocation1. J. Anim. Sci. 79, 2580–2593. https://doi.org/10.2527/2001.79102580x

Velez, J.C., Donkin, S.S., 2005. Feed restriction induces pyruvate carboxylase but not phosphoenolpyruvate carboxykinase in dairy cows. J. Dairy Sci. 88, 2938–2948. https://doi.org/10.3168/jds.S0022-0302(05)72974-X

Vossebeld, F., van Knegsel, A.T.M., Saccenti, E., 2022. Phenotyping metabolic status of dairy cows using clustering of time profiles of energy balance peripartum. J. Dairy Sci. 105, 4565–4580. https://doi.org/10.3168/JDS.2021-21518

Wathes, D.C., Cheng, Z., Bourne, N., Taylor, V.J., Coffey, M.P., Brotherstone, S., 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domest. Anim. Endocrinol. 33, 203–225. https://doi.org/10.1016/j.domaniend.2006.05.004

Wathes, D.C., Cheng, Z, Salavati, M, Buggiotti, L, et. al 2021. Relationships between metabolic profiles and gene expression in liver and leukocytes of dairy cows in early lactation. J. Dairy Sci. 104, 2021. https://doi.org/10.3168/jds.2020-19165

Wiseman, A., Redden, M., McGee, A., Spencer, C., Reuter, R., Horn, G., Lalman, D., 2019. Effects of timing of weaning on energy utilization in primiparous beef cows and post-weaning performance of their progeny1. J. Anim. Sci. 97, 1198–1211. https://doi.org/10.1093/jas/skz019

Wood, P.D.P., 1967. Algebraic Model of the Lactation Curve in Cattle. Nature 216, 164–165. https://doi.org/10.1038/216164a0

Xu, W., van Knegsel, A.T.M., Vervoort, J.J.M., Bruckmaier, R.M., van Hoeij, R.J., Kemp, B., Saccenti, E., 2019. Prediction of metabolic status of dairy cows in early lactation with on-farm cow data and machine learning algorithms. J. Dairy Sci. 102, 10186–10201. https://doi.org/10.3168/jds.2018-15791

Yonny, M.E., García, E.M., López, A., Arroquy, J.I., Nazareno, M.A., 2016. Measurement of malondialdehyde as oxidative stress biomarker in goat plasma by HPLC-DAD. Microchem. J. 129, 281–285. https://doi.org/10.1016/j.microc.2016.07.010

VIII. Anexos

ANEXO I: COMPENDIO DE TRABAJOS DERIVADOS DE ESTA TESIS

Publicaciones

- **Orquera-Arguero, K. G**., D. Villalba, M. Blanco, J. R. Bertolín, J. Ferrer, and I. Casasús.2022. Modelling beef cows' individual response to short nutrient restriction in different lactation stages. Animal. 16: 100619. https://doi.org/10.1016/j.animal.2022.100619
- **Orquera-Arguero, K. G**., M. Blanco, J. R. Bertolín, J. Ferrer, and I. Casasús.2023. Performance and milk fatty acid profile of beef cows with a different energy status with 3 short nutrient restriction and refeeding. Journal Animal Science 101: 1-11. https://doi.org/10.1093/jas/skad053.
- **Orquera-Arguero, K. G**., I. Casasús, J. Ferrer, and M. Blanco. 2023. Beef cows' performance and metabolic response to short nutritional challenges in different months of lactation. Research in Veterinary Science 159: 26-34. https://doi.org/10.1016/j.rvsc.2023.04.002.
- **Orquera-Arguero, K. G**., I. Casasús, D. Villalba, J. Ferrer, and M. Blanco. Adaptive response of beef cows to successive nutritional challenges (finalizando redacción).

Comunicaciones en congresos internacionales

- Casasús I., **Orquera K**., Bertolín J.R., Ferrer J., Blanco M. (2019). "Performance and oxidative status of beef cows facing short nutritional challenges during lactation". 70th Annual Meeting of European Federation of Animal Science. Gante (Bélgica). Book of abstracts no. 25: 617. Agosto 2019. http://hdl.handle.net/10532/4777
- Casasús I., Bertolín J. R., **Orquera K**., Ferrer J., Blanco M. (2020). "Milk fatty acid profiles of beef cows in response to a short feed restriction during lactation ". Annual Meeting of the American Dairy Science Association, Virtual Annual Meeting. Journal of Dairy Science, 103 (Suppl. 1): 188. Junio 2020. http://hdl.handle.net/10532/5054
- **Orquera K**., Ripoll G., Blanco M., Ferrer J., Bertolín J., Casasús I. (2020). "Indicators of body fat mobilisation of lactating beef cows under short nutritional challenges". 71st Annual Meeting of European Federation of Animal Science, Virtual meeting. Book of abstracts no. 26: 368. Diciembre 2020. http://hdl.handle.net/10532/5187
- **Orquera K**., Ortigues-Marty I., Thollon N., Casasús I., Sepchat B., De La Torre A. (2020). "The effect of routine practices on the behaviour of beef cows according to their feeding management ". 71st Annual Meeting of European Federation of Animal

Science, Virtual meeting. Book of abstracts no. 26: 145. Diciembre 2020. http://hdl.handle.net/10532/5191

- **Orquera, K**., Blanco, M., Bertolín, J.R., Ferrer, J., Casasús, I. (2021). "Energy balance and feed restriction effects on milk fatty acids and metabolic profile of beef cows". 72nd Annual Meeting of European Federation of Animal Science. Davos (Suiza). Book of abstracts no. 27: 137. Septiembre 2021. http://hdl.handle.net/10532/5516
- **Orquera, K**., Blanco, M., Ferrer, J., Casasús, I. (2021). "Daily activity budget of beef cows under occasional feed restriction periods throughout lactation". 72nd Annual Meeting of European Federation of Animal Science. Davos (Suiza). Book of abstracts no. 27: 615. Septiembre 2021. http://hdl.handle.net/10532/5520

Comunicaciones en congresos nacionales

- **Orquera, K**., Blanco, M., Bertolín, J.R., Ferrer, J. y Casasús, I. (2019). "Respuesta productiva y metabólica de vacas nodrizas ante una subnutrición breve e intensa al inicio de la lactación". XVIII Jornadas de Producción Animal AIDA. pág: 194-196. Mayo 2019. http://hdl.handle.net/10532/4667
- **Orquera K**., Blanco M., Bertolín J.R., Ferrer J., Casasús I. (2019). "Efectos de una restricción nutricional aguda sobre los parámetros productivos y metabólicos de vacas nodrizas en lactación". VI Jornadas Doctorales de Campus Iberus. Junio 2019.
- **Orquera, K**., Sanz, A., Ferrer, J., Blanco M. Casasús, I. (2021). "Efecto del balance energético sobre la ciclicidad de vacas nodrizas en el segundo mes de lactación". XIX Jornadas de Producción Animal AIDA. Formato virtual. pág 139. Junio 2021. http://hdl.handle.net/10532/5447

Animal 16 (2022) 100619

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Modelling beef cows' individual response to short nutrient restriction in different lactation stages

K.G. Orquera-Arguero [a,b], D. Villalba [c], M. Blanco [a,b], J. Ferrer [a,b], I. Casasús [a,b,*]

[a] Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059 Zaragoza, Spain
[b] Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Zaragoza, Spain
[c] Departament de Ciència Animal, Universitat de Lleida, Avinguda Alcalde Rovira Roure 191,25198, Lleida, Spain

ARTICLE INFO

Article history:
Received 28 January 2022
Revised 13 July 2022
Accepted 18 July 2022

Keywords:
Adaptation
Metabolites
Milk yield
Nutritional challenge
Perturbation

ABSTRACT

Short-term nutrient restrictions can occur naturally in extensive beef cattle production systems due to low feed quality or availability. The aims of the study were to (1) model the curves of milk yield, plasma non-esterified fatty acids (**NEFAs**) and β-hydroxybutyrate (**BHB**) contents of beef cows in response to short nutritional challenges throughout lactation; (2) identify clusters of cows with different response profiles; (3) quantify differences in cows' response between the clusters and lactation stages. Data of BW, body condition score (**BCS**), milk yield, NEFA, and BHB plasma concentration from 31 adult beef cows (626 ± 48 kg at calving) were used to study the effect of 4-day feed restriction repeated over months 2, 3 and 4 of lactation. On each month, all cows received a single diet calculated to meet the requirements of the average cow: 100 % requirements for 4 days (d-4 to d-1, basal period), 55 % requirements on the next 4 days (d0 to d3, restriction period) and 100 % requirements for 4 days (d4 to d7, refeeding period). Natural cubic splines were used to model the response of milk yield, NEFA and BHB to restriction and refeeding in the 3 months. The new response variables [baseline value, peak value, days to peak and to regain baseline, and areas under the curve (**AUC**) during restriction and refeeding] were used to cluster cows according to their metabolic response (**MR**) into two groups: Low MR and High MR. The month of lactation affected all the traits, and basal values decreased as lactation advanced. Cows from both clusters had similar BW and BCS values, but those in the High MR cluster had higher basal milk yield, NEFA and BHB contents, and responded more intensely to restriction, with more marked peaks and AUCs. Reaction times were similar, and baseline values recovered during refeeding in both clusters. Our results suggest that the response was driven by cows' milk potential rather than size or body reserves, and despite high-responding cattle's higher milk yield, they were able to activate metabolic pathways to respond to and recover from the challenge.

Implications

Modelling beef cows' short-term response to feed restriction and refeeding for 3 months of lactation allowed us to identify two groups of cows with different magnitudes for their responses (milk loss and fat mobilisation). Their coping strategies changed as lactation advanced. Identifying cows which, even with a high milk yield, show a better response is potentially interesting for future breeding programmes.

Introduction

Beef cattle managed under extensive conditions depend on the local availability of feed resources, which vary throughout the year in quality and quantity terms. This results in seasonal mobilisation patterns and the replenishment of body reserves, which might limit animal performance in critical physiological stages (Noya et al., 2019). The fact that cows face perturbations prevents them from fully expressing their production potential, with wide variability in individual coping strategies. In temperate climates, beef herds are housed in the winter (Blanco et al., 2008), and management is often simplified by group-feeding cows with a single diet irrespectively of their individual requirements. In these circumstances, animals' ability to cope with a nutritional challenge is particularly relevant.

This individual variability has been addressed in cows by testing different models to quantify the gap between the potential and disturbed performance that natural or induced perturbations cause (Codrea et al., 2011; Bjerre-Harpøth et al., 2012; Adriaens et al., 2021; De La Torre et al., 2022) as an indicator of not only animals' resilience but also their capacity to be minimally affected by

* Corresponding author.
E-mail address: icasasus@cita-aragon.es (I. Casasús).

https://doi.org/10.1016/j.animal.2022.100619
1751-7311/

perturbations and to rapidly return to the previous state (Berghof et al., 2019). When disturbances happen during lactation, complex homeostatic and homeorhetic mechanisms concur to maintain a physiological equilibrium while redirecting nutrient partitioning towards milk production (Bauman and Currie, 1980). In dairy cows, the major source of milk yield variation in animals lies in their ability to partition nutrients towards the mammary gland (Baumgard et al., 2017). This process is mediated by the somatotropic axis, with increased growth hormone and decreased insulin production in higher-yielding cows, which promotes glucose-sparing mechanisms and the mobilisation of body reserves in peripheral tissue (Knight et al., 2004). Pareek et al. (2007) found differences in this endocrine regulation of nutrient partitioning between dairy and beef breeds in relation to their different milk secretion and body mass accretion potentials.

To ensure adequate nutrient supply for milk production, lipolysis releases non-esterified fatty acids (**NEFAs**) from adipose tissue, which can be oxidised in the liver into ketone bodies like β-hydroxybutyrate (**BHB**) (Bell, 1995). Both metabolites have been proposed to assess the degree and effects of a negative energy balance (**EB**) in ruminants (Kessel et al., 2008; Gross et al., 2011), whereas BW and body condition score (**BCS**) changes are poor indicators in dairy cattle (Pedernera et al., 2008). With feed restriction, negative EB is associated with decreased milk yield and higher NEFA and BHB concentrations, and the magnitude of these effects depends on the lactation stage, and also on restriction severity and duration (Leduc et al., 2021).

The joint analyses of milk yield dynamics and other traits are useful for analysing the drivers of their concomitant changes (Ben Abdelkrim et al., 2021b). Multitrait clustering in different lactation phases has been used to identify distinct strategies to cope with metabolic challenges (Friggens et al., 2016; De Koster et al., 2019). In the long term, this has provided data to characterise dairy cows according to their ability to prioritise nutrient use among different life functions (Ollion et al., 2016), but this approach has not been used in beef cows. Therefore, the objectives of this study were to (1) model beef cows' response of milk yield and plasma NEFA and BHB concentrations to short feed restriction and refeeding in three lactation stages; (2) cluster cows according to their metabolic response (MR); (3) determine differences between groups of cows and lactation stages. We hypothesised that beef cows would respond differently to restriction depending on their potential milk yield, and eventually on their size and fat reserves, and different coping strategies would be elicited as lactation advanced.

Material and methods

Experimental design

This experiment was conducted at the CITA La Garcipollera Research Station (Spain, 42°37′N, 0°30′W, 945 m a.s.l.). It involved 31 Parda de Montaña lactating beef cows [626 ± 47.7 kg BW, 2.8 ± 0.22 BCS and 7.5 ± 2.91 years at calving]. Cow-calf pairs were loose-housed in straw-bedded pens (7 or 8 cows/pen, 10 × 20 m) equipped with individual feeders for forage and ALPRO automatic concentrate feeding stations (Alfa Laval Agri, Tumba, Sweden). Calves were penned in cubicles and allowed to suckle twice daily for 30 min at 0600 h and 1400 h. The study consisted of three feeding periods repeated over the second, third and fourth lactation months. During each lactation month, cows received a diet that was calculated to meet 100 % of their requirements for 4 days (d-4 to d-1, basal period), then, they were restricted for 4 days (d0 to d3, restriction period) with a diet that met only 55 % of their requirements and were returned to the 100 % energy diet for 4 days (d4 to d7, refeeding period). On the first day (d0) of the restriction period, cows were in milk for 31, 58 and 87 (±5.5) days (**DIM**; months 2, 3, and 4 of lactation, respectively) (Fig. 1).

Cows were fed a flat-rate regime during lactation. Diets were calculated by considering the net energy and metabolisable protein requirements for the maintenance and lactation of a standard cow (BW 615 kg, milk yield 8.5 kg/d) using INRA equations (INRA, 2007). During the basal and refeeding periods, all the cows received 8.0 kg of hay (as a fed basis) daily, and only 7.0 kg of hay during the restriction period, offered daily at 0800 h as a single meal in individual feeders. Cows were tied up for approximately 2 h until they finished their ration. The ALPRO feeding stations were programmed to offer 3.0 kg (as fed)/day of concentrate to all the cows during the basal and refeeding periods. The individual intake was recorded daily. Animals had free access to water and mineral blocks.

Measurements, sampling and chemical analyses

Samples of the offered feedstuffs were collected daily to determine their chemical composition and nutritive value (Table 1). All the analyses of feedstuffs were run in duplicate. Official methods were used to determine the contents of DM, ash and CP (Nitrogen analyser, Model NA 2100, CE Instruments, Thermoquest SA, Barcelona, Spain) (Association of Official Analytical Chemists (AOAC), 2000). The methods of Van Soest et al. (1991) were followed to obtain the contents of NDF, ADF and ADL using a fibre analyser (model Ankom 200/220, Ankom, Macedon, NY, USA). In the forage samples, NDF was assayed with heat-stable amylase. Lignin was analysed on ADF residue by the solubilisation of cellulose with sulphuric acid. All the values were corrected for ash-free content. The feed values were calculated from the measured chemical composition of diets using INRA equations (INRA, 2007).

In the 3 months of lactation, during the basal period (d-4 and d-2), the BCS was assessed on a scale from 0 to 5 based on the estimation of fat covering loin, ribs and tailhead. Milk yield was estimated (d-4, d-2 and daily from d1 to d7) by the weigh-suckle-weigh technique (Le Neindre and Dubroeucq, 1973), calculated using the milk consumed by the calf during both daily sucklings. Cows were weighed and bled on the same days at 0700 h, after suckling and before the hay was offered. Blood samples were collected from the coccygeal vein using test tubes with EDTA and heparin (BD Vacutainer, BD, Plymouth, UK) for the NEFA analysis and the BHB analysis, respectively. They were immediately centrifuged (3 500 rpm for 20 min at 4 °C). Plasma was collected and frozen at −20 °C until further analyses. Randox kits (Randox Laboratories ltd, Crumlin, UK) were used to determine the BHB plasma concentration (kinetic enzymatic method, sensitivity: 0.100 mmol/L) and the NEFA concentration (colorimetric method, sensitivity: 0.072 mmol/L). The mean intra- and interassay CVs were 6.8 % and 6.8 % for BHB and 4.0 % and 4.9 % for NEFA, respectively.

Calculations and statistical analysis

The statistical analysis involved three steps:

Step 1: Modelling the individual response. The curve predicted for each trait (milk yield, NEFA, BHB) on the day of the experiment was modelled using natural cubic splines. A natural cubic spline with K knots is represented by K basis functions. Each basis function is a third-degree polynomial specified in the Hermite form. Compared to other splines, a natural cubic confers additional constraints; i.e. function is linear beyond boundary knots. This frees up four degrees of freedom, which can be spent more profitably by sprinkling more knots in the interior region (Perperoglou et al., 2019). Each parameter that defines the natural cubic spline basis with eight knots was estimated for each cow within each month using

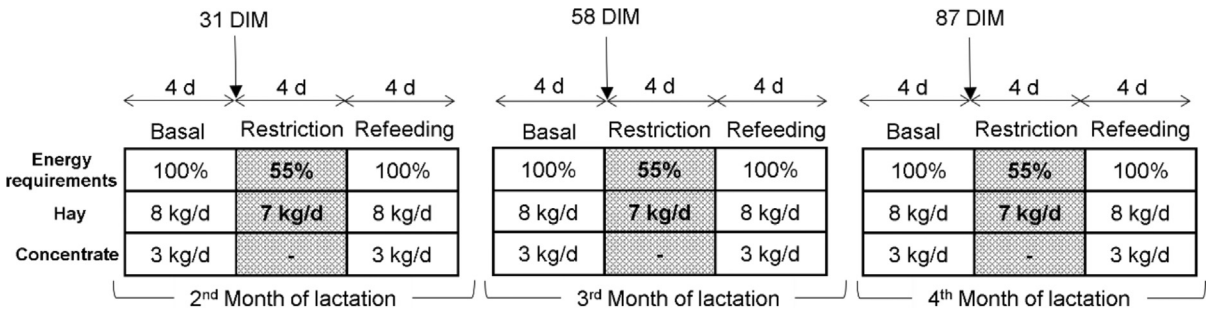


Fig. 1. Schematic representation of the timeline of three short nutritional challenges of the beef cows throughout lactation. DIM: days in milk.

Table 1
Chemical composition and nutritive value (mean ± SD) of the feedstuffs received by the beef cows during each month of lactation.

Item	Month 2	Month 3	Month 4
Hay			
Chemical composition			
DM, g/kg	919 ± 12.1	922 ± 11.7	918 ± 10.5
Ash, g/kg DM	98 ± 12.7	86 ± 24.4	78 ± 3.9
CP, g/kg DM	97 ± 25.7	109 ± 18.3	85 ± 8.1
NDF, g/kg DM	558 ± 59.2	570 ± 52.4	614 ± 21.2
ADF, g/kg DM	334 ± 33.5	324 ± 32.9	333 ± 15.9
Lignin, g/kg DM	41 ± 4.0	35 ± 12.8	28 ± 4.1
Nutritive Value			
Net energy, MJ/kg DM	5.4 ± 0.54	5.5 ± 0.54	5.4 ± 0.54
Metabolisable protein, g PDI/kg DM	81 ± 17.9	79 ± 12.8	59 ± 5.7
Concentrate			
Chemical composition			
DM, g/kg	907 ± 2.4	906 ± 4.0	911 ± 11.1
Ash, g/kg DM	68 ± 1.3	68 ± 1.4	69 ± 2.1
CP, g/kg DM	173 ± 3.5	167 ± 4.7	169 ± 4.2
NDF, g/kg DM	246 ± 17.4	256 ± 23.2	254 ± 18.2
ADF, g/kg DM	102 ± 4.5	114 ± 11.1	120 ± 10.5
Lignin, g/kg DM	25 ± 7.5	29 ± 8.8	33 ± 6.6
Nutritive Value			
Net energy, MJ/kg DM	7.5 ± 0.34	7.3 ± 0.34	7.5 ± 0.34
Metabolisable protein, g PDI/kg DM	123 ± 2.4	119 ± 3.3	120 ± 3.0

a non-linear mixed model with the random effect of the cow. The basal level of each cow within a month was also modelled with a mixed model, which included only the intercept, the linear random regression coefficients and the data from the basal and refeeding periods. Splines were obtained using command ns in the library splines of R (R Development Core Team, 2014). Mixed models were solved using command nlme in library lme4 of R.

The new response variables obtained from the fitted curve for milk yield and plasma metabolites (NEFA and BHB) are depicted in Fig. 2a and 2b, respectively. These response variables were 1) baseline: estimated values without feed restriction according to a linear interpolation from the basal to the refeeding period; 2) peak: the maximum difference between the actual daily value and the baseline value. For milk yield, the peak was the maximum daily milk loss, whereas it was the maximum daily increment compared to baseline values for NEFA and BHB; 3) days to peak: days from the start of restriction until the peak values were reached; 4) area under the curve (**AUC**) during restriction: the estimated total milk loss or the extra NEFA or BHB contents during restriction compared to the baseline values; 5) days to regain baseline: days from the start of restriction until the milk yield, and the NEFA or BHB contents reached the baseline again. 6) AUC during refeeding: the estimated total milk loss or extra NEFA or BHB contents during refeeding until the baseline values were regained.

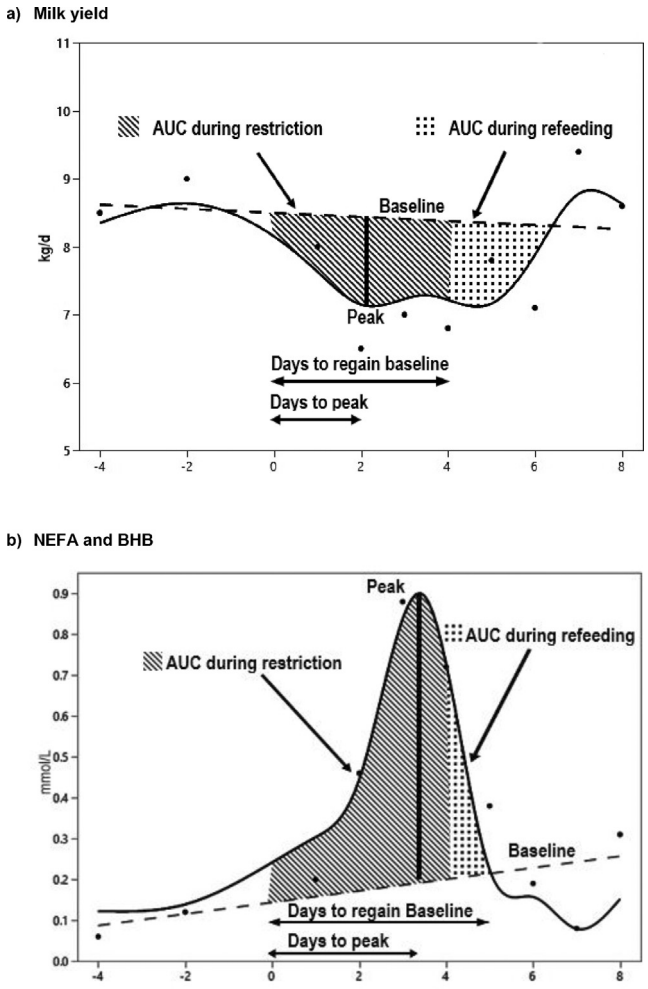


Fig. 2. Schematic representation of the piecewise model for describing the variables of the milk yield (2a) and non-esterified fatty acids and β-hydroxybutyrate (2b) beef cows' response curves to a 4-day restriction and a 4-d refeeding period. AUC: area under the curve; NEFAs: non-esterified fatty acids; BHB: β-hydroxybutyrate.

Step 2: Multivariate analysis. The new response variables obtained in step 1 for each trait, individual cow and month were employed to perform a multivariate analysis using the Factor Mine statistical package of the R software. First of all, a principal component analysis (**PCA** function) was used to identify the variables which accounted for most of the variability in the response among individuals. Then, hierarchical clustering on these principal components (**HCPC** function) was carried out to group the cows with a similar response pattern. The optimum number of clusters was calculated automatically by the algorithm.

Step 3: Effect of cluster and lactation stage on performance and MR. The phenotypic values and the new response variables during the three lactation months were studied according to the clusters obtained in the previous step using the SAS statistical package v 9.4 (SAS Institute Inc., Cary, NC, USA). Mixed linear models (MIXED procedure) were employed after taking cluster, month, and their interaction as fixed effects, and cow as the random effect. The least square means and associated SE were obtained, and multiple comparisons were adjusted with Tukey correction. The Pearson correlations (r) between the response variables were obtained following the CORR procedure. The results were considered significant when $P < 0.05$, and trends were discussed when $0.05 \leq P < 0.10$.

Results

The first three principal components obtained in the PCA accounted for 48 % of the total variance. The first one (Dim 1, 25.6 % of variance) was positively associated with the peaks and AUCs of NEFA, and negatively with the peaks and AUCs of milk yield during restriction (Fig. 3a). The second principal component (Dim 2, 12.5 % of variance) was associated positively with the AUCs of NEFA during both restriction and refeeding, and negatively with peaks and AUCs of milk yield and BHB during restriction. Finally, the third principal component was associated positively with the peak and AUC of milk yield in months 2 and 4, and with days to regain the baseline values of all the traits in month 2, and negatively with peak and AUC of BHB in month 4 (data not shown). The clustering analysis generated two clusters which differed in their MR, named Low MR (n = 16) and High MR (n = 15) (Fig. 3b). The cows in the Low MR cluster had lower energy requirements and a less negative EB and showed a poorer response to restriction in terms of milk yield and plasma NEFA and BHB concentrations. The cows in the High MR cluster showed a stronger response (Fig. 4).

Considering individual DM intake, on average diets met 91 %, 61 % and 93 % of the net energy requirements and 100 %, 58 % and 103 % of the metabolisable protein requirements during the basal, restriction and refeeding periods, respectively. Cow BW and BCS during the basal period did not differ between MR clusters (591 vs 590 kg in the Low MR and the High MR, respectively, $P = 0.91$; 2.80 vs 2.70 BCS points, respectively, $P = 0.18$). Both traits were affected by lactation stage and were higher in month 2 than thereafter (599, 588 and 584 kg in months 2, 3 and 4, respectively, $P < 0.001$; 2.81, 2.73 and 2.71, respectively, $P < 0.001$). The milk yield response to feed restriction and subsequent refeeding according to the MR cluster and the month of lactation is shown in Table 2. The MR cluster affected the baseline values and the response to restriction ($P \leq 0.04$), but not the recovery pattern in the refeeding phase. The High MR cows had a higher baseline milk yield and AUC values during restriction and tended to have greater peak milk loss. The month of lactation affected all the response variables during restriction ($P \leq 0.02$), but not during refeeding. A lower baseline yield was observed in month 4, and peak loss was greater in month 3 than in month 4, with intermediate values in month 2. The peak was reached more quickly, and total milk loss (AUC during restriction) was greater in month 3, with similar values in months 2 and 4.

The response of the plasma NEFA and BHB concentrations is shown in Table 3. For NEFA, the MR cluster affected the baseline values, peak and AUC during restriction ($P \leq 0.001$), with higher values obtained by the High MR cows. No differences were observed in the days to peak or to regain the baseline. All the NEFA response variables were affected by the month of lactation ($P \leq 0.04$). The baseline values were lower in month 4 compared

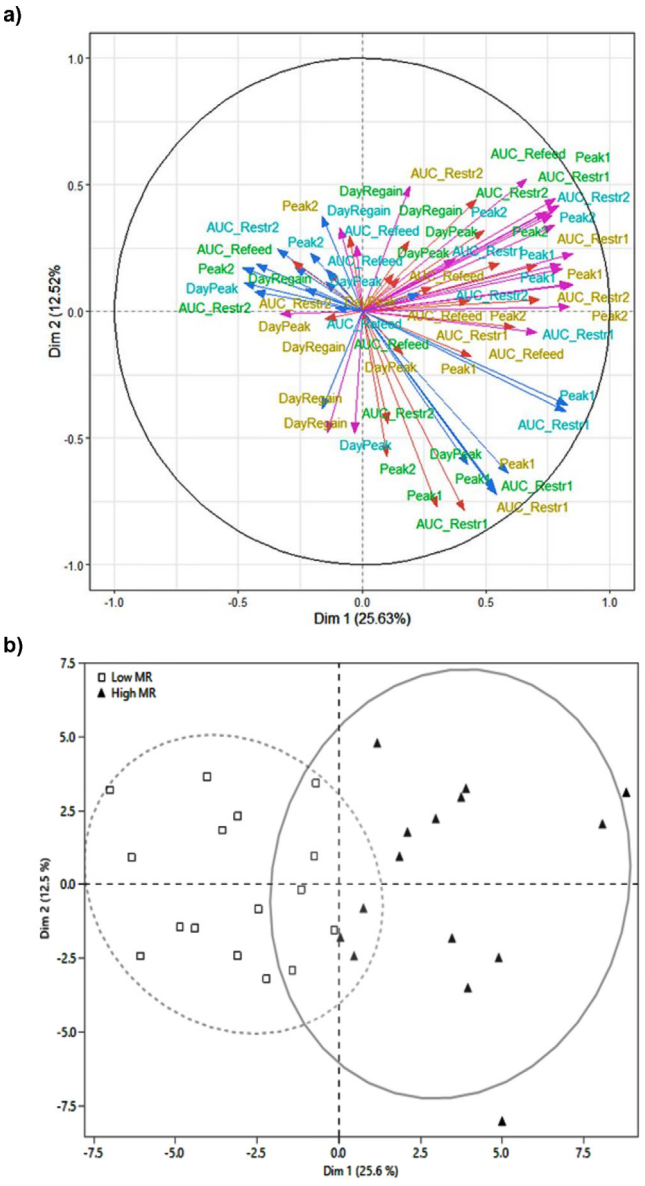


Fig. 3. Variable factor map of the first two Principal Components (3a). Variables related to milk yield (blue arrows), plasma non-esterified fatty acids (pink arrows) and plasma β-hydroxybutyrate (red arrows), and months of lactation 2, 3, and 4 for yellow, green, and blue text labels, respectively. Distribution of the cows into the two generated metabolic response (MR) clusters (3b).

to the other two months. Peak concentrations during restriction decreased significantly from lactation month 2 to lactation month 4 and were reached more quickly in month 4 than in the others. The days to regain baseline were also affected by month, with faster recovery in months 2 and 4 than in month 3. Only the AUC during refeeding was affected by the interaction between the MR cluster and the month of lactation (Fig. 5a). Regarding the BHB response, the baseline values and the AUC during restriction were higher in the High MR than in the Low MR cluster ($P \leq 0.02$). The month of lactation affected both parameters and the AUC during refeeding, which were lower in month 3 ($P \leq 0.03$), and tended to affect the days to regain the baseline ($P = 0.06$). Finally, the peak was affected by the interaction between the MR cluster and the month of lactation ($P = 0.03$), and the differences between the MR clusters were only significant in month 2, but not thereafter. Furthermore, the peak BHB in the Low MR cows remained stable

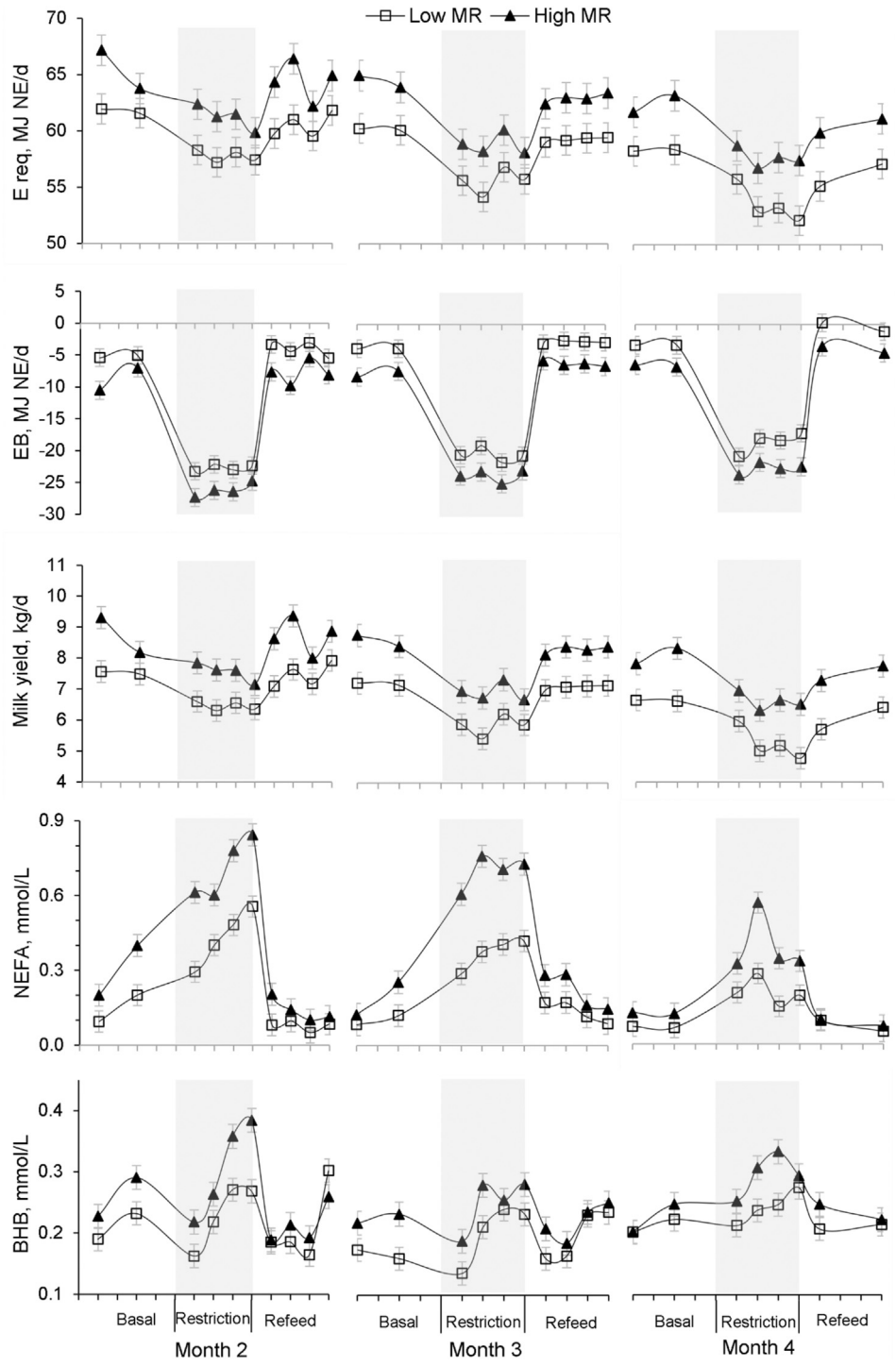


Fig. 4. Energy requirements (E req), energy balance (EB), milk yield, and plasma non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) concentrations of Low and High metabolic response (MR) beef cows during the experiment. Means are plotted, and the vertical bars indicate the SE.

throughout lactation, whereas the values in their High MR counterparts were higher in month 2 than later (Fig. 5b).

The significant correlations among the response variables of the milk yield, NEFA and BHB concentrations, all months considered, are shown in Fig. 6. Within trait, the AUC during restriction correlated strongly with the peak ($P < 0.001$), but not with the days to peak. For milk yield, the baseline values correlated negatively with the peak and AUC during restriction ($P < 0.001$). Milk loss (AUC) during refeeding correlated positively with the peak and AUC during restriction, but negatively with days to peak and to regain baseline ($P < 0.001$). For NEFA, the baseline values correlated positively with the peak and AUCs during restriction and refeeding ($P < 0.001$). The AUC during refeeding correlated strongly with the peak and AUC during restriction, and only moderately with days to peak and regain the baseline ($P < 0.001$). Regarding BHB, the AUC during refeeding correlated positively with the peak and AUC during restriction ($P < 0.001$). In the three traits, the correlations between days to peak and days to regain baseline were not significant. Across traits, the milk yield baseline values correlated moderately with the NEFA peak and AUC during restriction and the BHB baseline values, and weakly with the NEFA baseline values and the BHB peak ($P \leq 0.03$). The NEFA peak correlated weakly

Table 2
Effect of metabolic response (MR) cluster and month of lactation on the milk yield response of beef cows to a 4-day restriction and a 4-day refeeding period.

	MR Cluster (Cl)		Month (M)				*P*-values[1]	
Item	Low MR	High MR	2	3	4	RSD	Cl	M
Baseline, kg/d	6.94^{y}	8.27^{x}	8.10^{a}	7.80^{a}	6.92^{b}	0.584	0.002	0.001
Peak*, kg/d	−1.32	−1.56	−1.45^{ab}	−1.61^{b}	−1.27^{a}	0.463	0.068	0.020
Days to peak, d	2.57	2.63	2.80^{a}	1.78^{b}	3.22^{a}	0.990	0.813	0.001
$AUC^{\dagger}_{restriction}$*, kg	−3.80^{y}	−4.81^{x}	−4.01^{a}	−5.21^{b}	−3.70^{a}	1.656	0.036	0.002
Days to regain baseline, d	5.93	5.74	5.65	5.98	5.87	0.935	0.326	0.376
$AUC^{\dagger}_{refeeding}$*, kg	−0.83	−0.74	−0.68	−0.82	−0.86	0.798	0.644	0.647

Within a variable, least square means with different superscripts ($^{x, y}$) differ between MR clusters with $P < 0.05$; least square means with different superscripts ($^{a, b}$) differ among months with $P < 0.05$.
† area under the curve; *deviation from baseline.
[1] the interaction was not significant for any variable ($P > 0.05$).

Table 3
Effect of metabolic response (MR) cluster and month of lactation on plasma non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) response of beef cows to a 4-day restriction and a 4-day refeeding period.

	MR Cluster (Cl)		Month (M)				*P*-values[1]	
Item	Low MR	High MR	2	3	4	RSD	Cl	M
NEFA								
Baseline, mmol/l	0.09^{y}	0.15^{x}	0.13^{a}	0.15^{a}	0.08^{b}	0.049	0.001	0.001
Peak*, mmol/l	0.26^{y}	0.51^{x}	0.54^{a}	0.38^{b}	0.24^{c}	0.129	0.001	0.001
Days to peak, d	2.94	3.05	3.38^{a}	3.09^{a}	2.51^{b}	0.583	0.453	0.001
$AUC^{\dagger}_{restriction}$*, mmol × d/l	0.68^{y}	1.42^{x}	1.36^{a}	1.17^{a}	0.62^{b}	0.396	0.001	0.001
Days to regain baseline, d	5.74	5.74	5.55^{b}	6.08^{a}	5.59^{b}	0.869	0.991	0.036
$AUC^{\dagger}_{refeeding}$*, mmol × d/l	0.13^{y}	0.21^{x}	0.24^{a}	0.23^{a}	0.04^{b}	0.094	0.001	0.001
BHB								
Baseline, mmol/l	0.220^{y}	0.248^{x}	0.238^{ab}	0.222^{b}	0.243^{a}	0.031	0.024	0.026
Peak, mmol/l	0.07^{y}	0.11^{x}	0.12^{a}	0.07^{b}	0.08^{b}	0.068	0.002	0.003
Days to peak, d	3.20	3.11	3.29	3.08	3.09	0.815	0.574	0.540
$AUC^{\dagger}_{restriction}$*, mmol × d/l	0.04^{y}	0.13^{x}	0.10^{a}	0.02^{b}	0.13^{a}	0.135	0.011	0.006
Days to regain baseline, d	5.30	5.21	4.91	5.29	5.56	1.064	0.662	0.062
$AUC^{\dagger}_{refeeding}$*, mmol × d/l	−0.003	0.01	0.01^{a}	−0.02^{b}	0.02^{a}	0.045	0.175	0.001

Within a variable, least square means with different superscripts ($^{x, y}$) differ between MR clusters with $P < 0.05$; least square means with different superscripts ($^{a, b, c}$) differ among months with $P < 0.05$.
† area under the curve; *deviation from baseline.
[1] the interaction was significant for NEFA $AUC_{refeeding}$ ($P = 0.01$) and BHB Peak ($P = 0.03$).

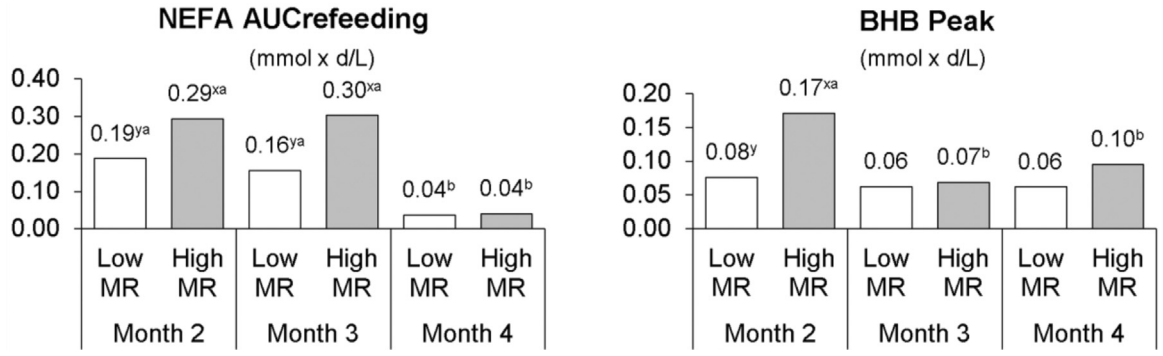


Fig. 5. Effect of the metabolic rate (MR) cluster and month of lactation on non-esterified fatty acids (NEFAs) $AUC_{refeeding}$ (5a) and β-hydroxybutyrate (BHB) peak (5b) in beef cows in response to a 4-d restriction and a 4-d refeeding period. For each response variable, means with different superscripts ($^{x, y}$) differ between MR clusters within month ($P < 0.05$) and with different superscripts ($^{a, b}$) differ among months within MR clusters with ($P < 0.05$). $AUCr_{efeeding}$: area under the curve during the refeeding period.

with the BHB peak and negatively with the milk yield peak ($P \leq 0.03$), but the milk yield and BHB peaks did not correlate. The AUCs of milk yield and NEFA during restriction were negatively correlated ($P \leq 0.003$), but not with those of BHB.

Discussion

Response curves

Different mathematical models have been used to characterise milk yield in dairy cows, from traditional models describing the shape of the lactation curve to individually adjusted polynomial curves based on well-established statistical models (Harder et al., 2019). Fewer modelling studies have been conducted on beef cattle because it is not routinely measured in common practice (Cortés-Lacruz et al., 2017; Sepchat et al., 2017; Sapkota et al., 2020). Animal performance can be affected by perturbations caused by climate, management or diseases, which can compromise both animal nutrition and welfare. Several studies have evaluated the response of ruminant females to natural (Poppe et al., 2020; Adriaens et al., 2021) or induced (Codrea et al., 2011; Friggens et al., 2016; Barreto-Mendes et al., 2022) perturbations, and found wide interindividual variations. They have analysed deviations

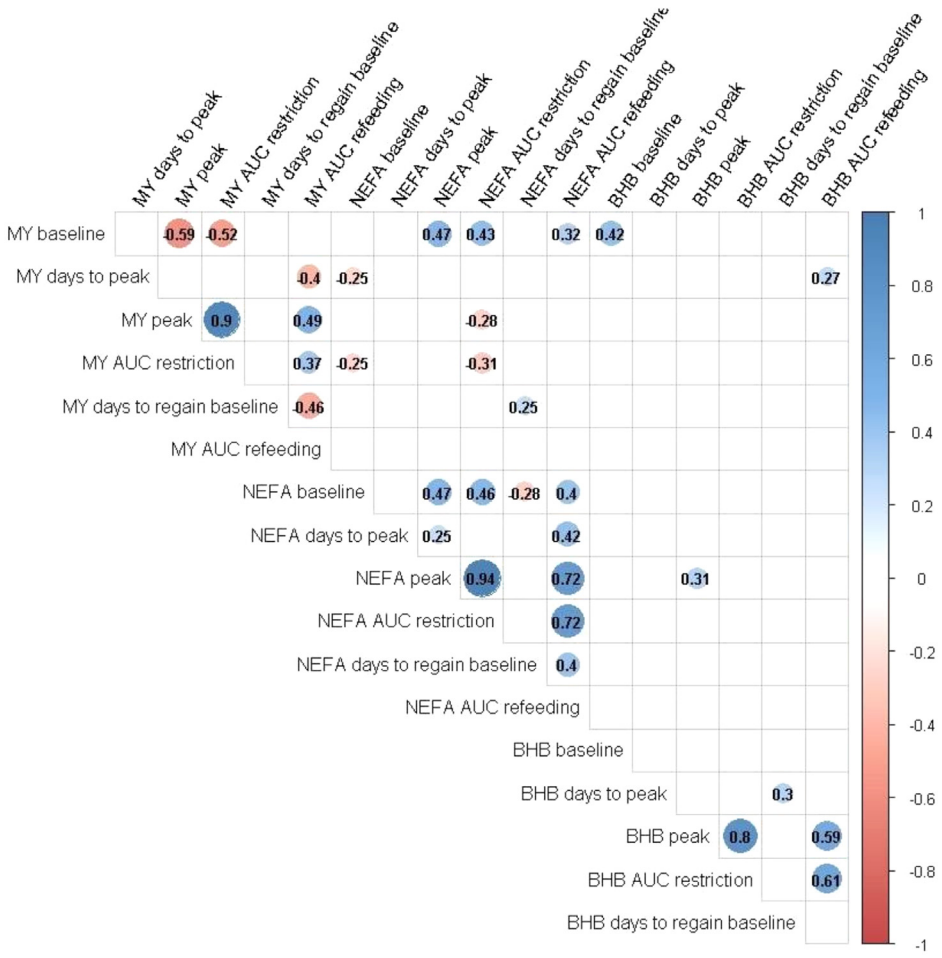


Fig. 6. Significant Pearson correlations between the response variables of milk yield (MY) and the plasma non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) concentrations in beef cows. AUC: area under the curve.

from a theoretical unperturbed lactation curve (Ben Abdelkrim et al., 2021a), which corresponds to the baseline in our study, and they have described the response while conducting challenges and in the recovery phase. Although most studies have modelled milk yield, this methodology could be extrapolated to other biological time-series data (Codrea et al., 2011), which are increasingly available with the rise of in-line measurement technologies. Ben Abdelkrim et al. (2021b) used a similar model to simultaneously predict the dynamics of milk yield and BW response over time, and to explore the relation between them, as we do herein with plasma NEFA and BHB.

Effect of the metabolic response cluster

The clustering analysis identified two distinct groups of cows that differed mainly in terms of their milk yield and NEFA response, and less markedly in their BHB response to nutritional challenges. Both BW and the BCS were similar in the two clusters throughout lactation, which implies that body size or fat reserves did not affect the response, which would be driven mainly by milk yield and the concomitant metabolic effort to sustain it. These findings are similar to those reported by Pedernera et al. (2008), who found that BW and BCS changes did not accurately reflect the extent of mobilisation of dairy cows' body reserves in early lactation. Schuh et al. (2019) reported that the BCS affected reserve mobilisation intensity, with higher NEFA and BHB serum concentrations in the cows with a high BCS, but this was not the case in our study. Breed or parity (Adriaens et al., 2021; Ben Abdelkrim et al., 2021a) can also influence individual responses to perturbations, but they did not differ between the MR clusters.

The size of the response was related to basal performance. All the basal values were higher in the High MR than in the Low MR profile, which coincides with Friggens et al. (2016). At the individual level, significant correlations were observed between the basal values and the response during restriction (peaks and AUCs) for milk yield and NEFA, but not for BHB. Berghof et al. (2019) have also indicated that high-performing animals can be more sensitive to perturbations. Interestingly, these differences were only observed in the magnitude of the response, but not in the time taken to react and recover, which reflects the plasticity of cows' response.

The impact of feed restriction on milk yield can widely range (from −7% to −71 %) depending on restriction severity and duration, and also on the lactation stage (Leduc et al., 2021). Here, the absolute milk loss was higher in the High MR than in the Low MR cows, but peak milk loss in relative terms was 19 % of the basal milk yield for both groups. When comparing Holstein and Montbéliarde cows, with different prechallenge milk yields, Billa et al. (2020) also observed a similar relative response to a 6-day 50 %-feed restriction between them. The MR cluster did not affect the time taken to reach the peak here (mean 2.6 days) or to regain the baseline (5.8 days), which implies that responses were larger, but not faster, in the High MR than in the Low MR cows. Both reaction times were shorter than those observed in

natural (Adriaens et al., 2021) or induced (Bjerre-Harpøth et al., 2012) perturbations in dairy cows, which is likely due to the lower milk yield and the associated metabolic load of beef cows.

Homeorhetic controls regulate different metabolic adaptations to support lactation. Of them, growth hormone and insulin are key mediating factors responsible for the partition of nutrients away from body storage and towards the mammary gland (Knight et al., 2004; Baumgard et al., 2017). Although the hormones involved in this partitioning were not herein investigated, we observed significant effects of feed restriction on the plasma metabolites that result from their action, which were more evident in NEFA than in BHB. With poor nutrient supply, cows mobilise adipose tissue by releasing circulating NEFA so they are either converted into milk triglycerides in the udder or oxidised in the liver as an energetic substrate (Bell, 1995). All the NEFA response variables had almost doubled in the High MR than in the Low MR cluster, which denotes that the cows with higher milk yields had greater basal fat mobilisation and were able to further increase lipolysis during the nutritional challenge. Excessive lipid mobilisation can surpass the liver's metabolic capacity to oxidise NEFA, and ketone bodies such as BHB are produced (Mann et al., 2016). Thus, the High MR cows also had a higher BHB peak and AUC during restriction than the Low MR cows. Threshold values of 0.60 mmol NEFA/l (Jorjong et al., 2014) and 1.2 mmol BHB/L (Li et al., 2012) are associated with the risk of clinical ketosis in dairy cows. Regarding NEFA, they were reached only by the High MR cows during the peaks of months 2 and 3, but not by the Low MR cows, and never for BHB, which suggests that circulating NEFA supplied enough energy to meet the metabolic demands induced by nutrient restriction.

The response profiles observed herein suggest that the High MR cows had a higher potential milk yield and were able to efficiently partition more nutrients towards milk synthesis than the Low MR cows. Elgersma et al. (2018) considered that dairy cows with fewer milk yield fluctuations under natural perturbations were more resilient because the minor variance in performance genetically correlated with better health and longevity. Conversely, we can conclude that the High MR cows were able to establish homeorhetic mechanisms in the short term (Bauman and Currie, 1980) with sufficient intensity to ensure that, despite their more negative EB, they continued to display better lactation performance and recovered after the challenge. Ollion et al. (2016) have described different profiles in dairy cows depending on their lactation performance, reproduction and ability to maintain their reserves, the most determinant life functions among which trade-offs have often been identified. They found that milk yield was an important driver of these profiles, as we observed in the present work, but not the only one given the wide individual variability in the strategies to prioritise nutrient allocation to these life functions.

Effect of the lactation stage

Previous studies have analysed the adaptations of lactating ruminants to feed restriction in different phases. Within-animal responses are repeatable between early- and mid-lactation in dairy cows (Gross and Bruckmaier, 2015), between consecutive lactations in dairy goats (Friggens et al., 2016) and between two consecutive feeding challenges of different duration in beef cattle (De la Torre et al., 2022), which indicate that variability may be genetically driven. Here, we clustered cows according to their response throughout lactation and analysed the month of lactation separately, finding a strong effect on most response variables. The general lack of interactions between MR cluster and month confirmed the validity of our approach.

To the best of our knowledge, no comparable studies are available on beef cows in different lactation stages. As stated above, the lactation curves of beef breeds are less well-known than those of dairy cattle. Sepchat et al. (2017) have described slow increases in milk production after calving, which peaked between the first and third lactation months. The curve was flatter than in dairy cows due to the balance between a calf's ability to drink milk and the dam's production potential. A recent meta-analysis by Sapkota et al. (2020) described earlier peak milk yields dairy-beef crosses (4–6 weeks) compared to pure beef cows (5–8 weeks), the latter showing a better persistency. The basal milk yield here was similar in months 2 and 3, which suggests that the peak was reached before week 8, and then decreased in month 4. The basal values agreed with previous observations in multiparous Parda de Montaña cows, as in Blanco et al. (2008), regardless of suckling management, calf sex or supplementation (Cortés-Lacruz et al., 2017).

The impact of feed restriction on milk yield was higher in month 3 than in months 2 and 4, as shown by the greater peak loss (in both absolute and relative terms, 21 % vs 18 %), which was attained more quickly, and the total milk loss. With an induced short-term feed restriction, Bjerre-Harpøth et al. (2012) found a similar milk loss in relation to prechallenge values (30 %) in early-, mid- and late lactation with dairy cows, unlike our results. In response to natural perturbations, effects were severer, developed more quickly and recovered more slowly in early- to mid-lactation than in later stages (Adriaens et al., 2021). Conversely, we found that the lactation stage did not affect the recovery rate during refeeding, as observed by Codrea et al. (2011).

Whereas the milder effect of nutrient restriction in later stages (i.e. in month 4) was supported by the above-mentioned literature, the stronger impact in month 3 than in month 2 was not expected given the similar energy and protein intake. We hypothesise that, as the basal milk yield was similar, but both BW and the BCS were lower in month 3, these beef cows' coping strategies in month 3 were not sufficient to buffer the effect of feed restriction on milk production. The basal NEFA concentrations were similar in months 2 and 3, and were higher than those of month 4, but the peak values of NEFA and BHB decreased steadily, and were reached more quickly for NEFA, as lactation progressed. All this indicates decreasing lipid mobilisation. Apparently, despite the metabolic demand for milk yield still being high in month 3, these beef cows' response to homeorhetic controls was not sufficient to ensure adequate nutrient supply to support milk synthesis under the feed restriction. Baumgard et al. (2017) indicated that when a negative EB occurs, the dairy cows selected for higher milk yield are able to partition more nutrients away from storage and towards mammary utilisation. The opposite would be the case in our study, where that response would be less intense in beef cows with a lower genetic capacity for milk production. This is supported by the findings of Pareek et al. (2007), who compared the response to a metabolic challenge between breeds of different genetic merits for milk yield, and found that dairy cows had lower insulin levels, a lower EB, but greater milk production efficiency than beef cows which, in turn, had a higher potential for body energy and protein accretion.

Regarding the BHB peak, the interaction between month and the MR cluster implied that lipid mobilisation was insufficient only in month 2 for the High MR cows, and the ketogenesis from NEFA resulted in a greater BHB peak in response to feed restriction in early lactation. The higher metabolic load in earlier lactation stages has been described in dairy cows, with natural NEFA peaks 1–2 weeks postpartum and a delayed response in BHB peaks at 2–3 weeks (Kessel et al., 2008; Gross et al., 2011), which decrease thereafter. In Parda de Montaña beef cows fed at 75 % (Alvarez-Rodríguez et al., 2009) or 100 % (Noya et al., 2019) of their requirements, NEFA peaked at 0.27–0.35 mmol/l up to week 5 postpartum and then decreased to reach 0.08 mmol/l in month 4, whereas BHB

contents remained constant (approx. 0.20 mmol/l) throughout lactation (Rodríguez-Sánchez et al., 2018).

This effect of month on the basal values could condition the coping strategies which cows apply to face undernutrition in different stages. Bjerre-Harpøth et al. (2012) found decreasing basal NEFA concentrations from early to late lactation, and high BHB contents only in early lactation. When short-term energy deficit was induced, the relative changes in NEFA during restriction increased throughout lactation, while BHB only responded in early lactation. Other studies report that plasma NEFA concentrations are less responsive to feed restriction in late lactation (Carlson et al., 2006; Gross et al., 2011), when even a drastic energy restriction may not increase the BHB concentration if there are not sufficient NEFAs for ketogenesis. According to our results, in a recent review on the effects of feed restriction on dairy cows, Leduc et al. (2021) found that NEFA increased (+14 % to + 3475 %) in most studies, while the effect on BHB was less consistent (+26 % to + 721 % in only 14 of the 23 studies).

Conclusion

Changes in the performance and plasmatic indicators of lipolysis and ketogenesis of beef cows in response to short-term feed restriction can be modelled using spline curves, which allows different MR profiles to be established. The extent, but not the speed, of the individual response was driven primarily by basal milk yield, but adaptation strategies changed as lactation advanced, and as the nutrient demand for milk production and concomitant fat mobilisation decreased. Although long-term performance should also be evaluated, identifying animals that can respond to a nutritional challenge by establishing mechanisms to minimise the impact on their performance is key to develop breeding programmes for enhanced beef cows' resilience.

Ethics approval

The Animal Ethics Committee of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) approved all experimental procedures (protocol no. CEEA-03-2018-01), which followed the guidelines of the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author ORCIDs

Karina G. Orquera-Arguero: 0000-0002-6805-9063
Daniel Villalba: 0000-0001-8919-0450
Mireia Blanco: 0000-0003-3875-4935
Javier Ferrer: 0000-0002-4048-3786
Isabel Casasús: 0000-0003-3943-5311

Author contributions

Conceptualization: **IC, MB, DV**; Methodology: **IC, MB, KO**; Investigation; **KO, JF, IC**; Formal analysis: **KO, DV, MB**; Supervision: **IC, MB**; Writing-Original draft: **KO**, Writing-Review & Editing: **IC, MB, DV**; Project administration: **IC**; Funding acquisition: **IC**.

Declaration of interest

None.

Acknowledgements

The authors thank the farm staff at CITA La Garcipollera Research Station for their technical support.

Financial support statement

Work was supported by the European Union's Horizon 2020 research and innovation program (GenTORE, grant agreement No. 727213) and the Government of Aragón (Grant Research Group Funds A14_20R, predoctoral contract of K. G. Orquera-Arguero).

References

Adriaens, I., van den Brulle, I., D'Anvers, L., Statham, J.M.E., Geerinckx, K., De Vliegher, S., Piepers, S., Aernouts, B., 2021. Milk losses and dynamics during perturbations in dairy cows differ with parity and lactation stage. Journal of Dairy Science 104, 405–418.

Alvarez-Rodríguez, J., Palacio, J., Casasús, I., Revilla, R., Sanz, A., 2009. Performance and nursing behaviour of beef cows with different types of calf management. Animal 3, 871–878.

AOAC, 2000. Official methods of analysis. The Association of Official Analytical Chemists (AOAC), Arlington, VA, USA.

Barreto-Mendes, L., De La Torre, A., Ortigues-Marty, I., Cassar-Malek, I., Pires, J., Blanc, F., 2022. How to approach the resilience of livestock exposed to environmental challenges? Quantification of individual response and recovery by means of differential calculus. Animal - Open Space 1, 100008.

Bauman, D., Currie, B., 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. Journal of Dairy Science 63, 1514–1529.

Baumgard, L.H., Collier, R.J., Bauman, D.E., 2017. A 100-Year Review: Regulation of nutrient partitioning to support lactation. Journal of Dairy Science 100, 10353–10366.

Bell, A., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of Animal Science 73, 2804–2819.

Ben Abdelkrim, A., Puillet, L., Gomes, P., Martin, O., 2021a. Lactation curve model with explicit representation of perturbations as a phenotyping tool for dairy livestock precision farming. Animal 15, 100074.

Ben Abdelkrim, A., Tribout, T., Martin, O., Boichard, D., Ducrocq, V., Friggens, N.C., 2021b. Exploring simultaneous perturbation profiles in milk yield and body weight reveals a diversity of animal responses and new opportunities to identify resilience proxies. Journal of Dairy Science 104, 459–470.

Berghof, T.V.L., Poppe, M., Mulder, H., 2019. Opportunities to improve resilience in animal breeding programs. Frontiers in Genetics 9, 692.

Billa, P.A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J.A.A., 2020. Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. Journal of Dairy Science 103, 3133–3146.

Bjerre-Harpøth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., Moyes, K.M., 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. Journal of Dairy Science 95, 2362–2380.

Blanco, M., Villalba, D., Ripoll, G., Sauerwein, H., Casasús, I., 2008. Effects of preweaning concentrate feeding on calf performance, carcass and meat quality of autumn-born bull calves weaned at 90 or 150 days of age. Animal 2, 779–789.

Carlson, D.B., Litherland, N.B., Dann, H.M., Woodworth, J.C., Drackley, J.K., 2006. Metabolic effects of abomasal L-carnitine infusion and feed restriction in lactating Holstein cows. Journal of Dairy Science 89, 4819–4834.

Codrea, M., Højsgaard, S., Friggens, N.C., 2011. Differential smoothing of time-series measurements to identify disturbances in performance and quantify animal response characteristics: An example using milk yield profiles in dairy cows. Journal of Animal Science 89, 3089–3098.

Cortés-Lacruz, X., Casasús, I., Revilla, R., Sanz, A., Blanco, M., Villalba, D., 2017. The milk yield of dams and its relation to direct and maternal genetic components of weaning weight in beef cattle. Livestock Science 202, 143–149.

De Koster, J., Salavati, M., Grelet, C., Crowe, M.A., Matthews, E., O'Flaherty, R., Opsomer, G., Foldager, L., Hostens, M., 2019. Prediction of metabolic clusters in early-lactation dairy cows using models based on milk biomarkers. Journal of Dairy Science 102, 2631–2644.

De La Torre, A., Barreto-Mendes, L., Pires, J.A.A., Cassar-Malek, I., Ortigues-Marty, I., Blanc, F., 2022. Exploration of robustness indicators using adaptive responses to short-term feed restriction in suckling primiparous beef cows. Animal 16, 100556.

Elgersma, G.G., de Jong, G., van der Linde, R., Mulder, H.A., 2018. Fluctuations in milk yield are heritable and can be used as a resilience indicator to breed healthy cows. Journal of Dairy Science 101, 1240–1250.
Friggens, N.C., Duvaux-Ponter, C., Etienne, M.P., Mary-Huard, T., Schmidely, P., 2016. Characterizing individual differences in animal responses to a nutritional challenge: Toward improved robustness measures. Journal of Dairy Science 99, 2704–2718.
Gross, J.J., Bruckmaier, R.M., 2015. Repeatability of metabolic responses to a nutrient deficiency in early and mid lactation and implications for robustness of dairy cows. Journal of Dairy Science 98, 8634–8643.
Gross, J., van Dorland, H.A., Bruckmaier, R.M., Schwarz, F.J., 2011. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. Journal of Dairy Science 94, 1820–1830.
Harder, I., Stamer, E., Junge, W., Thaller, G., 2019. Lactation curves and model evaluation for feed intake and energy balance in dairy cows. Journal of Dairy Science 102, 7204–7216.
INRA, 2007. Alimentation des bovins, ovins et caprins. Besoins des Animaux-Valeur des aliments, Editions Quae, Versailles, France.
Jorjong, S., van Knegsel, A.T.M., Verwaeren, J., Lahoz, M.V., Bruckmaier, R.M., De Baets, B., Kemp, B., Fievez, V., 2014. Milk fatty acids as possible biomarkers to early diagnose elevated concentrations of blood plasma nonesterified fatty acids in dairy cows. Journal of Dairy Science 97, 7054–7064.
Kessel, S., Stroehl, M., Meyer, H.H.D., Hiss-Pesch, S., Sauerwein, H., Schwarz, F., Bruckmaier, R.M., 2008. Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. Journal of Animal Science 86, 2903–2912.
Knight, C., Alamer, M., Sorensen, A., Nevison, I., Flint, D., Vernon, R., 2004. Metabolic safety-margins do not differ between cows of high and low genetic merit for milk production. Journal of Dairy Research 71, 141–153.
Le Neindre, P., Dubroeucq, H., 1973. Observations sur l'estimation de la production laitière des vaches allaitantes per la pesée du veau avant et après la tétée. Annales de Zootechnie 22, 413–422.
Leduc, A., Souchet, S., Gelé, M., Le Provost, F., Boutinaud, M., 2021. Effect of feed restriction on dairy cow milk production: a review. Journal of Animal Science 99, 1–12.
Li, P., Li, X.B., Fu, S.X., Wu, C.C., Wang, X.X., Yu, G.J., Long, M., Wang, Z., Liu, G.W., 2012. Alterations of fatty acid β-oxidation capability in the liver of ketotic cows. Journal of Dairy Science 95, 1759–1766.
Mann, S., Nydam, D.V., Lock, A.L., Overton, T.R., McArt, J.A.A., 2016. Short communication: Association of milk fatty acids with early lactation hyperketonemia and elevated concentration of nonesterified fatty acids. Journal of Dairy Science 99, 5851–5857.
Noya, A., Casasús, I., Achiaga, J., Sanz, A., 2019. Long-term effects of maternal subnutrition in early pregnancy on cow-calf performance, immunological and physiological profiles during the next lactation. Animals 9, 936.
Ollion, E., Ingrand, S., Delaby, L., Trommenschlager, J.M., Colette-Leurent, S., Blanc, F., 2016. Assessing the diversity of trade-offs between life functions in early lactation dairy cows. Livestock Science 183, 98–107.
Pareek, N., Voigt, J., Bellmann, O., Schneider, F., Hammon, H.M., 2007. Energy and nitrogen metabolism and insulin response to glucose challenge in lactating German Holstein and Charolais heifers. Livestock Science 112, 115–122.
Pedernera, M., García, S.C., Horagadoga, A., Barchia, I., Fulkerson, W.J., 2008. Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. Journal of Dairy Science 91, 3896–3907.
Perperoglou, A., Sauerbrei, W., Abrahamowicz, M., Schmid, M., 2019. A review of spline function procedures in R. BMC Medical Research Methodology 19, 46.
Poppe, M., Veerkamp, R.F., van Pelt, M.L., Mulder, H.A., 2020. Exploration of variance, autocorrelation, and skewness of deviations from lactation curves as resilience indicators for breeding. Journal of Dairy Science 103, 1667–1684.
R Development Core Team, 2014. R: A language and environment for statistical computing (https://www.R-project.org/). R Foundation for Statistical Computing, Vienna, Austria.
Rodríguez-Sánchez, J.A., Sanz, A., Ferrer, J., Casasús, I., 2018. Influence of postweaning feeding management of beef heifers on performance and physiological profiles through rearing and first lactation. Domestic Animal Endocrinology 65, 24–37.
Sapkota, D., Kelly, A.K., Crosson, P., White, R.R., McGee, M., 2020. Quantification of cow milk yield and pre-weaning calf growth response in temperate pasture-based beef suckler systems: A meta-analysis. Livestock Science 241, 104222.
Schuh, K., Sadri, H., Häussler, S., Webb, L.A., Urh, C., Wagner, M., Koch, C., Frahm, J., Dänicke, S., Dusel, G., Sauerwein, H., 2019. Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated *v.* normal body condition at dry-off. Animal 13, 1478–1488.
Sepchat, B., D'hour, P., Agabriel, J., 2017. Production laitière des vaches allaitantes : caractérisation et étude des principaux facteurs de variation. INRAE Productions Animales 30, 139–152.
Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Research in Veterinary Science 159 (2023) 26–34

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Beef cows' performance and metabolic response to short nutritional challenges in different months of lactation

Karina G. Orquera-Arguero [a,b], Isabel Casasús [a,b], Javier Ferrer [a], Mireia Blanco [a,b,*]

[a] Departamento de Ciencia Animal, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059 Zaragoza, Spain
[b] Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Zaragoza, Spain

ARTICLE INFO

Keywords:
Feed restriction
Milk yield
Negative energy balance
Mobilisation

ABSTRACT

Lactating cows can react to changes in nutrient availability with a range of behavioural and physiological mechanisms, which may differ among lactation stages. We investigated the effects of short feed restriction and refeeding periods on beef cows' performance and metabolic status in different months of lactation. For this, Parda de Montaña beef cows [$n = 31$; 626 ± 47.7 kg body weight (BW)] were subjected to short nutritional restriction and refeeding cycles, which were repeated in months 2, 3 and 4 of lactation. Each month, cows were consecutively fed a diet to meet 100% of their energy and protein requirements during a 4-day basal period, 55% during a 4-day restriction period, and again 100% during a 4-day refeeding period. The performance (energy balance, BW, milk yield and composition) and plasma metabolite concentrations (glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), urea and malondialdehyde) were measured daily. Most of the traits were significantly affected by the interaction between feeding period and lactation month. Feed restriction induced milk yield loss, decreased milk protein and increased milk urea contents to different extents. The plasma NEFA concentrations rose with restriction in months 2, 3 and 4 but BHB and urea concentrations increased only in month 4. Most of these metabolites lowered to basal values during refeeding. These results suggest that beef cows use different adaptation strategies to cope with nutritional challenges as lactation advances, body fat mobilisation predominates in early lactation and protein catabolism prevails at later stages.

1. Introduction

Beef cows managed in temperate grassland systems depend very much on forage availability and quality during the grazing season, and also in the winter when they are usually group-fed preserved forages. Under these conditions, they face a dynamically changing nutrient supply, which can be inadequate to meet their requirements during some key physiological periods (Mulliniks and Beard, 2019). Projected climate changes, including more frequent extreme weather events, will further affect the quantity and nutritive value of the feed available throughout the production cycle (Henry et al., 2018). To successfully cope with these challenges, effective strategies need to be developed at both the animal and farm levels (Blanc et al., 2006).

Lactating cows respond to limiting nutritional environments with the mobilisation of body tissues and a range of behavioural and physiological mechanisms that involve modifications in nutrient allocation towards the different metabolic functions, whose priority differs depending on lactation stage (Bjerre-Harpøth et al., 2012; Murrieta et al., 2010). In order to disentangle the mechanisms that determine this metabolic flexibility in response to environmental change, the nutritional perturbations involving both short- and long-term feed restriction-refeeding cycles have been widely studied in dairy cows (Abdelatty et al., 2017; Gross et al., 2011a; Pires et al., 2019). In beef cattle, several papers have assessed cows' performance and metabolic response to long-term underfeeding (Alvarez-Rodríguez et al., 2009; Fiems et al., 2015), but adaptation to short-term nutrient restrictions has only been recently considered (De La Torre et al., 2022; Orquera-Arguero et al., 2022). Animals' ability to respond to and recover after short-term disturbances, defined as resilience (Friggens et al., 2022), is key for their performance in variable environments.

In dairy cows, the adaptive response to underfeeding usually implies reduced milk yield, and milk composition may, or may not, be affected depending on the length and intensity of restriction, among other factors (Boutinaud et al., 2019; Kvidera et al., 2017; Leduc et al., 2021). In order

* Corresponding author at: Departamento de Ciencia Animal, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059 Zaragoza, Spain.
E-mail address: mblanco@cita-aragon.es (M. Blanco).

https://doi.org/10.1016/j.rvsc.2023.04.002
Received 22 February 2023; Received in revised form 21 March 2023; Accepted 6 April 2023
Available online 7 April 2023

to overcome the negative energy balance (EB), cows will mobilise their body reserves, including both fat and protein. The mobilisation of body fat releases non-esterified fatty acids (NEFA) into the blood stream, which can be oxidised in the liver into ketone bodies, such as β-hydroxybutyrate (BHB), as energy fuel (Bell, 1995). Complementary, NEFA can be esterified to triglycerides and accumulate in the liver, or taken up by the mammary gland, where they account for a significant fraction of milk fat synthesis. When the oxidative metabolism is altered, excessive reactive oxygen species (ROS) production leads to oxidative stress (Abuelo et al., 2015), for which malondialdehyde (MDA), a degradation product of lipid peroxidation, has been proposed as a biomarker (Castillo et al., 2006). The catabolism of the protein mainly from the skeletal muscle yields glucogenic amino acids, and affects plasma glucose and urea concentrations (Ingvartsen et al., 2003). In *ad libitum*-fed dairy cows, body protein catabolism starts in the transition period (from 3 weeks before calving) and extends up to 5 weeks after calving, while fat reserves are mobilised up to 12 weeks postpartum, when feed intake matches milk yield requirements and endocrine status limits mobilisation (Sadri et al., 2023). This period can be shorter in lower milk-yielding breeds (Jorge-Smeding et al., 2021). When faced with temporary nutrient restriction, lactation stage plays a key role in the physiological adaptive response because the priority and requirements of the mammary gland change as lactation evolves by modifying the allocation of nutrients to milk synthesis (Boutinaud et al., 2019; Gross and Bruckmaier, 2019). Furthermore when cows are refed, the post-challenge recovery rate can be faster in later lactation stages (Bjerre-Harpøth et al., 2012). This information is not available in beef cows, where the influence of lactation stage on nutrient allocation may differ from that of dairy cows due to their lower milk yield and different feeding management because they are rarely fed to appetite and are often placed in limited nutrient environments (Mulliniks and Beard, 2019).

The aim of this experiment was to determine lactating beef cows' response to short-term feed restriction and refeeding periods in three different months of lactation both on the productive and physiological levels. We hypothesised that cows would respond to nutritional perturbations by reducing their milk yield and modifying their lipid and protein metabolism differently as lactation progressed.

2. Material and methods

The Animal Ethics Committee of the research centre approved all the experimental procedures (protocol no CEEA-03-2018-01), which followed the EU Directive 2010/63 guidelines on the protection of animals used for experimental and other specific purposes.

2.1. Animal management, experimental and diet design

The experiment was conducted at CITA La Garcipollera Research Station in the Pyrenees mountain area (Spain, 42°37′ N, 0°30′ W, 945 m a.s.l.) using 31 lactating Parda de Montaña beef cows [body weight (BW) (mean ± SD): 626 ± 47.7 kg; body condition score (BCS): 2.8 ± 0.22 (0–5 scale); age: 7.5 ± 2.91 yr]. Cows were randomly allocated in pens (7 or 8 cows/pen, 10 × 20 m) equipped with individual feeders for forage and automatic feeding stations (ALPRO, Alfa Laval Agri, Tumba, Sweden) for concentrate. Calves were stocked in straw-bedded cubicles adjacent to their dams. They were allowed to suckle their dams daily for two 30-min periods at 06:00 h and 14:00 h. All the cows received the same ration, which was composed of different quantities of hay and concentrate. The chemical composition and nutritive value of feedstuffs are presented in Table 1 (for detailed information see Orquera-Arguero et al., 2022). Diets were calculated by considering the net energy and metabolisable protein requirements for the maintenance and lactation (INRA, 2007) of a standard cow with a BW of 615 kg and a milk yield of 8.5 kg/d. From calving to the end of the experiment all the cows were fed a diet that met 100% standard cow energy and protein requirements, except for 3 restriction periods when they were fed a diet to meet 55% standard cow energy and protein requirements. The experiment consisted of three consecutive 4-day feeding periods, which were repeated over months 2, 3 and 4. Every month, the trial started with 4 days on which cows had access to the abovementioned diet, which met 100% of their requirements (basal period). For the next 4 days, they were fed a diet that met 55% requirements (restriction period). On the last 4 days, once again they received the formulated diet to meet their 100% requirements (refeeding period). On the first day of restriction periods, cows were in milk for 31 (month 2), 58 (month 3), and 87 (month 4) days.

Table 1
Chemical composition and nutritive value (mean ± standard deviation) of the feedstuffs offered to the beef cows.

	Hay	Concentrate
Chemical composition		
Dry matter (DM), g/kg	920 ± 10.9	908 ± 6.7
Ash, g/kg DM	87.5 ± 17.3	68.3 ± 1.6
Crude protein, g/kg DM	97.1 ± 20.5	170 ± 4.7
Neutral detergent fibre, g/kg DM	581 ± 51.0	252 ± 19.2
Acid detergent fibre, g/kg DM	330 ± 27.3	112 ± 11.5
Lignin, g/kg DM	34.9 ± 9.30	29.3 ± 8.10
Nutritive Value		
Net energy, MJ/kg DM	5.4 ± 0.13	7.4 ± 0.36
Metabolizable protein, g PDI[1] /kg DM	73 ± 12.1	121 ± 2.9

[1] true protein digestible in the small intestine.

The diet fed to meet 100% energy and protein requirements was composed of 7.4 kg dry matter (DM) hay and 2.7 kg DM concentrate. During restriction, cows received 6.4 kg DM hay to meet 55% of their energy and protein requirements. Throughout the experiment, water and mineral blocks were supplied *ad libitum*. Hay was offered daily as a single meal at 08:00 h in individual feeders with cows tied up for approximately 2 h until they had finished their ration. The ALPRO feeding stations were programmed to offer concentrate to all the cows during the basal and refeeding periods. The individual hay and concentrate intakes were recorded daily.

2.2. Measurements and samplings

All the cow measurements were taken daily in the morning before hay-feeding, and during each feeding period (basal, restriction, refeeding) in experiment months 2, 3 and 4. Cows were weighed on an electronic scale. Milk yield was estimated by the weight-suckle-weight technique of the calf (Le Neindre and Dubroeucq, 1973) as the sum of the milk consumed in both sucklings. After the morning suckling, a composite 50-mL milk sample was manually collected per cow from all four teats, after discarding 3 streams of milk per teat. After calf removal, cows were administered an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) 5 min before the manual extraction to facilitate the letdown of residual milk. Milk samples were preserved with sodium azide (PanReac, Barcelona, Spain) and refrigerated at 4 °C until further analyses. Cow blood samples were collected from the coccygeal vein in heparinised tubes (BD Vacutainer Becton-Dickenson and Company, Plymouth, UK) to determine BHB and MDA, and in tubes containing EDTA (BD Vacutainer Becton-Dickenson and Company) to analyse glucose, NEFA and urea concentrations. Immediately after collection, blood samples were centrifuged at 3500 rpm for 20 min at 4 °C, and plasma was frozen at −20 °C until further analyses.

2.3. Chemical analyses

In milk samples, lactose, fat, protein and urea contents, and somatic cell count, were determined with an infrared scan (Milkoscan 7 RM, Foss Electric Ltd., Hillerød, Denmark). Randox kits (Randox Laboratories Ltd., Country Antrim, UK) were employed to determine the plasma

concentrations of NEFA (colorimetric method, sensitivity: 0.072 mmol/L) and BHB (kinetic enzymatic method, sensitivity: 0.100 mmol/L). An automatic analyser (Gernon, RAL S.A., Barcelona, Spain) was used to measure the plasma concentrations of glucose (enzymatic-colorimetric method, sensitivity: 0.06 mmol/L) and urea (kinetic method, sensitivity: 0.056 mmol/L). The mean intra- and interassay coefficients were for NEFA: 4.0% and 4.9%, BHB: 6.8% and 6.8%; glucose: 2.2% and 2.4%; urea: 4.4% and 5.5%.

The plasma concentration of MDA, used as an indicator of oxidative status, was determined by liquid chromatography as described in Bertolín et al. (2019). An Acquity UPLC H-Class liquid chromatograph (Waters, Milford, Massachusetts, USA), equipped with a silica-based bonded phase column (Acquity UPLC HSS PFP, 100 mm × 2.1 mm × 1.8 μm, Waters), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ detector, Waters) and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters), were utilised. The intra- and interassay coefficients of variation were 4.6% and 7.3% for MDA, respectively.

2.4. Calculations and statistical analyses

The INRA system (INRA, 2007) was used to estimate the individual EB as the difference between inputs (net energy (NE) intake) and outputs (NE for maintenance and NE for lactation). The NE intake was estimated from the individual DM intake (DMI) and feedstuffs' energy contents. The NE for maintenance was calculated from the individual metabolic BW, and the NE for production was obtained using the milk yield, fat, and protein contents in milk.

Statistical analyses were performed by the SAS statistical package v 9.4 (SAS Institute Inc., Cary, NC, USA) and the R software. Normal data distribution was assessed with the Shapiro-Wilk test ($P > 0.05$). Normality could not be confirmed for the somatic cell count values. Therefore, analyses were run on the log-transformed data. Parameters were analysed with mixed models by taking feeding period (basal, restriction, refeeding), lactation month (months 2, 3 and 4), and their interaction, as fixed effects, and cow as the random effect. Degrees of freedom were adjusted with the Kenward-Roger correction. The least square means and associated standard errors were obtained and multiple comparisons were adjusted with Tukey correction. The Pearson's correlations between variables were obtained and presented on heatmaps for all the data and separately per feeding period using the CORRPLOT package of R (R Development Core Team, 2021). The level of significance for all the tests was $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$.

3. Results

The interaction between feeding period and lactation month affected all the parameters ($P < 0.05$ to $P < 0.001$), except milk yield, which only tended to be affected by this interaction ($P < 0.10$) and somatic cell count ($P > 0.05$). For each parameter, the basal values during the three lactation months, and then the effects of restriction and refeeding during the three lactation months are presented.

3.1. Cow performance

On average, 91%, 61% and 93% of the net energy requirements and 100%, 58% and 103% of the metabolisable protein requirements were met during the basal, restriction and refeeding periods, respectively. Cows' EB, BW, milk yield and milk composition are depicted in Fig. 1 according to feeding period and lactation month. The calculated basal EB improved progressively from month 2 to month 4 ($P < 0.01$). According to the experimental design, cows' EB was more negative during restriction than during the basal period in the three lactation months ($P < 0.001$). During refeeding, the EB returned to basal values in lactation months 2 and 3, but went even higher, close to a neutral EB, in lactation month 4 ($P < 0.001$). Basal BW decreased between months 2 and 4 ($P < 0.001$). BW diminished with restriction in the three lactation months (by −2.3%, −2.0% and − 1.7% in months 2, 3 and 4, respectively). During refeeding, BW lowered by a further 1% in month 2 ($P < 0.001$), but remained unchanged in months 3 and 4 ($P > 0.05$).

The basal milk yield was higher in months 2 and 3 than in month 4 ($P < 0.05$ to $P < 0.001$). Milk yield decreased with restriction in the three lactation months by −14%, −19% and − 20% in months 2, 3 and 4, respectively ($P < 0.001$). Milk yield increased during refeeding and reached the basal values in months 2 and 3, but stayed below the basal values in lactation month 4 (by −8%; $P = 0.03$). Regarding milk composition in the basal phase, lactose, fat and urea contents were not affected by lactation month ($P > 0.05$), whereas protein content was higher in month 2 than in the subsequent months ($P < 0.001$), and somatic cell counts were lower in month 2 than thereafter (99, 135 and 131 × 10^3 cells/mL in months 2, 3 and 4, respectively, $P < 0.05$).

Feed restriction did not affect milk lactose in month 2, but lowered in months 3 and 4 (by −1.9 and − 1.5%, respectively) and then increased during refeeding in the three lactation months ($P < 0.001$). Milk fat content was similar regardless of feeding periods ($P > 0.05$). Protein content lowered with restriction in months 2 and 3 (by −5% and − 4%, respectively; $P < 0.001$), but was not affected in month 4 ($P > 0.05$). It remained stable during refeeding in months 2 and 4, but increased to reach the basal values in month 3. Milk urea content increased during restriction in the three months by +8%, +21%, and + 37% in months 2, 3 and 4, respectively ($P < 0.05$), and decreased during refeeding, even below the basal values in month 2 and to the basal values in months 3 and 4 ($P < 0.001$). The highest somatic cell counts were obtained during refeeding (128, 159 and 186 × 10^3 cells/mL in the basal, restriction and refeeding period, respectively, $P < 0.05$).

3.2. Plasma metabolic profile

The plasma concentrations of NEFA, BHB, glucose, urea and MDA are presented in Fig. 2. Lactation month did not affect the basal concentrations of BHB and urea ($P > 0.05$), but affected those of NEFA, glucose and MDA ($P < 0.001$). The basal NEFA concentrations were higher in month 2 than in month 4 ($P < 0.001$). The basal glucose concentrations were lower in month 3 than in months 2 and 4 ($P \leq 0.001$). The basal MDA concentrations were higher in month 2 than in the subsequent months ($P < 0.001$).

Regarding the effect of feeding period, NEFA concentrations increased to different extents due to restriction in the three months (by +157%, +269% and + 212% in months 2, 3 and 4, respectively; $P < 0.001$), whereas refeeding lowered NEFA concentrations to below the basal value in month 2 ($P < 0.001$) and to basal values in months 3 and 4. The BHB concentration rose with restriction in the three months, but only significantly in month 4, by +14% ($P = 0.11$), +17% ($P = 0.11$) and + 23% ($P < 0.001$) in months 2, 3 and 4, respectively. During refeeding, BHB decreased and reached basal values in months 2 and 4. Glucose concentration dropped during restriction in month 2 ($P = 0.01$), with no changes thereafter ($P > 0.05$). During refeeding, it decreased in month 2 ($P < 0.001$), increased in month 3 ($P < 0.001$) and remained unchanged in month 4 ($P > 0.05$). The urea concentration rose significantly during restriction, but only in lactation month 4 (by +18%; $P < 0.001$), and lowered during refeeding below the basal values in months 2 and 4 ($P < 0.01$). The MDA concentration did not change with restriction and was only affected by refeeding in month 4, with higher values than during the basal period ($P = 0.03$).

The significant overall correlations with $r \geq 0.25$ between the performance parameters and plasma metabolites are shown in Fig. 3, whereas the correlations during each feeding period are depicted in Suppl. Fig. 1. The overall correlations were weak ($r = 0.25$ to 0.39) or moderate ($r = 0.40$ to 0.59), but were strong within feeding periods ($r = 0.60$ to 0.79) and very strong ($r \geq 0.80$) ($P < 0.001$). BW correlated positively with milk yield and negatively with the EB. Milk urea content

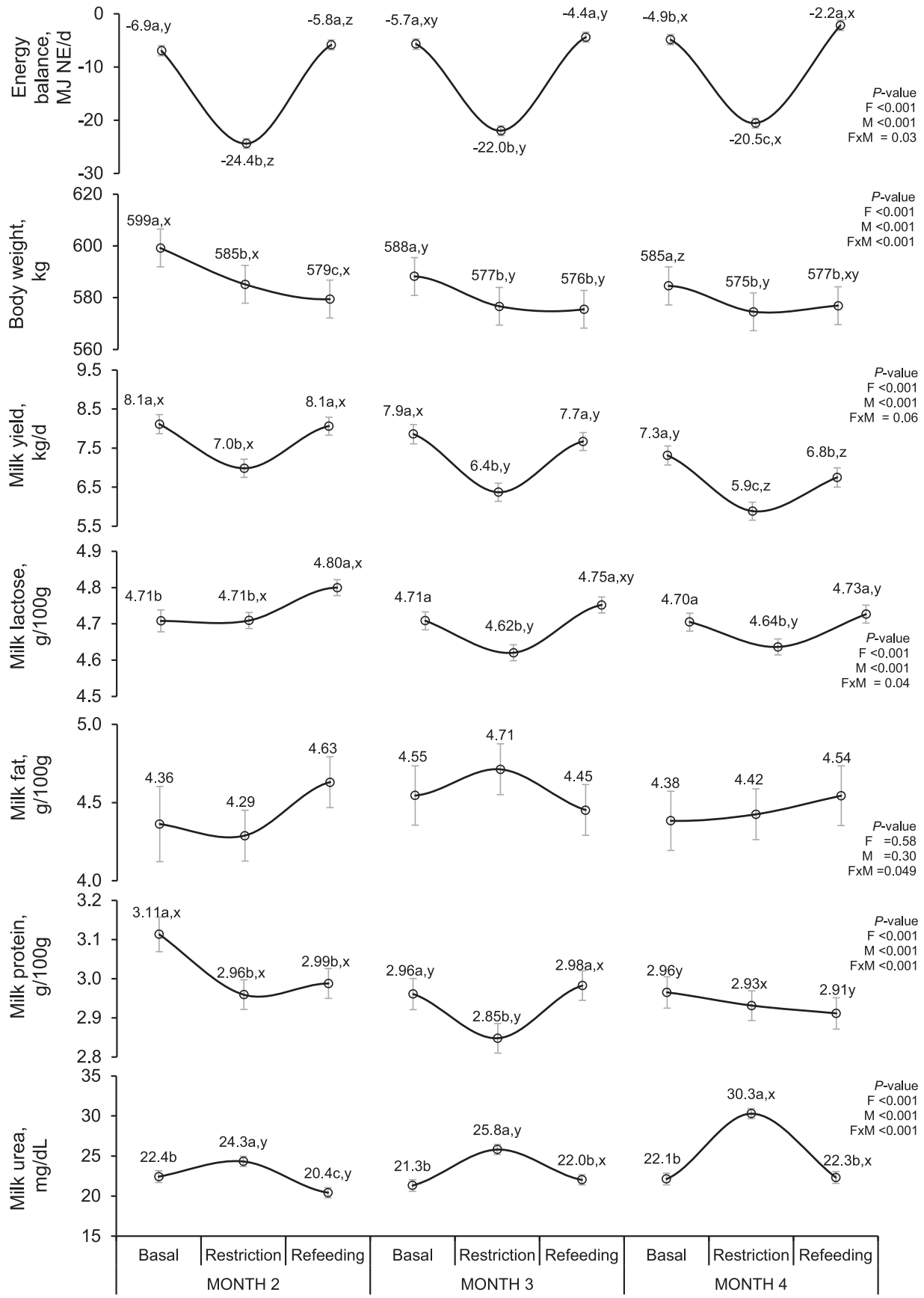


Fig. 1. Effect of feeding period (F: Basal, Restriction, Refeeding) and lactation month (M: 2, 3, 4) on energy balance (EB), BW, milk yield and milk composition. Within a parameter and month, the means with a different letter (a,b,c) indicate differences due to feeding period ($P < 0.05$). Within a parameter and feeding period, the means with a different letter (x,y,z) denote differences due to lactation month ($P < 0.05$).

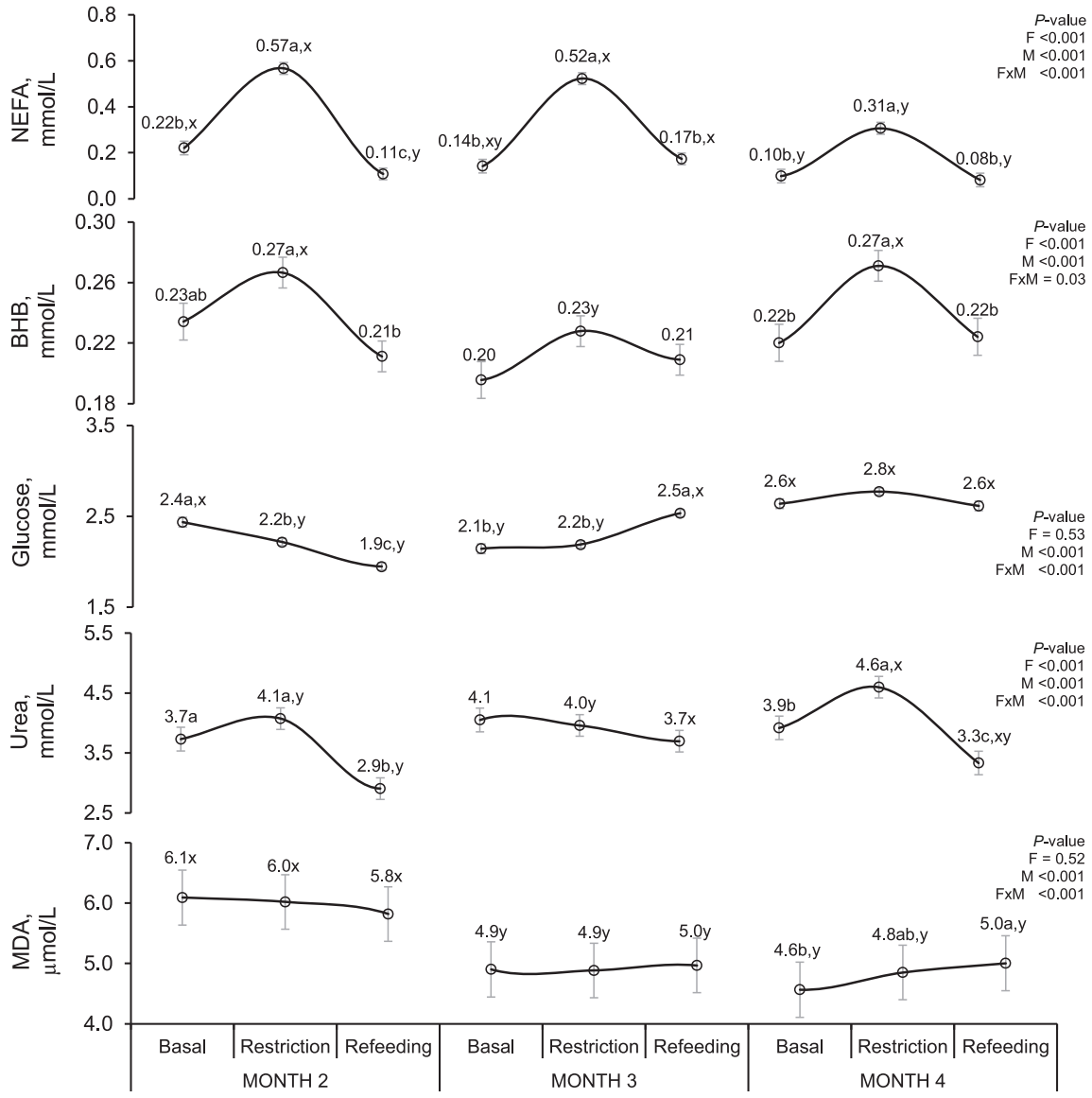


Fig. 2. Effect of feeding period (F: Basal, Restriction, Refeeding) and lactation month (M: 2, 3, 4) on plasma concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), glucose, urea and malondialdehyde (MDA).
Within a parameter and month, the means with a different letter (a,b,c) indicate differences due to feeding period ($P < 0.05$). Within a parameter and feeding period, the means with a different letters (x,y,z) denote differences due to lactation month ($P < 0.05$).

correlated negatively with milk yield and the EB ($P < 0.001$). Within the basal, restriction and refeeding periods, correlations were moderate between BW and the EB ($r = -0.52$ to -0.64), and were very strong between milk yield and the EB ($r = -0.87$ to -0.93). The plasma NEFA concentration correlated negatively with the EB and milk protein content, and positively with milk urea content ($P < 0.001$). The BHB concentration correlated negatively with the EB and positively with milk urea and the plasma concentrations of glucose, urea and MDA ($P < 0.001$). The plasma urea concentration correlated negatively with the EB and positively with milk urea content and plasma glucose concentration ($P < 0.001$). Within feeding periods, the plasma urea concentration correlated positively with BW, milk yield and milk protein during the basal period and with milk protein during the refeeding period ($P < 0.001$). The plasma MDA concentration correlated positively with BW, milk yield, milk protein content and plasma urea concentration, and negatively with the EB ($P < 0.001$).

4. Discussion

In the present experiment, restriction implied reductions of −36% in DMI, −42% in net energy intake and − 47% in protein intake on average. The restriction herein applied could be considered moderate according to the review by Leduc et al. (2021) because the reduction in DMI was <50%. Basal cow performance and some plasma metabolites differed among the three lactation months, as did their patterns of response to restriction and refeeding. This scenario suggests a change in the metabolic priority of different biological functions as lactation advanced.

4.1. Cow performance

BW loss between months 2 and 4 agrees with previous experiments with lactating Parda de Montaña cows (Blanco et al., 2009). Beef cows are rarely fed according to their theoretical requirements (Blanc et al., 2006). During lactation, they have to rely on the mobilisation of their body reserves to produce milk. In the present experiment, BW was only

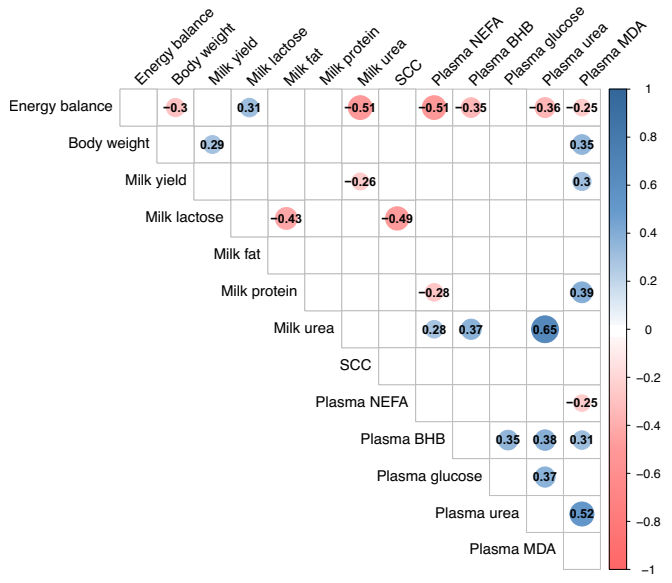


Fig. 3. Significant Pearson's rank correlations[1] between cow performance and plasma metabolites in all the lactation months.
[1]Only the significant correlations ($P < 0.05$) are presented and the correlations between equal variables are omitted. SCC: somatic cell count; NEFA: non-esterified fatty acids; BHB: β-hydroxybutyrate; MDA: malondialdehyde.

mildly affected by a short feed restriction, similarly to the −4 to −5% BW loss reported after a 4-day 50% DMI restriction in beef cows (De La Torre et al., 2022) and dairy cows (Ferraretto et al., 2014; Kvidera et al., 2017). This BW loss could be linked with the decrease in DMI, gut fill loss and mobilisation of body reserves (Gross et al., 2011a; Laeger et al., 2012). Incomplete BW recovery in the 4-day refeeding phase implies that a longer recovery period is needed; *e.g.* 10 days in beef cows after a similar restriction to that herein applied (De La Torre et al., 2022) or at least 1 to 2 weeks in dairy cows with severer restrictions that cause greater BW loss (−10%; Billa et al., 2020; Pires et al., 2019).

The lower basal milk yield values with progressing lactation agree with previous data on Parda de Montaña cows (Casasús et al., 2004; Dervishi et al., 2017), and suggest that the peak milk yield had already been reached at the start of the experiment, in month 2, as described by Sapkota et al. (2020) for beef cows. In the present study, the reduced milk yield caused by feed restriction falls in line with those reported by other studies of comparable lengths and restriction severities in beef cows (−12%; De La Torre et al., 2022) and dairy cows (−13 to −20% in Abdelatty et al., 2017; Laeger et al., 2012; Nielsen et al., 2003). The milk loss magnitude was lower in month 2, when cows displayed the most negative EB, than thereafter. Several homeorhetic mechanisms involved in nutrient partitioning regulation concur to maintain milk yield during feed restriction periods or metabolic imbalance, *e.g.* decreased glucose use, increased body lipids use and the mobilisation of protein reserves as energy sources (Bauman and Currie, 1980; Ingvartsen et al., 2003). However, these regulation processes are stage-dependent and the adaptive response diminishes with advancing lactation (Blanc et al., 2006). Our results indicate that the metabolic priority of the mammary gland in feed-restricted beef cows decreased after month 2. This would be supported by the shift in nutrient partitioning away from the udder towards subcutaneous adipose tissue, as observed on 60 d postpartum in beef cows by Murrieta et al. (2010). The milk yield response to refeeding was fast, with full recovery occurring within 4 days in months 2 and 3, but not in month 4. The lower milk synthesis priority in this later stage may increase the necessary recovery time. A quick response to refeeding has also been reported in low-producing beef cows (2 days for full recovery; De La Torre et al., 2022), but more days are required for full recovery with high-producing dairy cows in early lactation (7 to 8 days; Bjerre-Harpøth et al., 2012; Pires et al., 2019).

Concerning milk composition, the basal milk protein and lactose contents were similar, but fat content was higher than those previously reported in Parda de Montaña cows with a similar milk yield (Casasús et al., 2004; Dervishi et al., 2017). This difference was probably related to the sampling method. In this study, milk samples were manually obtained after calves had suckled (alveolar milk). In the above-mentioned studies, they were collected by machine milking before calves had access to their dam (cisternal milk). The fat concentration in cisternal milk is lower than in alveolar milk, whereas milk protein content is minimally affected (Sarikaya et al., 2005). The basal milk composition was similar in the three months, except for the higher protein content in early lactation. In dairy cows, lactose regulates milk osmolality and generally remains constant throughout lactation, while milk fat and protein tend to decrease from peak lactation in response to improved nutritional status and lower milk yield (Gross and Bruckmaier, 2019). All this was confirmed in our experiment for lactose and protein, but not for fat. This was probably due to the smaller differences in the EB and milk yield among months here than those observed in high-producing dairy cows. Furthermore, the stable basal milk urea throughout lactation agrees with the results reported in beef cows in the first three months of lactation (Wiseman et al., 2019) and in early-, mid- and late-lactating dairy cows (Bjerre-Harpøth et al., 2012).

Milk composition was affected by nutritional perturbation to different extents. Lactose content lowered with restriction and increased during refeeding, which agrees with previous reports in dairy cows that only needed 2 days to recover basal values after restriction had ended (Bjerre-Harpøth et al., 2012; Hervé et al., 2019; Sigl et al., 2013). The negative correlation herein observed between lactose content and somatic cell count has been associated with inflammatory reactions in milk secretory cells (Cinar et al., 2015). However in our study, somatic cell count was always below the threshold for subclinical mastitis (200×10^3 cells/mL; Dervishi et al., 2017).

Milk fat originates from either dietary or mobilisation fatty acids, which are taken up from the bloodstream, or by *de novo* synthesis in the mammary gland (Chilliard et al., 2000). Here milk fat content was not affected by feed restriction, which is consistent with previous results in dairy cows restricted at 50–60% during 4–5 days with 10–22% milk yield loss (Abdelatty et al., 2017; Carlson et al., 2006; Gross et al., 2011a). Other experiments with 30–50% milk loss report increases in milk fat content during feed restriction (Agenäs et al., 2003; Bjerre-Harpøth et al., 2012), which are associated with an increment in the long-chain fatty acids that arise from body fat mobilisation (Gross et al., 2011b). Apparently fat mobilisation and the concurrent rise in circulating NEFA would not have been enough to increase milk fat content in our study, but could have made the proportion of long *vs.* short- and medium-chain fatty acids higher, as observed by Orquera-Arguero et al. (2023).

Milk protein may decrease with feed restriction, but changes in milk urea depend on the nature of restriction (Leduc et al., 2021) given the influence by feed intake, but also by urea transfer from blood to milk, and *vice versa* (Spek et al., 2016). Here we observed reductions in milk protein (in months 2 and 3) and increments in milk urea contents in response to simultaneous reduction in dietary energy and protein supply. These findings agree with other experiments with 50% nutritional restriction, *e.g.* -7% milk protein and + 21% milk urea content in Carlson et al. (2006), −5.6% milk protein in Gross et al. (2011a). The higher milk urea content during restriction, especially in month 4, and its negative correlation with the EB suggests that protein catabolism took place in this phase to compensate for reduced energy intake, and this adaptation mechanism was more intense in later lactation stages. Body protein mobilisation to obtain glucose as an energy substrate increases circulating urea, which can be diffused from the blood stream to mammary glands (Spek et al., 2016). When restriction ended, basal values were regained after four refeeding days in most cases, except for milk protein in month 2. This suggests quicker recovery than that observed in high-producing dairy cows (Billa et al., 2020; Bjerre-

Harpøth et al., 2012; Pires et al., 2019).

4.2. Plasma metabolic profile

Plasma metabolites have commonly been used as indicators of energy, protein and oxidative status (Castillo et al., 2006; van Knegsel et al., 2007). The basal values herein observed were similar to those reported in lactating Parda de Montaña cows fed their 100% requirements in the case of NEFA, BHB and urea (Alvarez-Rodríguez et al., 2009), but were lower than those of glucose (Rodríguez-Sánchez et al., 2018). The fact that basal NEFA decreased from month 2 to month 4 indicates that the lipid mobilisation needed to support the energy demand for milk yield decreased throughout lactation, as shown in dairy cattle (Gross et al., 2011a; Jorge-Smeding et al., 2021). Basal BHB remained stable, as noted by Rodríguez-Sánchez et al. (2018) in beef cows, but were unlike the results of Bruckmaier and Gross (2017) in Holstein cows, where BHB peaked between 2 and 3 weeks postpartum and decreased thereafter, which suggest more metabolic stress for dairy cows in early lactation.

Feed restriction in months 2 and 3 increased the plasma NEFA concentrations to >2-fold their basal values, which came close to the compromised metabolic status threshold in dairy cows (0.57–0.60 mmol/L; Ospina et al., 2010), but induced a milder response in month 4. This supports the high priority of nutrient partitioning towards the mammary gland in response to reduced energy supply in earlier lactation stages, when body fat is largely mobilised and NEFA are released to provide energy for milk synthesis. Orquera-Arguero et al. (2022) observed wide variability in this response among beef cows, with more marked increments in cows' BW and milk yield. Plasma BHB responded to reduced nutrient intake to a much lesser degree (+15 to 20%), and only significantly so in month 4, and remained far below the risk threshold for subclinical ketosis (>1.2 mmol/L) (Benedet et al., 2019). The greater increments in NEFA than in BHB concentrations in response to reduced feed supply agree with previous studies with similar restrictions in dairy cows (Kvidera et al., 2017; Moyes et al., 2009; Pires et al., 2019), but they did not even change in Charolais cows with lower milk yield BHB (De La Torre et al., 2022). Both metabolites reacted quickly to refeeding, and basal values had recovered within 4 days, which agrees with other studies in beef (De La Torre et al., 2022) and dairy cattle (Abdelatty et al., 2017; Gross et al., 2011a), regardless of lactation stage (Billa et al., 2020; Bjerre-Harpøth et al., 2012).

The response of plasma glucose to diet changes was not consistent across lactation stages in the present study because it only decreased with restriction in month 2. The stronger effect on early lactation has been ascribed by Bjerre-Harpøth et al. (2012) to greater physiological imbalance, and could be driven by higher mammary glucose uptake for lactose synthesis (Gross et al., 2011a). In beef cows, no relevant changes were observed when feed was reduced at 54 or 75 days from calving (De La Torre et al., 2022). The literature reports conflicting results on the effect of moderate feed restrictions on glycaemia, which may decrease or remain stable, and has been considered a poor indicator of energy status in cows because gluconeogenesis can balance its concentration (Leduc et al., 2021).

Plasma urea is influenced by a wide variety of interrelated factors, such as dietary protein intake and muscle tissue breakdown when energy supply is insufficient (Puppel and Kuczyńska, 2016). Protein mobilisation from skeletal muscle releases glucogenic amino acids, which are used to supply glucose (Ingvartsen et al., 2003) and to generate urea during the process (Agenäs et al., 2006). The concentrations herein noted fell within the range reported for adequately nourished cows (1.8 to 7 mmol/L; Agenäs et al., 2006), and basal values remained stable throughout lactation, as observed by Bjerre-Harpøth et al. (2012) in early-, mid- and late-lactating cows. The lack of effect of feed restriction in months 2 and 3 agrees with previous reports in beef (De La Torre et al., 2015) or dairy cows (Hervé et al., 2019; Laeger et al., 2012), although other authors have found reduced blood urea in feed-restricted cows (Kvidera et al., 2017). The fact that restriction elicited a rise in the plasma urea concentration in month 4, when protein intake did not differ from previous months, implies that a certain degree of protein catabolism took place during restriction. This resulted in stable glycaemia in this month, as observed by Fiems et al. (2007) in energy-restricted beef cows. Apparently in late lactation, cows rely less on the mobilisation of fat reserves and more on the mobilisation of lean mass as a strategy to cope with a short-term nutritional challenge.

The metabolic adaptation to a negative EB can intensify the NEFA oxidation processes in the liver, and can result in both increased ROS production and oxidative stress developing (Turk et al., 2008), which occur with an imbalance between ROS production and antioxidant availability (van Knegsel et al., 2014). The values obtained in the present experiment are far below the concentrations reported by Castillo et al. (2006) for Holstein cows, which lowered from 69 to 29 μmol/L in the 8 first weeks of lactation. In our case, the higher MDA concentrations in early lactation (month 2) than thereafter, as observed by Castillo et al. (2006) in dairy cows, are likely the consequence of the higher plasma NEFA concentrations available for oxidation (Abuelo et al., 2015; Shi et al., 2015), with which they correlated.

5. Conclusions

Short-term restriction-refeeding periods resulted in both productive and metabolic adaptations in lactating beef cows. The most relevant responses to feed restriction were a drop in milk yield and an increase in the plasma NEFA concentrations, although their magnitude of change decreased as lactation advanced. In early postpartum, the mobilisation of fat reserves partially buffered the impact of a moderate feed restriction on milk yield. In later stages, when priority for milk production decreased, body protein reserves were also mobilised and longer recovery times were needed to compensate for a less effective response. Our results show that beef cows use different metabolic strategies to face nutritional perturbations depending on lactation stage.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.04.002.

Funding

This work was supported by the European Union's Horizon 2020 Research and Innovation Program (GenTORE, grant agreement No. 727213) and the Government of Aragón (Grant Research Group Funds, pre-doctoral contract of K. G. Orquera- Arguero).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the farm staff at CITA-La Garcipollera Research Station for their technical support.

References

Abdelatty, A.M., Iwaniuk, M.E., Garcia, M., Moyes, K.M., Teter, B.B., Delmonte, P., Kadegowda, A.K.G., Tony, M.A., Mohamad, F.F., Erdman, R.A., 2017. Effect of short-term feed restriction on temporal changes in milk components and mammary lipogenic gene expression in mid-lactation Holstein dairy cows. J. Dairy Sci. 100, 4000–4013. https://doi.org/10.3168/jds.2016-11130.

Abuelo, A., Hernández, J., Benedito, J.L., Castillo, C., 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 99, 1003–1016. https://doi.org/10.1111/jpn.12273.

Agenäs, S., Dahlborn, K., Holtenius, K., 2003. Changes in metabolism and milk production during and after feed deprivation in primiparous cows selected for different milk fat content. Livest. Prod. Sci. 83, 153–164. https://doi.org/10.1016/S0301-6226(03)00096-4.

Agenäs, S., Heath, M.F., Nixon, R., Wilkinson, J.M., Phillips, C., 2006. Indicators of undernutrition in cattle. Anim. Welf. 15.
Alvarez-Rodríguez, J., Palacio, J., Casasús, I., Revilla, R., Sanz, A., 2009. Performance and nursing behaviour of beef cows with different types of calf management. Animal 3, 871–878. https://doi.org/10.1017/S175173110900408X.
Bauman, D., Currie, B., 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63, 1514–1529. https://doi.org/10.3168/jds.S0022-0302(80)83111-0.
Bell, A., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73, 2804–2819. https://doi.org/10.2527/1995.7392804x.
Benedet, A., Manuelian, C.L., Zidi, A., Penasa, M., De Marchi, M., 2019. β-hydroxybutyrate concentration in blood and milk and its associations with cow performance. Animal 13, 1676–1689. https://doi.org/10.1017/S175173111900034X.
Bertolín, J.R., Joy, M., Blanco, M., 2019. Malondialdehyde determination in raw and processed meat products by UPLC-DAD and UPLC-FLD. Food Chem. 298, 125009 https://doi.org/10.1016/j.foodchem.2019.125009.
Billa, P.A., Faulconnier, Y., Larsen, T., Leroux, C., Pires, J.A.A., 2020. Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. J. Dairy Sci. 103, 3133–3146. https://doi.org/10.3168/jds.2019-17466.
Bjerre-Harpøth, V., Friggens, N.C., Thorup, V.M., Larsen, T., Damgaard, B.M., Ingvartsen, K.L., Moyes, K.M., 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different stages of lactation. J. Dairy Sci. 95, 2362–2380. https://doi.org/10.3168/jds.2011-4419.
Blanc, F., Francois, B., Agabriel, J., D'hour, P., Chilliard, Y., 2006. Adaptive abilities of the females and sustainability of ruminant livestock systems. A review. https://doi.org/10.1051/animres:2006040 55 doi: 10.1051/animres:2006040.
Blanco, M., Villalba, D., Ripoll, G., Sauerwein, H., Casasús, I., 2009. Effects of early weaning and breed on calf performance and carcass and meat quality in autumn-born bull calves. Livest. Sci. https://doi.org/10.1016/j.livsci.2008.05.003.
Boutinaud, M., Herve, L., Quesnel, H., Lollivier, V., Finot, L., Dessauge, F., Chanat, E., Lacasse, P., Charton, C., Guinard-Flament, J., 2019. The cellular mechanisms underlying mammary tissue plasticity during lactation in ruminants. Animal 13, s52–s64. https://doi.org/10.1017/S1751731119000624.
Bruckmaier, R.M., Gross, J.J., 2017. Lactational challenges in transition dairy cows. Anim. Prod. Sci. 57, 1471–1481.
Carlson, D.B., Litherland, N.B., Dann, H.M., Woodworth, J.C., Drackley, J.K., 2006. Metabolic effects of abomasal L-carnitine infusion and feed restriction in lactating Holstein cows. J. Dairy Sci. 89, 4819–4834. https://doi.org/10.3168/jds.S0022-0302(06)72531-0.
Casasús, I., Sanz, A., Villalba, D., Ferrer, R., Revilla, R., 2004. Intake capacity of two breeds of suckler cattle of different milk yield potential and validation of prediction models. Livest. Prod. Sci. 89, 195–207. https://doi.org/10.1016/j.livprodsci.2004.02.003.
Castillo, C., Hernández, J., Valverde, I., Pereira, V., Sotillo, J., Löpez Alonso, M., Benedito, J.L., 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. Res. Vet. Sci. 80, 133–139. https://doi.org/10.1016/j.rvsc.2005.06.003.
Chilliard, Y., Ferlay, A., Mansbridge, R.M., Doreau, M., 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Anim. Res. 49, 181–205. https://doi.org/10.1051/animres:2000117.
Cinar, M., Serbester, U., Ceyhan, A., Gorgulu, M., 2015. Effect of somatic cell count on milk yield and composition of first and second lactation dairy cows. Ital. J. Anim. Sci. 14, 105–108. https://doi.org/10.4081/ijas.2015.3646.
De La Torre, A., Recoules, E., Blanc, F., Ortigues-Marty, I., D'Hour, P., Agabriel, J., 2015. Changes in calculated residual energy in variable nutritional environments: an indirect approach to apprehend suckling beef cows' robustness. Livest. Sci. 176, 75–84. https://doi.org/10.1016/j.livsci.2015.03.008.
De La Torre, A., Barreto-Mendes, L., Pires, J.A.A., Cassar-Malek, I., Ortigues-Marty, I., Blanc, F., 2022. Exploration of robustness indicators using adaptive responses to short-term feed restriction in suckling primiparous beef cows. animal 16, 100556. https://doi.org/10.1016/J.ANIMAL.2022.100556.
Dervishi, E., Blanco, M., Rodríguez-Sánchez, J.A., Sanz, A., Calvo, J.H., Casasús, I., 2017. Milk yield and genomewide expression profiling in the mammary gland of beef primiparous cows in response to the dietary management during the pre- and postweaning periods. J. Anim. Sci. 95, 4274–4287. https://doi.org/10.2527/jas2017.1736.
Ferraretto, L.F., Gencoglu, H., Hackbart, K.S., Nascimento, A.B., Dalla Costa, F., Bender, R., Guenther, J.N., Shaver, R.D., Wiltbank, M.C., 2014. Effect of feed restriction on reproductive and metabolic hormones in dairy cows. J. Dairy Sci. 97, 754–763. https://doi.org/10.3168/jds.2013-6925.
Fiems, L.O., Vanacker, J.M., De Boever, J.L., Van Caelenbergh, W., Aerts, J.M., De Brabander, D.L., 2007. Effect of energy restriction and re-alimentation in Belgian Blue double-muscled beef cows on digestibility and metabolites. J. Anim. Physiol. Anim. Nutr. (Berl). 91, 54–61. https://doi.org/10.1111/j.1439-0396.2006.00641.x.
Fiems, L.O., De Boever, J.L., Ampe, B., Vanacker, J.M., De Campeneere, S., Sonck, B., 2015. Effect of energy restriction and dietary protein level during the re-alimentation period on feed efficiency in double-muscled Belgian Blue cows. Livest. Sci. 174, 46–52. https://doi.org/10.1016/j.livsci.2015.01.017.
Friggens, N., Adriaens, I., Boré, R., Cozzi, G., Jurquet, J., Kamphuis, C., Leiber, F., Lora, I., Sakowski, T., Statham, J.M.E., Haas, Y., 2022. Resilience: reference measures based on longer-term consequences are needed to unlock the potential of precision livestock farming technologies for quantifying this trait. Peer Commun. J. 2 https://doi.org/10.24072/pcjournal.136.
Gross, J.J., Bruckmaier, R.M., 2019. Metabolic challenges and adaptation during different functional stages of the mammary gland in dairy cows: perspectives for sustainable milk production. J. Dairy Sci. 102, 2828–2843. https://doi.org/10.3168/jds.2018-15713.
Gross, J.J., van Dorland, H.A., Bruckmaier, R.M., Schwarz, F.J., 2011a. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. J. Dairy Sci. 94, 1820–1830. https://doi.org/10.3168/jds.2010-3707.
Gross, J.J., Van Dorland, H.A., Bruckmaier, R.M., Schwarz, F.J., 2011b. Milk fatty acid profile related to energy balance in dairy cows. J. Dairy Res. 78, 479–488. https://doi.org/10.1017/S0022029911000550.
Henry, B.K., Eckard, R.J., Beauchemin, K.A., 2018. Adaptation of ruminant livestock production systems to climate changes. Animal 12, s445–s456. https://doi.org/10.1017/S1751731118001301.
Hervé, L., Quesnel, H., Véron, M., Portanguen, J., Gross, J., Bruckmaier, R.M., Boutinaud, M., 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfoliation in dairy cows. J. Dairy Sci. 102 https://doi.org/10.3168/jds.2018-15398.
Ingvartsen, K.L., Dewhurst, R.J., Friggens, N.C., 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest. Prod. Sci. 83, 277–308. https://doi.org/10.1016/S0301-6226(03)00110-6.
INRA, 2007. Alimentation des bovins, ovins et caprins. Besoins des Animaux-Valeur des aliments, Quae Versailles. ed. Paris, France.
Jorge-Smeding, E., Astigarraga, L., Loza, C., Carriquiry, M., Rico, D.E., Oxiley, A., 2021. Characterisation of productive performance, body reserve dynamics and energy metabolic parameters of Holstein Friesian and Normande dairy cows managed in a low-input pastoral dairy system. Anim. Prod. Sci. doi. https://doi.org/10.1071/AN21250.
Kvidera, S.K., Horst, E.A., Fernandez, M.V.S., Abuajamieh, M., Ganesan, S., Gorden, P.J., Green, H.B., Schoenberg, K.M., Trout, W.E., Keating, A.F., 2017. Characterizing effects of feed restriction and glucagon-like peptide 2 administration on biomarkers of inflammation and intestinal morphology. J. Dairy Sci. 100, 9402–9417.
Laeger, T., Görs, S., Metges, C.C., Kuhla, B., 2012. Effect of feed restriction on metabolites in cerebrospinal fluid and plasma of dairy cows. J. Dairy Sci. 95, 1198–1208. https://doi.org/10.3168/jds.2011-4506.
Le Neindre, P., Dubroeucq, H., 1973. Observations sur l'estimation de la production laitière des vaches allaitantes per la peseè du veau avant et aprés la tétée. Ann. Zootech. 22, 413–422.
Leduc, A., Souchet, S., Gelé, M., Le Provost, F., Boutinaud, M., 2021. Effect of feed restriction on dairy cow milk production: a review. J. Anim. Sci. 99, 1–12. https://doi.org/10.1093/jas/skab130.
Moyes, K.M., Drackley, J.K., Salak-Johnson, J.L., Morin, D.E., Hope, J.C., Loor, J.J., 2009. Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation. J. Dairy Sci. 92, 4301–4316. https://doi.org/10.3168/jds.2009-2170.
Mulliniks, J., Beard, J., 2019. Sustainable and economically viable management options for cow/calf production through enhanced beef cow metabolic efficiency. J. Anim. Sci. 97, 1398–1406. https://doi.org/10.1093/jas/sky476.
Murrieta, C.M., Hess, B.W., Lake, S.L., Scholljegerdes, E.J., Rule, D.C., 2010. Body condition score and day of lactation regulate fatty acid metabolism in milk somatic cells and adipose tissue of beef cows. Livest. Sci. 131, 65–72. https://doi.org/10.1016/J.LIVSCI.2010.02.028.
Nielsen, N.I., Ingvartsen, K.L., Larsen, T., 2003. Diurnal variation and the effect of feed restriction on plasma and milk metabolites in TMR-fed dairy cows. J. Vet. Med. Ser. A Physiol. Pathol. Clin. Med. 50, 88–97. https://doi.org/10.1046/j.1439-0442.2003.00496.x.
Orquera-Arguero, K.G., Villalba, D., Blanco, M., Ferrer, J., Casasús, I., 2022. Modelling beef cows' individual response to short nutrient restriction in different lactation stages. Animal 16, 100619. https://doi.org/10.1016/j.animal.2022.100619.
Orquera-Arguero, K.G., Blanco, M., Bertolín, J.R., Ferrer, J., Casasús, I., 2023. Performance and milk fatty acid profile of beef cows with a different energy status with short nutrient restriction and refeeding. J. Anim. Sci. skad053 https://doi.org/10.1093/jas/skad053.
Ospina, P.A., Nydam, D.V., Stokol, T., Overton, T.R., 2010. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: critical thresholds for prediction of clinical diseases. J. Dairy Sci. 93, 546–554. https://doi.org/10.3168/jds.2009-2277.
Pires, J.A.A., Pawlowski, K., Rouel, J., Delavaud, C., Foucras, G., Germon, P., Leroux, C., 2019. Undernutrition modified metabolic responses to intramammary lipopolysaccharide but had limited effects on selected inflammation indicators in early-lactation cows. J. Dairy Sci. 102, 5347–5360. https://doi.org/10.3168/jds.2018-15446.
Puppel, K., Kuczyńska, B., 2016. Metabolic profiles of cow's blood; a review. J. Sci. Food Agric. 96, 4321–4328. https://doi.org/10.1002/jsfa.7779.
R Development Core Team, 2021. R: A language and environment for statistical computing.
Rodríguez-Sánchez, J.A., Sanz, A., Ferrer, J., Casasús, I., 2018. Influence of postweaning feeding management of beef heifers on performance and physiological profiles through rearing and first lactation. Domest. Anim. Endocrinol. 65, 24–37. https://doi.org/10.1016/j.domaniend.2018.05.001.
Sadri, H., Ghaffari, M.H., Sauerwein, H., 2023. Invited review: muscle protein breakdown and its assessment in periparturient dairy cows. J. Dairy Sci. 106, 822–842. https://doi.org/10.3168/JDS.2022-22068.

Sapkota, D., Kelly, A.K., Crosson, P., White, R.R., McGee, M., 2020. Quantification of cow milk yield and pre-weaning calf growth response in temperate pasture-based beef suckler systems: a meta-analysis. Livest. Sci. 241, 104222 https://doi.org/10.1016/j.livsci.2020.104222.

Sarikaya, H., Werner-Misof, C., Atzkern, M., Bruckmaier, R.M., 2005. Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. J. Dairy Res. 72, 486–492. https://doi.org/10.1017/S0022029905001317.

Shi, X., Li, D., Deng, Q., Li, Y., Sun, G., Yuan, X., Song, Y., Wang, Z., Li, X., Li, X., Liu, G., 2015. NEFAs activate the oxidative stress-mediated NF-κB signaling pathway to induce inflammatory response in calf hepatocytes. J. Steroid Biochem. Mol. Biol. 145, 103–112. https://doi.org/10.1016/j.jsbmb.2014.10.014.

Sigl, T., Gellrich, K., Meyer, H.H.D., Kaske, M., Wiedemann, S., 2013. Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation – metabolism, productivity and effect of a short-term feed restriction. J. Anim. Physiol. Anim. Nutr. (Berl). 97, 278–296. https://doi.org/10.1111/j.1439-0396.2011.01268.x.

Spek, J.W., Dijkstra, J., Bannink, A., 2016. Influence of milk urea concentration on fractional urea disappearance rate from milk to blood plasma in dairy cows. J. Dairy Sci. 99, 3880–3888. https://doi.org/10.3168/jds.2015-9421.

Turk, R., Juretić, D., Gereš, D., Svetina, A., Turk, N., Flegar-Meštrić, Z., 2008. Influence of oxidative stress and metabolic adaptation on PON1 activity and MDA level in transition dairy cows. Anim. Reprod. Sci. 108, 98–106. https://doi.org/10.1016/j.anireprosci.2007.07.012.

van Knegsel, A.T.M., van den Brand, H., Dijkstra, J., Kemp, B., 2007. Effects of dietary energy source on energy balance, metabolites and reproduction variables in dairy cows in early lactation. Theriogenology 68, S274–S280. https://doi.org/10.1016/j.theriogenology.2007.04.043.

van Knegsel, A.T.M., Hammon, H.M., Bernabucci, U., Bertoni, G., Bruckmaier, R.M., Goselink, R.M.A., Gross, J., Kuhla, B., Metges, C.C., Parmentier, H.K., 2014. Metabolic adaptation during early lactation: key to cow health, longevity and a sustainable dairy production chain. CAB Rev. 9, 1–15.

Wiseman, A., Redden, M., McGee, A., Spencer, C., Reuter, R., Horn, G., Lalman, D., 2019. Effects of timing of weaning on energy utilization in primiparous beef cows and post-weaning performance of their progeny1. J. Anim. Sci. 97, 1198–1211. https://doi.org/10.1093/jas/skz019.

Journal of Animal Science, 2023, **101**, 1–16
https://doi.org/10.1093/jas/skad053
Advance access publication 16 February 2023
Ruminant Nutrition

Performance and milk fatty acid profile of beef cows with a different energy status with short nutrient restriction and refeeding

Karina G. Orquera-Arguero,[†,‡] Mireia Blanco,[†,‡] Juan R. Bertolín,[†,‡] Javier Ferrer,[†] and Isabel Casasús[†,‡,1]

[†]Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Avda. Montañana 930, 50059, Zaragoza, Spain
[‡]Instituto Agroalimentario de Aragón – IA2 (CITA-Universidad de Zaragoza), Zaragoza, Spain

[1]Corresponding author: icasasus@cita-aragon.es

Abstract

Our study objective was to determine the effect of a short feed restriction (4 d) and subsequent refeeding (4 d) on the performance and metabolism of beef cows with a different nutritional status by particularly focusing on their milk fatty acid (**FA**) profile, to consider its potential use as biomarker of metabolic status. Thirty-two Parda de Montaña multiparous lactating beef cows were individually fed a diet based on the average cow's net energy (**NE**) and metabolizable protein requirements. At 58 d in milk (**DIM**, day 0), cows underwent a 4 d feed restriction (55% requirements, restriction period). Before and after the restriction, diets met 100% of their requirements (basal and refeeding periods). Cow performance, milk yield and composition, and plasma metabolites, were determined on day –2, 1, 3, 5, 6, and 8. Cows were classified into two status clusters according to their pre-challenge performance and energy balance (**EB**) (Balanced vs. Imbalanced). All traits were statistically analyzed considering the fixed effect of status cluster and feeding period or day, with cow as a random effect. Imbalanced cows were heavier and had a more negative EB ($P < 0.001$), but similar milk yield, milk composition, and circulating metabolites (except for greater urea) than Balanced cows ($P > 0.10$). Milk contents of C18:1 cis-9, monounsaturated FA (**MUFA**), and mobilization FA were greater ($P < 0.05$), whereas saturated FA (**SFA**) and de novo FA were lesser in Imbalanced than Balanced cows ($P < 0.05$). Restriction decreased body weight (**BW**), milk yield, and milk protein compared to the basal period, but increased milk urea and plasma nonesterified fatty acids (**NEFA**) ($P < 0.001$). Milk contents of SFA, de novo, and mixed FA decreased immediately during the restriction, while MUFA, polyunsaturated FA and mobilization FA increased ($P < 0.001$). Basal milk FA contents were recovered on day 2 of refeeding, and all their changes strongly correlated with differences in EB and NEFA ($P < 0.05$). The general lack of interactions between status clusters and feeding periods implied that the response mechanisms to diet changes did not differ between cows with a different pre-challenge nutritional status.

Lay Summary

Lactating cows can undergo periods with a negative energy balance due to feed shortages, which trigger metabolic adaptations to support cow maintenance and milk yield. We explored beef cows' response to a short feed restriction (4 d, 55% of their energy and protein requirements) and subsequent refeeding (4 d, 100% of their energy and protein requirements) in the second month of lactation. We analyzed the effect on their performance and metabolism by placing special emphasis on milk production and milk fatty acid composition in two beef cow groups with a different nutritional status before the challenge. When cows faced a food restriction, both groups had similar changes in productive and metabolic traits. These changes are similar to those occurring in restricted dairy cows, but of lesser magnitude due to the lower milk yield and associated metabolic load of beef cows. The milk fatty acid profile, rarely analyzed in beef cows, proved to be an accurate indicator of their metabolic status.

Key words: beef cows, induced feed restriction, metabolites, milk fatty acid profile, refeeding

Abbreviations: BCS, body condition score; BHB, β- hydroxybutyrate; BW, body weight; DIM, days in milk; DM, dry matter; DMI, dry matter intake; EB, energy balance; FA, fatty acids; FAME, fatty acid methyl esters; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; NE, net energy; NEFA, nonesterified fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Introduction

Wide seasonal variations in the availability and quality of feeding resources in extensive ruminant systems imply that animals are often subjected to underfeeding-refeeding cycles (Bocquier and González-García, 2010). When undernutrition occurs in lactating cows, both homeostatic and homeorhetic controls bring about adaptations to help to maintain balance and to supply nutrients to the mammary gland (Bauman and Currie, 1980) to support the high metabolic priority of milk production. Strategies to cope with the physiological imbalance caused by feed restriction depend, among other factors, on: restriction duration and its severity (Leduc et al., 2021); lactation stage (Orquera-Arguero et al., 2022); individual variability (Gross et al., 2011a; Bjerre-Harpøth et al., 2012). In beef cows, the impacts of restriction and refeeding on cow metabolism have been well assessed in the long term (Fiems et al., 2015), and only recently with short-term restrictions (De La Torre et al., 2022; Orquera-Arguero et al., 2022).

Received October 28, 2022 Accepted February 14, 2023.

Furthermore, ad libitum or individual feeding strategies are commonly used in dairy cattle, where individual concentrate allocation based on milk yield can improve the energy balance (**EB**) and cow performance (Lawrence et al., 2016), while other studies report no milk yield differences (Henriksen et al., 2019). On extensive beef cow farms, feeding management is often simplified by adopting a flat-rate regime (Manninen et al., 2004), which involves all cows receiving the same diet irrespectively of their individual requirements. This common feeding can cause disruptive situations under an eventual restriction in nutrient intake, with the most sensitive individuals, those with greater requirements, being the most affected (Bocquier and González-García, 2010). Clustering analyses have been used to group dairy cows according to their performance, plasma metabolites, hormones, and milk traits to identify animals with different strategies to face metabolic challenges (De Koster et al., 2019; Xu et al., 2019; Orquera-Arguero et al., 2022), which could facilitate herd management decisions.

Major changes occur in adipose tissue in response to a negative EB, which results in the mobilization of body reserves and an increase in circulating nonesterified fatty acids (**NEFA**) and ketones to provide energy and precursors for milk synthesis (Baumgard et al., 2017). Plasma concentrations of these and other metabolites, such as malondialdehyde (**MDA**), associated with oxidative status (Castillo et al., 2006) or urea as an indicator of protein metabolism (Bittante, 2022), have been used as biomarkers of cow metabolic load. In the last few years, milk composition traits have been examined as non-invasive indicators of dairy cows' nutritional status (Gross and Bruckmaier, 2019; Billa et al., 2020) because they can be cost-efficiently and routinely measured from test-day milk samples (Mäntysaari et al., 2019). Of them, milk fatty acid (**FA**) contents are promising indicators of energy status in dairy cows (Khiaosa-ard et al., 2020) given that FA C4:0 to C14:0 are synthesized *de novo* in the mammary gland, whereas those longer than C18:0 and around 50% of C16:0 originate from diet and lipid mobilization (Chilliard et al., 2000; Palmquist, 2009). In fact C16:0, C18:0, and 18:1 cis-9 are the most abundant FA in plasma and body fat stores (Hostens et al., 2012), and their concentrations and ratios are closely related to the EB in dairy cows (Dórea et al., 2017), but no information on this is available in beef cows. We hypothesized that the response to restriction and refeeding would be driven by each cow's weight, milk yield, and nutritional status before the challenge. Therefore, the main objectives of this study were to: 1) evaluate the effects of a negative EB induced by a short feed restriction on the performance, metabolites, and milk FA profile in two groups of beef cows classified according to their previous performance; 2) confirm the potential use of milk FA composition as a biomarker of metabolic status in beef cows.

Materials and Methods

The Animal Ethics Committee of the Research Centre approved the experimental procedures (protocol no. CEEA-03-2018-01), which followed the guidelines of EU Directive 2010/63 on the protection of animals used for experimental and other specific purposes (EU, 2010). The experiment was conducted in the Pyrenees Mountain area at the CITA La Garcipollera Research Station (Spain, 42°37′ N, 0°30′ W, 945 m a.s.l.).

Animal management, diets, and experimental design

The study was conducted with 32 multiparous Parda de Montaña beef cows (at calving: body weight [**BW**]: 626 ± 47.7 kg; body condition score [**BCS**, on a 5-point scale]: 2.8 ± 0.22; age: 7.5 ± 2.91 yr). One cow was removed from the study due to physical injury. After calving, cows were randomly allocated in pens (eight cows/pen, 10 × 20 m) equipped with individual feeders for forage (200-l fiberglass boxes in front of self-locking feeding places) and automatic feeding stations (ALPRO Herd Management 7.0, DeLaval) for concentrate. Calves were penned in straw-bedded cubicles adjacent to their dams. They were allowed to suckle their dams twice daily for 30 min at 06:00 h and 14:00 h.

Cows were fed a flat-rate regime during lactation. They all received the same amount of feed. Diets were calculated by considering the net energy (**NE**) and metabolizable protein requirements for the maintenance and lactation of a standard cow (615 kg BW; milk yield: 8.5 kg/d) using INRA equations (INRA, 2007). From calving to the start of the experiment 2 mo later, cows were fed a formulated diet to meet 100% standard cow energy requirements (Table 1).

The experiment was conducted at the end of the second lactation month and involved three consecutive periods, where day 0 was taken as the first day of restriction (days in milk [**DIM**]: 58 ± 6.3). Cows were first fed a diet that met 100% of their energy and metabolizable protein requirements (day −2 to −1, basal period), then 55% of those requirements for 4 d (day 0 to 3, restriction period) and, finally, 100% again on the following 4 d (day 4 to 7, refeeding period). Diets consisted of 8.0 kg hay and 3.0 kg of concentrate (as-fed basis) during the basal and refeeding periods, and 7.0 kg

Table 1. Chemical composition, fatty acids (FA) composition and nutrition value (mean ± SD) of the feedstuffs offered to beef cows

Parameter	Hay	Concentrate
Chemical composition		
DM[3], g/kg	922 ± 11.7	906 ± 4.0
Ash, g/kg DM	86.4 ± 24.4	68.3 ± 1.4
Crude protein, g/kg DM	109 ± 18.3	167 ± 4.7
Neutral detergent fiber, g/kg DM	570 ± 52.4	256 ± 23.2
Acid detergent fiber, g/kg DM	324 ± 32.9	114 ± 11.1
Lignin, g/kg DM	35.2 ± 12.8	29.4 ± 8.8
FA composition		
C16:0, g/100 g ID FAME[1]	32.2 ± 2.37	19.2 ± 0.60
C18:0, g/100 g ID FAME[1]	14.1 ± 2.02	5.3 ± 0.02
C18:1 cis-9, g/100 g ID FAME[1]	4.5 ± 1.15	23.6 ± 0.32
C18:2 n-6, g/100 g ID FAME[1]	15.7 ± 3.30	44.4 ± 1.78
C18:3 n-3, g/100 g ID FAME[1]	26.6 ± 10.17	1.8 ± 0.31
Total, mg ID FAME[1]/g DM	18.5 ± 2.99	65.7 ± 2.15
Nutritive value		
Net energy, MJ/kg DM	5.5 ± 0.15	7.3 ± 0.41
Metabolizable protein, g PDI[2]/kg DM	81 ± 17.9	123 ± 2.4

[1]Identified fatty acid methyl esters.
[2]True protein digestible in the small intestine.
[3]DM, dry matter.

hay during the restriction period. Animals had free access to water and mineral blocks throughout the experiment.

Measurements

Samples of feedstuffs were collected daily (day −2 to 8) and lyophilized in a Genesis Freeze Dryer 25 (Hucoa Erlöss, SA/Thermo Fisher Scientific) to determine their chemical composition and FA profile. Hay was offered daily at 08:00 h as a single meal in individual troughs, where cows were tied up until they finished their ration, during approximately 2 h. ALPRO feeding stations were programmed to offer 3 kg of concentrate daily (as-fed basis) to all the cows during the basal and refeeding periods. Individual concentrate intake was recorded daily.

The BCS was recorded upon calving, 30 DIM, and on experimental period day −2 and 8. It was determined by a trained person on a 1–5 scale, based on estimating the fat covering ribs, loin, and tailhead (Lowman et al., 1976). Cows were weighed on an electronic scale upon calving and then at 07:00 h on 30 and 31 DIM and on experiment day −2, 1, 3, 5, 6, and 8. Milk yield was estimated on the same days by the weight-suckle-weight technique (Le Neindre and Dubroeucq, 1973). Calves were weighed before and after the two daily 30-min periods in which they had access to suckle their dams. The daily milk yield was estimated as the sum of the milk consumed by the calf in these two suckling periods. Milk samples were manually taken from each dam after the morning suckling. Five minutes before the manual extraction, all cows received an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) to accelerate the letdown of the residual milk. A 100-mL sample was collected to determine milk composition, added with sodium azide (PanReac) as a preservative and refrigerated at 4 °C until the analysis. To determine FA composition, a second 40-mL sample was collected, lyophilized, and stored at −20 °C until analyzed.

Cows were bled on the same experiment days described above to assess their metabolic profile. Blood samples were collected from the coccygeal vein at 07:00 h after suckling and before offering hay. Heparinized tubes (BD Vacutainer Becton-Dickenson and Company) were used for the β-hydroxybutyrate (**BHB**) and MDA determinations, and the tubes that contained K2 EDTA (BD Vacutainer Becton-Dickenson and Company) were used to analyze glucose, NEFA, and urea concentrations. Immediately after collection, blood samples were centrifuged at 3,500 rpm for 20 min at 4 °C. Plasma was collected and frozen at −20 °C until further analyses.

Analyses

Feedstuffs and milk

The chemical composition of feedstuffs was analyzed in duplicate as described in Orquera-Arguero et al. (2022). Briefly, dry matter (**DM**) and ash content were determined according to AOAC methods (AOAC, 2000). Nitrogen content was determined following the Dumas Procedure (index no. 968.06) with a nitrogen analyzer (Model NA 2100, CE Instruments, Thermoquest SA., Barcelona, Spain). Neutral detergent fiber, acid detergent fiber, and acid detergent lignin contents were analyzed following the sequential procedure of Van Soest et al. (1991) with an Ankom 200/220 fiber analyzer (Ankom Technology Corporation, Fairport, NY, USA). In milk samples, fat, protein, and urea contents were analyzed by an infrared scan (Milkoscan 7 RM, Foss Electric Ltd., Hillerød, Denmark). The FA of the freeze-dried feedstuffs were extracted and methylated as proposed by Sukhija and Palmquist (1988). The fatty acid methyl esters (**FAME**) of the freeze-dried milk samples were obtained as described by Kramer et al. (1997). Determination was done by gas chromatography with a flame ionization detector and Bruker Scion 436-GC (Bruker, Billerica, USA) equipped with a CP-8400 Autosampler (Bruker), a cyanopropyl capillary column SP-2560 (100 m × 0.25 mm ID × 0.20 µm thickness for feedstuffs and 200 m × 0.25 mm ID × 0.20 µm thickness for milk) (Sigma-Aldrich, Sant Louis, USA) and the Compass CDS software. FAME was ID using the GLC-532, GLC-401, GLC-643, GLC-642, GLC-463 C18:1 t11, C19:0, C23:0 (Nu-Chek-Prep Inc.), mixture BR1, mixture BR4 (Larodan Research Grade Lipids) standard references, and the relative retention times observed in the bibliography (Kramer et al., 1997; Shingfield et al., 2003; De La Fuente et al., 2015). Fatty acid quantification was performed as described in UNE-EN ISO 12966-4:2015 and expressed as a percentage of the total amount of identified FAME. The chemical composition and FA profile of the feedstuffs are presented in Table 1.

Blood metabolites

Glucose (enzymatic-colorimetric method, sensitivity: 0.06 mmol/L) and urea (kinetic method, sensitivity: 0.056 mmol/L) concentrations were determined in plasma with an automatic analyzer (Gernon, RAL S.A, Barcelona, Spain). The mean intra- and interassay CV were 1.5% and 1.9% for glucose and 3.2% and 4.8% for urea, respectively. Plasma BHB (kinetic enzymatic method, sensitivity: 0.100 mmol/L) and NEFA (colorimetric method, sensitivity: 0.072 mmol/L) were determined using Randox kits (Randox Laboratories Ltd., Country Antrim, UK). The mean intra- and interassay CV were respectively 3.3% and 3.7% for NEFA and 6.2% in both cases for BHB. Oxidative status was determined using MDA as a biomarker of lipid peroxidation. This indicator was determined by liquid chromatography using an Acquity UPLC H-Class liquid chromatograph (Waters, Milford, MA, USA) equipped with a silica-based bonded phase column (Acquity UPLC HSS PFP, 100 mm × 2.1 mm × 1.8 µm, Waters), an absorbance detector (Acquity UPLC Photodiode Array PDA eλ detector, Waters) and a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters). The quantification of MDA was done by fluorescence detection at $\lambda_{excitation}$ = 530 nm and $\lambda_{emission}$ = 550 nm following the chromatographic conditions described in Bertolín et al. (2019). The mean intra- and interassay CV were 4.6% and 7.3%, respectively.

Calculations

The chemical composition of feedstuffs was employed to calculate their NE content using INRA equations (INRA, 2007). Individual EB was estimated by calculating the difference between inputs (NE intake) and outputs (NE for maintenance and NE for lactation) (INRA, 2007). Net energy intake was estimated from the individual intake and energy contents of feedstuffs. Net energy for maintenance was calculated from the individual metabolic weight. Net energy for production was obtained using the milk yield, fat, and protein contents in milk.

In milk, FA were grouped according to their degree of saturation as saturated fatty acid (**SFA**), monounsaturated fatty acid (**MUFA**), and polyunsaturated fatty acid (**PUFA**)

according to their origin from de novo synthesis (C4:0–C15:1), of mixed origin (C16:0–C16:1), and from mobilization (≥C17:0) (Palmquist, 2009). The C18:1 cis-9 to C15:0 FA ratio was calculated to assess its relation with the EB and metabolic profile.

Statistical analyses

All the data were analyzed using the SAS statistical package v 9.4 (SAS Institute, Inc., Cary, NC, USA). Cows were assigned to clusters according to their resemblance in terms of Euclidean distance calculated using data from BW and BCS at calving and BW, BCS, milk yield, and EB at 30 and 31 DIM. A non-hierarchical clustering was performed using the k-means method (FASTCLUS procedure). The selection of the optimum number of clusters was based on cubic conglomerating criteria. Two clusters (hereafter referred to as status clusters) were obtained, namely Balanced and Imbalanced. An analysis of variance was performed on the classifying variables using a general linear model (GLM procedure) and taking the cluster as a fixed effect.

Cows' metabolic and production data were studied in two sets of analyses, which considered different time effects during the experiment: feeding period (basal, restriction, refeeding) and day (day −2 to 8). In both cases, mixed models for repeated measures (MIXED procedure) were used by considering the status cluster (Balanced and Imbalanced), time (feeding period or day), their interaction as fixed effects and cow as the random effect. The model used was $Y_{ijk} = \mu + S_j + T_k + S_j \times T_k + C_i + e_{ijk}$, where Y_{ijk} was the dependent variable at each time point for the ith cow; μ, the overall mean; S_j, the effect of the status cluster; T_k, the effect of time (either feeding period or day); C_i, the random effect of cow i and e_{ijk} was the experimental error. Degrees of freedom were adjusted with the Kenward–Roger correction to take into account missing values. The variance components structure was selected on the basis of the lowest Akaike and Bayesian information criteria. Least square means and associated standard errors were obtained, and multiple comparisons were adjusted with Tukey correction. Pearson's relations (*r*) between variables were obtained and presented on heatmaps for cow performance, plasma metabolites, and milk FA composition variables using the CORRPLOT package of R (R Core Team, 2021). The data set used for the correlation analyses corresponds to all traits and samples collected per cow at day −2, 1, 3, 5, 6, and 8 of experiment (n = 186 values per trait). The *P*-value for significance was set at $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$.

Results

The results of the status cluster and feeding period effects appear in the tables. The results of the status cluster and day effects are plotted in the figures. The clustering analysis resulted in two cow clusters, which differed in terms of their pre-experimental BW, milk yield, and EB (Table 2). Cows in the first cluster were classified as Balanced and those in the second cluster as Imbalanced. Balanced cows were lighter, had a lower milk yield and a less negative EB than Imbalanced cows in the second cluster ($P \leq 0.03$).

Cow performance

Dry matter intake (**DMI**) was only affected by feeding period ($P < 0.001$; Table 3). According to the experimental design, DMI was lower during the restriction than during the basal and refeeding periods ($P < 0.001$), and so were energy intakes (59.8, 34.9, and 59.8 MJ NE/d during the basal, restriction, and refeeding periods, respectively, $P < 0.001$) and metabolizable protein intakes (859, 471, and 859 g/d, respectively; $P < 0.001$). The BCS was affected by the status cluster (2.65 and 2.81 in Balanced and Imbalanced cows, respectively, $P < 0.001$), and tended to decrease between day −2 and day 8 (2.75 and 2.71, respectively, $P = 0.08$). Cow BW was affected by the interaction between status cluster and feeding period (Table 3) because restriction decreased BW in both groups ($P < 0.001$), but during refeeding BW decreased even more in Imbalanced cows ($P = 0.03$), whereas it was maintained in Balanced cows ($P \geq 0.23$). In any case, Balanced cows were lighter than their Imbalanced counterparts throughout the experiment ($P < 0.001$). Regarding daily changes, BW of Imbalanced cows lowered from the start (day −2) to the end of the experiment (day 8) ($P < 0.05$), while that of Balanced cows decreased until day 6 ($P < 0.01$), but then regained basal values on day 8 (Figure 1).

Table 2. Initial cow characteristics (30–31 d in milk) according to the status cluster[1]

Item	Balanced	Imbalanced	SEM	*P*-value
n	15	16	-	-
Body weight, kg	563	633	4.12	<0.001
Body condition score (scale 1 to 5)	2.8	2.9	0.04	0.18
Milk yield, kg/d	7.5	8.6	0.17	0.03
Energy balance, MJ NE[2]/d	−3.5	−10.0	0.77	<0.001

[1]Cows clustered according to the analysis based on pre-challenge cow traits and energy status.
[2]Net energy.

Milk yield was affected by the status cluster-feeding period interaction ($P < 0.001$, Table 3). Milk yield lowered similarly during the restriction in both status clusters (−18% and −17% for Balanced and Imbalanced cows, respectively). During refeeding, it increased again to the basal values for Imbalanced cows but did not fully recover for Balanced cows (−9%). Milk yield loss due to the restriction varied between −3% and −37% among cows. On average, Imbalanced cows had a numerically, but nonsignificantly greater milk yield (7.0 vs. 7.8 kg/d in Balanced vs. Imbalanced cows, respectively, $P = 0.10$). In fact, when analyzed by day Imbalanced cows showed faster milk yield regain during the refeeding period (Figure 1). Cow EB was affected by the status cluster and feeding period interaction ($P < 0.001$) because the difference between Balanced and Imbalanced cows was greater during the refeeding period than during the basal and restriction periods (Table 3). In both groups, EB was more negative during the restriction period than in the other periods ($P < 0.001$). This was confirmed when analyzed by day, where the differences between status clusters were only significant on day 5, 6, and 8 during the refeeding period (Figure 1). Milk fat content only tended to be affected by the status cluster, with a lower content in Balanced than in Imbalanced cows ($P = 0.09$; Table 4). Milk protein and milk urea contents were affected only by feeding period ($P < 0.001$; Table 4). Milk protein content was lesser

Table 3. Effect of the status cluster[1] and FP[2] on beef cows' performance

	Status cluster			*P*-value		
Item	Balanced	Imbalanced	RSD[3]	Status	FP	Status × FP
Dry matter intake, kg/d			0.16	0.98	<0.001	0.51
Basal	10.0[a]	10.1[a]				
Restriction	6.4[b]	6.5[b]				
Refeeding	10.1[a]	10.0[a]				
Body weight, kg			6.55	<0.001	<0.001	0.01
Basal	553[a, y]	621[a, x]				
Restriction	542[b, y]	611[b, x]				
Refeeding	543[b, y]	606[c, x]				
Milk yield, kg/d			0.70	0.10	<0.001	0.001
Basal	7.7[a]	8.2[a]				
Restriction	6.3[c]	6.9[b]				
Refeeding	7.0[b]	8.3[a]				
EB[4], MJ NE[5]/d			2.46	<0.001	<0.001	<0.001
Basal	0.1[b, x]	−5.4[a, y]				
Restriction	−20.3[c, x]	−25.3[b, y]				
Refeeding	2.8[a, x]	−5.1[a, y]				

[1]According to the clustering analysis based on pre-challenge cow traits and energy status.
[2]FP, feeding period.
[3]Residual standard deviation.
[4]Energy balance.
[5]Net energy.
[a,b,c]Different superscripts indicate differences between feeding periods ($P < 0.05$).
[x,y]Different superscripts indicate differences between status clusters ($P < 0.05$).

and milk urea content was greater during the restriction compared to the other periods ($P < 0.001$), which was corroborated by the negative correlation between milk urea and EB (Figure 2).

Blood metabolites

Plasma glucose concentration was affected only by feeding period ($P < 0.001$; Table 5). Glucose concentrations were similar during the basal and restriction periods, but rose during the refeeding period ($P < 0.001$). Plasma NEFA concentration was affected by feeding period ($P < 0.001$, Table 5), and increased during the restriction before decreasing during the refeeding period. When NEFA concentration was analyzed by day, an immediate response to diet changes was observed, with a rise after only 1 d on the restricted diet (day 1) and the basal values recovered after 1 d of refeeding (day 5) (Figure 3). Daily NEFA concentration in plasma correlated negatively with energy intake and EB ($P < 0.001$; Figure 2). Plasma BHB concentration was not affected by either the status cluster or the feeding period (Table 5). However, when analyzed by day, minor fluctuations in BHB concentrations occurred (Figure 3). Daily plasma BHB concentration weakly, but positively, correlated with both milk yield and glucose plasma concentration ($P < 0.001$; Figure 2).

Plasma urea concentrations were affected by both the status cluster ($P = 0.03$), with lesser values in Balanced than in Imbalanced cows, and the feeding period ($P < 0.001$; Table 5), with lesser concentrations during refeeding than the other periods. When plasma urea was analyzed daily (Figure 3), it decreased from day 1 of the restriction to day 6 of refeeding, and then increased and reached the basal values by the end of the experiment (day 8). Plasma urea concentration positively correlated with milk urea and plasma glucose and BHB concentrations ($P < 0.001$; Figure 2). Plasma MDA concentration tended to be affected by status cluster ($P = 0.07$; Table 5), and Balanced cows tended to have lesser concentrations than Imbalanced cows. Despite no clear differences being observed for feeding period, an increase in plasma MDA was observed by day 3 of the restriction as compared to previous basal values ($P < 0.05$) when analyzed by day (see Figure 3) and up to the start of the refeeding period (day 5 and 6). Basal values had recovered by the end of refeeding (day 8). Plasma MDA concentration positively correlated with glucose, BHB, and urea plasma concentrations ($P < 0.001$; Figure 2).

Diet FA intake and milk FA content

Diet FA intake were affected only by feeding period ($P < 0.001$), decreased during the restriction and increased to the basal intakes during refeeding (Table 6). Regarding the individual FA in milk, the status cluster tended to affect C16:0 ($P = 0.09$) and C18:1 cis-9 ($P = 0.002$), with greater concentrations in Imbalanced than in Balanced cows. All the major milk FA were affected by feeding period ($P < 0.001$). Restriction lowered the milk contents of C14:0 and C16:0 and increased those of C18:1 cis-9. During refeeding, C14:0 and C16:0 increased, while C18:0 and C18:1 cis-9 decreased. The time effect was confirmed when analyzing C14:0 and C16:0 on a daily basis. Feed restriction elicited an immediate response with nadir values on day 1 and 3, and then increased during refeeding. With C14:0, a status cluster and day interaction ($P = 0.01$) took place because of the slightly different recovery pattern noted during refeeding (Figure 4). The C18:1 cis-9 content increased steadily on d 1 and 3 of the restriction, and then decreased on the first day of refeeding (Figure

4). Milk contents of C14:0 and C16:0 positively correlated, whereas C18:1 cis-9 correlated negatively with EB ($P < 0.001$; Figure 2). Milk C14:0 correlated negatively and C18:1 cis-9 positively with NEFA plasma content ($P < 0.001$, Figure 2).

When FA were analyzed according to their degree of saturation, both SFA and MUFA were affected by the status cluster ($P < 0.05$) and the feeding period ($P < 0.001$), and PUFA only by feeding period ($P < 0.01$; Table 6). The milk FA profile of Balanced cows had greater SFA and lesser MUFA contents than that Imbalanced cows, whereas PUFA contents were similar in both status clusters. During the restriction, SFA content lowered, while MUFA and PUFA rose ($P < 0.001$). During refeeding, SFA increased but did not reach the basal values, MUFA decreased to the basal values and PUFA remained unchanged. When analyzed by day, the SFA basal values had recovered by day 6 and after 2 d on the refeeding diet (Figure 5). For PUFA, a status cluster and day interaction was observed ($P = 0.01$, Figure 5) because Balanced cows had not regained the basal values by day 8, whereas Imbalanced cows had. Altogether, milk SFA contents correlated highly and positively with total diet FA intake and cow EB ($P < 0.001$; Figure 2), while negative correlations were observed between milk MUFA content and both parameters ($P < 0.001$). SFA negatively

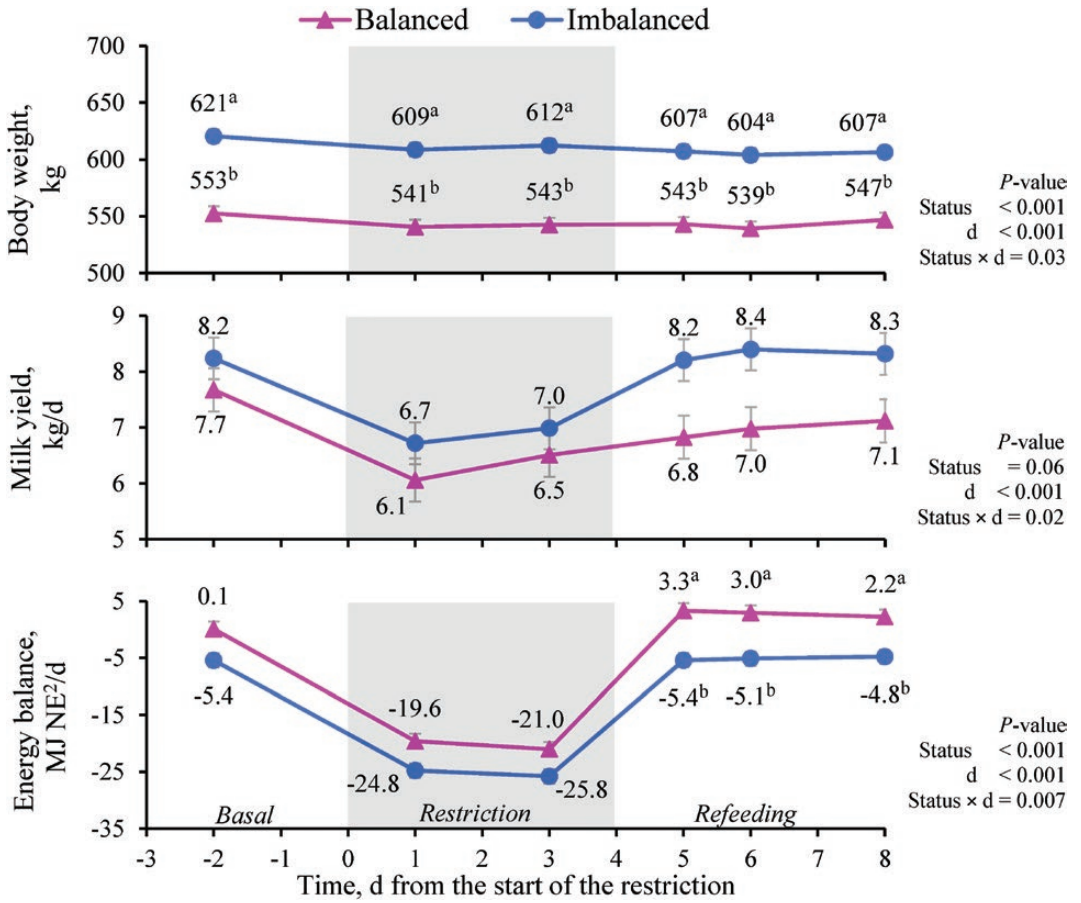


Figure 1. Effect of the status cluster[1] and day (d) on beef cows' body weight, milk yield, and energy balance. The gray area represents the 4 d feed restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error. [1]According to the clustering analysis based on pre-challenge cow traits and energy status. [2] Net energy. [a, b] Within a day, different superscripts indicate differences between status clusters ($P < 0.05$).

Table 4. Effect of the status cluster[1] and FP[2] on beef cows' milk composition

	Status cluster		FP				*P*-value[4]	
Item	Balanced	Imbalanced	Basal	Restriction	Refeeding	RSD[3]	Status	FP
Fat, g/100 g	4.28	4.77	4.58	4.57	4.41	0.80	0.09	0.37
Protein, g/100 g	2.91	2.91	2.93[a]	2.85[b]	2.95[a]	0.01	0.94	<0.001
Urea, mg/dL	22.8	24.5	22.7[b]	25.5[a]	22.8[b]	2.45	0.29	<0.001

[1]According to the clustering analysis based on pre-challenge cow traits and energy status.
[2]FP, feeding period.
[3]Residual standard deviation.
[4]The interaction was never significant ($P = 0.31$–0.94).
[a,b] Different superscripts indicate differences among feeding periods ($P < 0.05$).

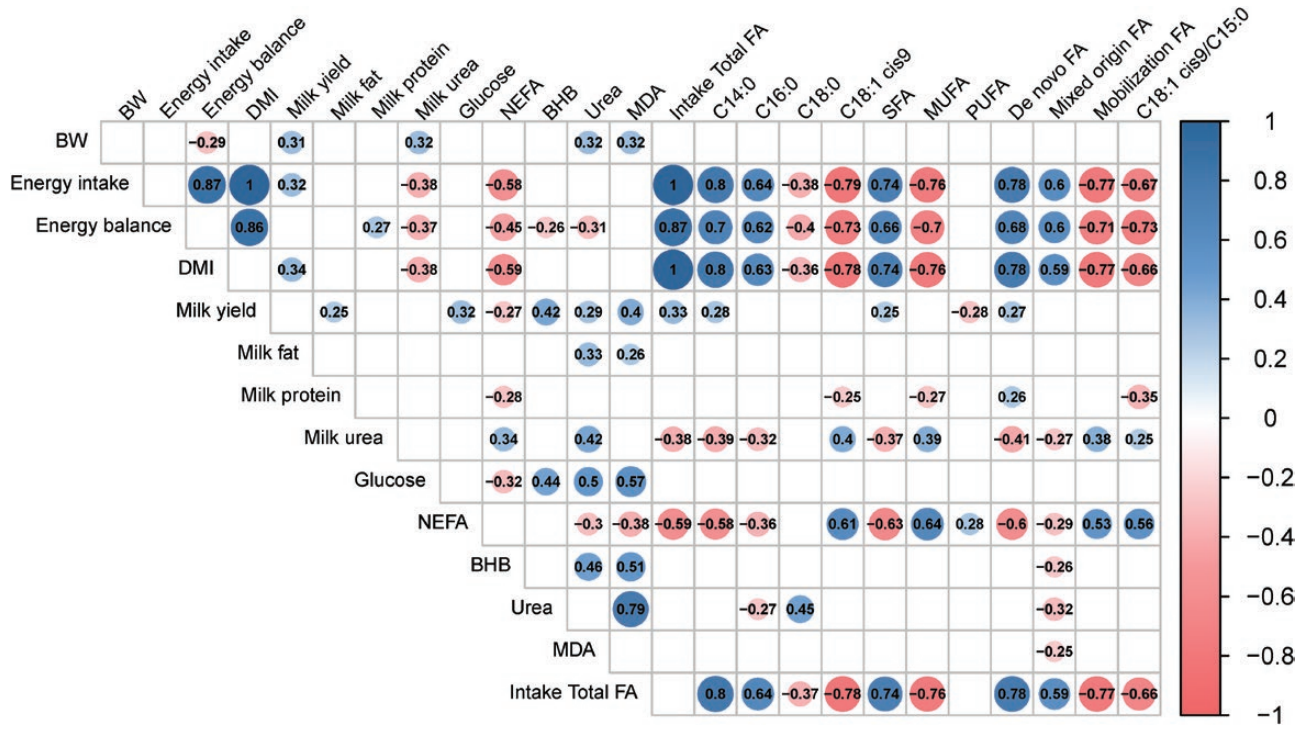


Figure 2. Significant Pearson's correlations ($P < 0.05$) among beef cow performance, metabolic profile variables and milk fatty acids (FA) composition. BHB, β- hydroxybutyrate; BW, body weight; DMI, dry matter intake; MDA, malondialdehyde; MUFA, monounsaturated FA; NEFA, nonesterified fatty acids; PUFA, polyunsaturated FA; SFA, saturated FA; de novo FA (C4:0 - C15:1), mixed origin FA (C16:0 - C16:1), and mobilization FA (≥ C17:0).

Table 5. Effect of the status cluster[1] and FP[2] on beef cows' plasma metabolite concentrations.

	Status cluster		FP				*P*-value[4]	
Item	Balanced	Imbalance	Basal	Restriction	Refeeding	RSD[3]	Status	FP
Glucose, mmol/L	2.18	2.31	2.10[b]	2.15[b]	2.48[a]	0.35	0.28	<0.001
NEFA[5], mmol/L	0.29	0.23	0.10[c]	0.49[a]	0.19[b]	0.17	0.33	<0.001
BHB[6], mmol/L	0.18	0.22	0.20	0.20	0.20	0.06	0.10	0.78
Urea, mmol/L	3.35	4.55	4.21[a]	4.08[a]	3.56[b]	0.84	0.03	<0.001
MDA[7], µmol/L	4.18	5.64	4.91	4.83	5.00	0.51	0.07	0.10

[1]According to the clustering analysis based on pre-challenge cow traits and energy status.
[2]FP, feeding period.
[3] Residual standard deviation.
[4] The interaction was never significant ($P = 0.08–0.92$).
[5] Nonesterified fatty acids.
[6] β- hydroxybutyrate.
[7] Malondialdehyde.
[a,b,c] Different superscripts indicate differences between feeding periods ($P < 0.05$).

and MUFA positively correlated with NEFA plasma contents ($P < 0.001$).

Regarding the effect on the FA grouped according to their origin, the status cluster affected de novo (C4:0–C15:1) and mobilization FA ($P < 0.05$), and tended to affect mixed origin FA (C16:0–C16:1) ($P = 0.09$) with Balanced cows having greater de novo FA contents, slightly greater mixed origin FA and lesser mobilization FA than Imbalanced cows (Table 6). Feeding period affected the three FA groups ($P < 0.001$). De novo and mixed origin FA decreased, while mobilization FA increased during the restriction before returning to the basal values during refeeding. When analyzed by day, an immediate effect was noted on de novo FA during the restriction in both status clusters, with low and constant values on day 1 and 3 (Figure 6). They thereafter increased during refeeding to the basal values on day 5 in both status clusters, but continued to rise even beyond the basal values on day 6 and 8 in Imbalanced cows. Similarly, the daily values of mixed origin FA lowered immediately with the restriction and increased from the start of refeeding irrespectively of the status cluster (Figure 6). Mobilization FA of both Balanced and Imbalanced cows sharply rose on the first day of restriction (day 1), decreased with refeeding below the basal values on day 6 and returned to the baseline values on day 8 (Figure 6). Daily individual EB correlated highly and positively with milk contents of de novo and mixed origin FA ($P < 0.001$; Figure 2), but negatively with mobilization FA ($P < 0.001$). De novo and

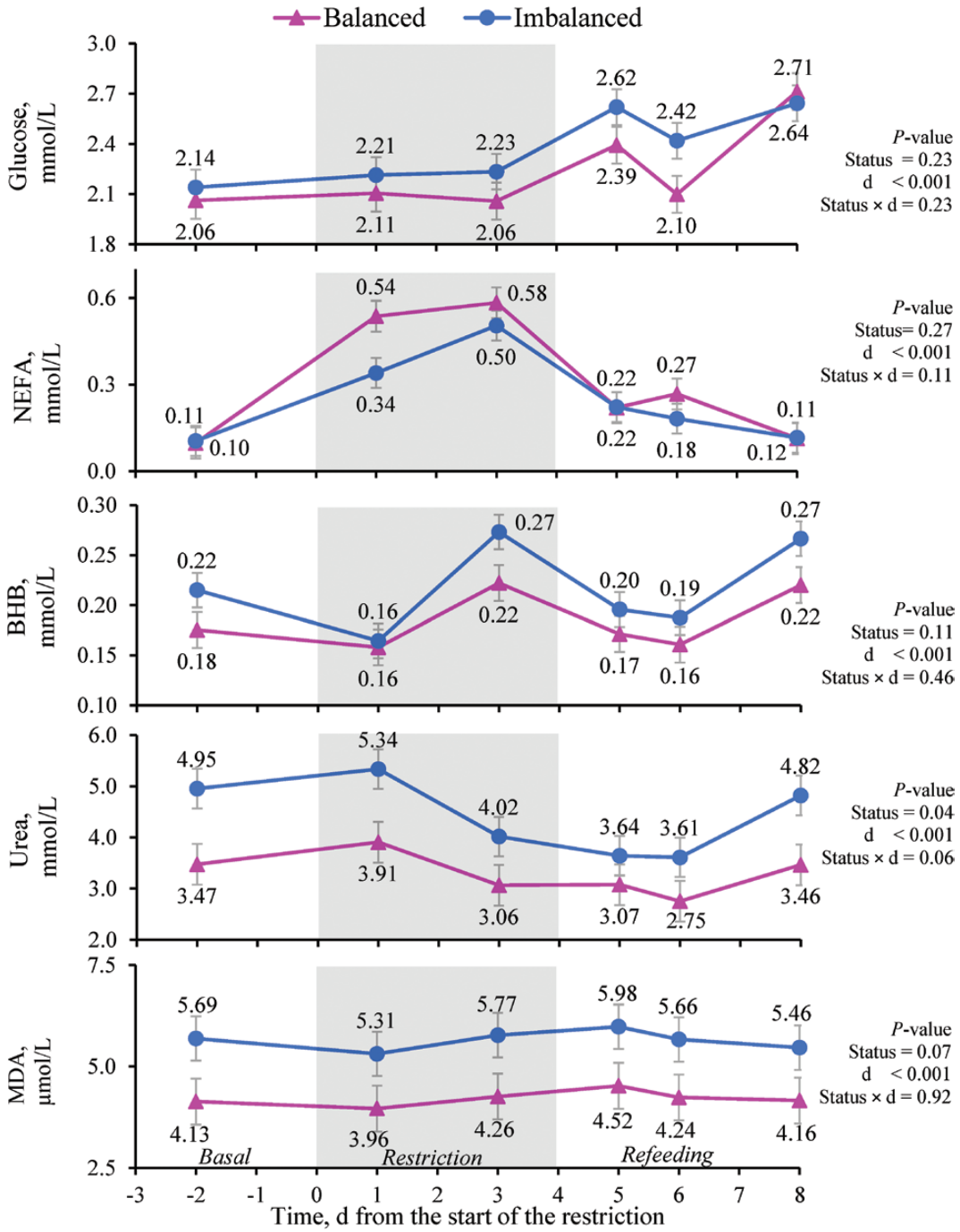


Figure 3. Effect of the status cluster[1] and the day (d) on the plasma metabolites[2] of the beef cows. The gray area represents the 4 d feed restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error. [1]According to the clustering analysis based on pre-challenge cow traits and energy status. [2]NEFA: nonesterified fatty acids; BHB: β- hydroxybutyrate (BHB); MDA: malondialdehyde.

mobilization FA obtained correlations of a different sign with NEFA plasma concentrations ($P < 0.001$).

The C18:1 cis-9 to C15:0 ratio was affected by the status cluster ($P = 0.001$), with greater values in Imbalanced cows than in their Balanced counterparts, and also by the feeding period ($P < 0.001$) with an increment during the restriction and a return to the basal values during the refeeding period (Table 6). This ratio correlated negatively with EB ($P < 0.001$) and positively with plasma NEFA concentrations ($P < 0.001$), but not with the other plasma metabolites (Figure 2).

Discussion

This study investigated the pattern of beef cows' adaptive responses in different energy statuses to a short, but intense, feed restriction, and subsequent refeeding. Their pre-challenge performance and energy status were established by retrospective cow classification according to their previous BW, milk yield, and EB. We obtained two distinct status clusters: Imbalanced cows were heavier, tended to have greater milk yields and a more negative EB, whereas Balanced cows fed the same diets were lighter, had lesser milk yields and a neutral EB. When subjected to nutrient restriction, and despite wide between-cow variability, most of

Table 6. Effect of the status cluster[1] and FP[2] on beef cows' dietary intake of FA[3] and on the major FA in milk, FA according to their saturation and origin, and the C18:1 cis-9 to C15:0 ratio

	Status cluster		FP				*P*-value[5]	
Item	Balanced	Imbalanced	Basal	Restriction	Refeeding	RSD[4]	Status	FP
Intake of dietary FA, g/d								
C16:0	64.3	64.1	77.2[a]	38.1[b]	77.2[a]	2.01	0.55	<0.001
C18:0	24.4	24.4	28.3[a]	16.6[b]	28.3[a]	0.56	0.74	<0.001
C18:1 cis-9	33.5	33.1	47.4[a]	5.2[b]	47.3[a]	2.47	0.34	<0.001
C18:2 n-6	72.4	71.6	98.9[a]	18.3[b]	98.8[a]	4.65	0.36	<0.001
C18:3 n-3	38.2	38.3	40.9[a]	33.0[b]	40.9[a]	0.19	0.12	<0.001
Total	248	247	312[a]	119[b]	312[a]	10.48	0.45	<0.001
Milk FA, g/100 g ID FAME [6]								
Individual FA								
C14:0	8.9	8.4	9.8[a]	6.2[b]	9.8[a]	1.16	0.10	<0.001
C16:0	26.7	25.9	27.3[a]	24.1[b]	27.4[a]	1.49	0.09	<0.001
C18:0	10.6	11	11.6[a]	11.4[a]	9.4[b]	1.14	0.31	<0.001
C18:1 cis-9	24.1	26.1	22.3[b]	30.2[a]	22.9[b]	2.55	0.002	<0.001
FA according to saturation								
Saturated FA	61.9	60.3	64.7[a]	55.6[c]	63.0[b]	2.95	0.04	<0.001
Monounsaturated FA	32.9	34.6	30.8[b]	38.8[a]	31.7[b]	2.6	0.01	<0.001
Polyunsaturated FA	5.2	5.1	4.5[b]	5.6[a]	5.4[a]	0.66	0.46	<0.001
FA according to origin								
De novo (C4:0 to C15:1)	22.1	20.8	23.4[a]	16.8[b]	24.1[a]	2.41	0.04	<0.001
Mixed origin (C16:0 + C16:1)	29.1	28.2	29.5[a]	26.7[b]	29.8[a]	1.48	0.09	<0.001
Mobilization (≥ C17:0)	48.8	51.0	47.2[b]	56.5[a]	46.1[b]	3.52	0.02	<0.001
C18:1 cis-9 to C15:0 ratio	16.6	19.2	15.5[b]	21.7[a]	16.5[b]	2.18	0.001	<0.001

[1]According to the clustering analysis based on pre-challenge cow traits and energy status.
[2]FP, feeding period.
[3]FA, fatty acid.
[4] Residual standard deviation.
[5] The interactions were not significant (P = 0.06–0.70).
[6] Identified fatty acid methyl esters.
[a,b,c] Different superscripts indicate differences among feeding periods ($P < 0.05$).

the parameters that describe cows' performance, plasma metabolites, and milk composition were affected by time (feeding period or day). A less marked effect was observed for the status cluster (Balanced vs. Imbalanced cows).

Cow performance

According to the experimental design, DMI (64%) and both energy (55%) and protein (53%) intake lowered during the restriction period, which resulted in lighter BW (−2%), lower milk yield (−17%), and less milk protein content (−3%) compared to the basal values. Milk fat content did not change, and milk urea content increased (+13%). The BW loss could be a consequence of the reduced DMI and the concomitant loss of gut fill, together with the mobilization of body reserves in response to the restriction (Gross et al., 2011a). This mobilization was probably larger for Imbalanced cows, which were heavier and had a lower EB throughout the study, which allowed them to cope with the metabolic challenge, but resulted in net BW loss at the end of the refeeding period.

The diminished milk yield during the restriction was associated with reduced energy supply, as observed in other studies. The −17% reduction herein observed for beef cows after a 4 d restriction at 55% of their requirements was similar to the −19% to −20% reduction after a 4–5 d restriction at 50–60% of previous intake for dairy cows (Carlson et al., 2006; Abdelatty et al., 2017). A greater (−30%) reduction was observed when dairy cows were restricted more intensely (48% of their requirements) for 4 d (Bjerre-Harpøth et al., 2012). In beef cattle, Charolais cows had −12% milk loss under a similar restriction condition, which was probably related to a less negative physiological imbalance (De La Torre et al., 2022). As observed here, all the aforementioned studies report a wide variation in cows' individual adaptive ability to counterbalance the feed restriction, which Orquera-Arguero et al. (2022) associated to the cows milk yield potential and capacity of mobilization of fat reserves.

Despite the fact that the basal milk yield did not differ between status clusters, it was not only numerically greater in Imbalanced cows, as observed by De Koster et al. (2019) in two groups of cows clustered according to their metabolic profiles, but also recovered more quickly when refeeding started. According to Baumgard et al. (2017), milk yield would be a major driver of the different partition of nutrients toward milk production or fat reserves in cows and would, therefore, condition their response to feed restriction. The slower recovery observed in Balanced cows resulted in their EB being even better during refeeding than during the basal period because energy intake exceeded their requirements for a numerically lesser milk yield. When analyzed by day, the basal values had recovered in both status clusters by the end

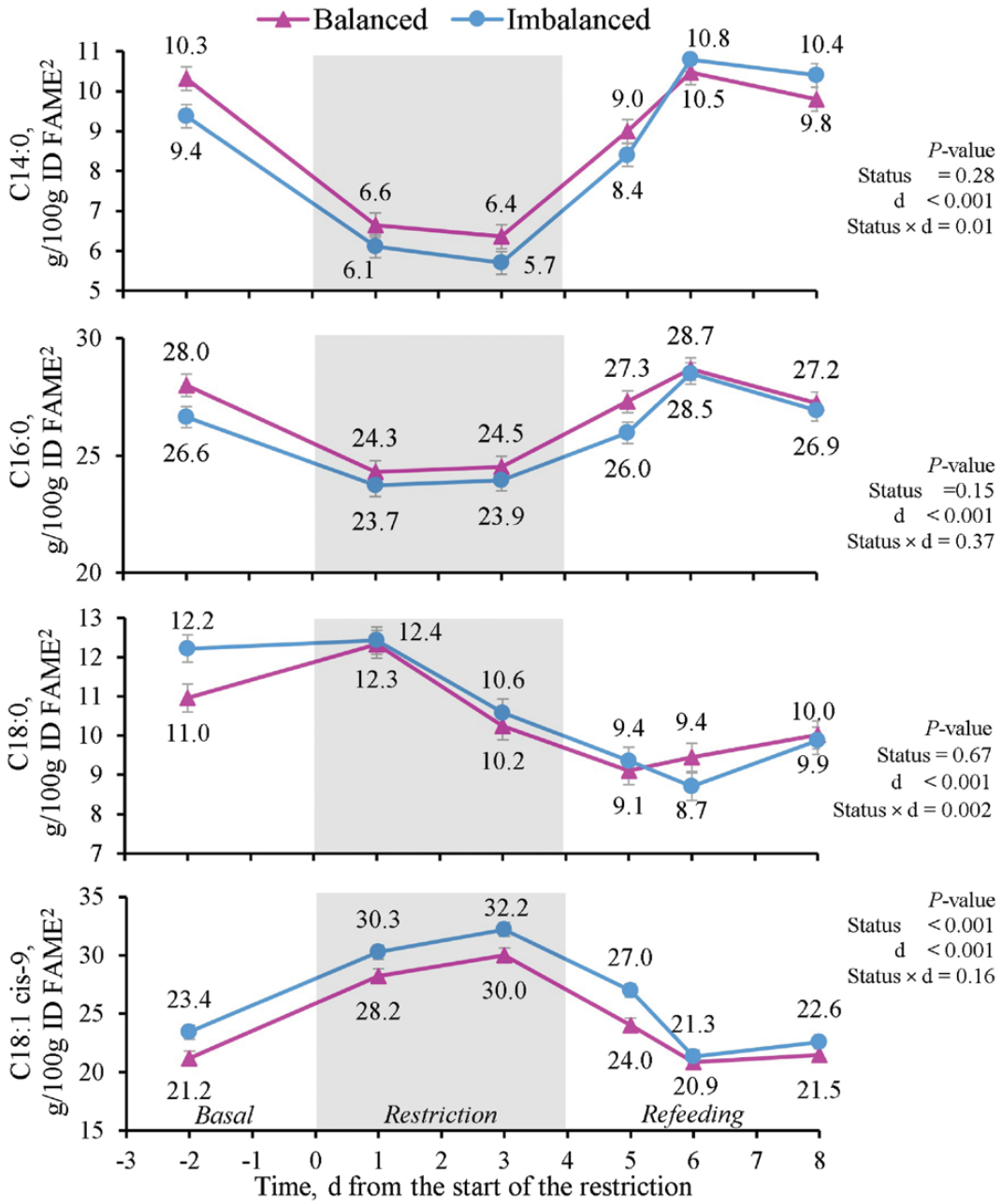


Figure 4. Effect of the status cluster[1] and day (d) on beef cows' milk concentrations of individual milk fatty acids: C14:0, C16:0, C18:0, and C18:1 cis-9. The gray area represents the 4 d feed restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error. [1] according to the clustering analysis based on pre-challenge cow traits and energy status. [2] identified fatty acid methyl esters.

of the refeeding period. This finding agrees with other studies in beef (De La Torre et al., 2022) and dairy (Gross et al., 2011a; Bjerre-Harpøth et al., 2012) cows, which reflects the plasticity of the cow response to a short nutritional challenge.

Several studies report greater milk fat content associated with a negative EB and body fat mobilization (Agenäs et al., 2003; Kessel et al., 2008), whereas others report no difference between cows with different fat mobilization intensities (Schuh et al., 2019). In the present study, no changes were observed in response to a short feed restriction, which agrees with the results of Carlson et al. (2006), who worked with dairy cows under similar conditions, although they also found increased plasma indicators of lipolysis (NEFA and BHB). As pointed out by Schuh et al. (2019), the fact that milk fat did not mirror the increase in circulating NEFA could be explained by them being partly diverted to other tissues to be used as an energy substrate rather than to the mammary gland to be converted into milk FA. Milk fat content tended to be greater in Imbalanced cows, which agrees with the observations made by Stoop et al. (2009) when comparing cows with different EB, which could reflect a longer-term difference in the nutritional status of cows with different BW and milk yields fed at a flat rate since lactation onset.

The immediate milk protein content reduction during the restriction period observed in similar studies with dairy cows (Gross et al., 2011a; Billa et al., 2020) can be ascribed to reduced dietary energy and protein intake, which compromise both microbial protein synthesis and by-pass protein flux to the intestine. Similarly, Bjerre-Harpøth et al. (2012) confirmed

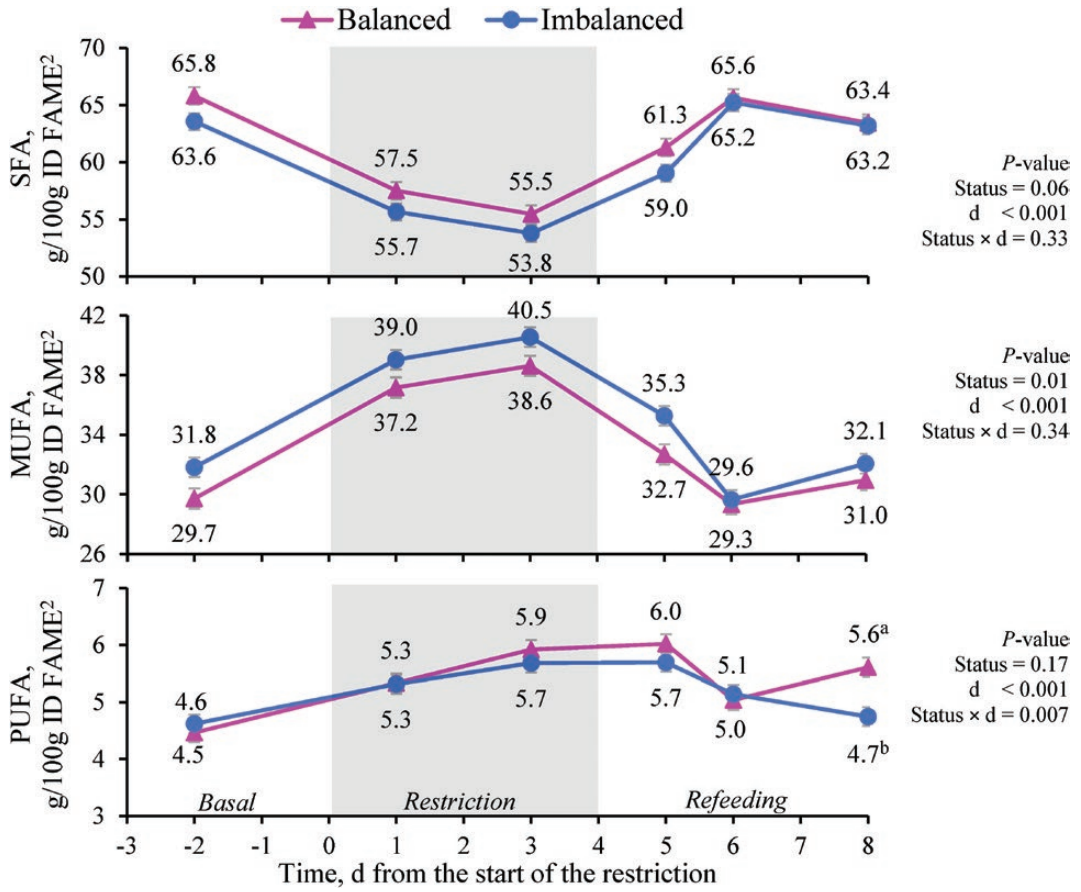


Figure 5. Effect of the status cluster[1] and day (d) on beef cows' milk concentrations of grouped fatty acids (FA) according to their saturation: saturated FA (SFA), monounsaturated FA (MUFA), and polyunsaturated FA (PUFA). The gray area represents the 4 d feed restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error. [1] according to the clustering analysis based on pre-challenge cow traits and energy status. [2] identified fatty acid methyl esters. [a, b] Within a day, different superscripts indicate differences between status clusters ($P < 0.05$).

that milk protein content lowered during the restriction and returned to the prerestriction content during refeeding regardless of the lactation stage. The rise in milk urea contents during feed restriction agrees with the observations made by Broderick (2003), who described that when dietary energy lowers, milk yields, and milk protein contents decrease, while milk urea increases, in response to the lower amino acid requirements for lesser milk secretion (Bittante, 2022).

Blood metabolites

In the present experiment, the metabolites associated with energy metabolism and oxidative status were not affected by the status cluster, except for greater plasma urea concentration in Imbalanced cows. Glucose, NEFA, and urea immediately responded to diet changes, while a delayed response was noted for BHB and MDA. Plasma glucose concentration strongly depended on the current energy and protein intake at a given time, and also on diet composition. They were all similar for both status clusters and, thus, their glucose concentration did not differ. Plasma glucose did not change during the restriction, although it was expected to decrease as a consequence of lower feed and energy intake. This lack of response could be due to the lower gluconeogenesis associated with lower ruminal propionic acid production (Kessel et al., 2008) caused by the lower proportion of concentrate in the restriction diet. However, circulating glucose also depends on uptake by mammary glands for milk lactose production, as observed in other studies (Agenäs et al., 2003; Carlson et al., 2006). The increment that occurred in the refeeding phase agrees with the observations made by Bjerre-Harpøth et al. (2012), for whom glucose also peaked at the start of refeeding due to metabolic readjustment.

An increase in circulating NEFA concentration is an indicator of adipose tissue catabolism in response to a negative EB to supply FA, which can be converted into milk triglycerides in the mammary gland or oxidized in the liver as an energy substrate (Bell, 1995). In the current study, NEFA did not differ among cows in both status clusters, probably because the actual difference in EB between them was too narrow to elicit a response. However, they responded immediately to the large differences in energy intake among feeding periods, with which they correlated. A critical threshold of 0.57 mmol NEFA/L was set by Ospina et al. (2010) as an early postpartum indicator of increased risk of clinical ketosis in dairy cows, which was only just reached by Balanced cows on day 3 in our experiment.

Excessive NEFA mobilization can impair the liver's metabolic capacity to completely oxidize them, which results in the production of ketone bodies, such as BHB, acetoacetate, and acetone (Jorjong et al., 2015; Mann et al., 2016). In our experiment, the tendency of a greater BHB concentration for

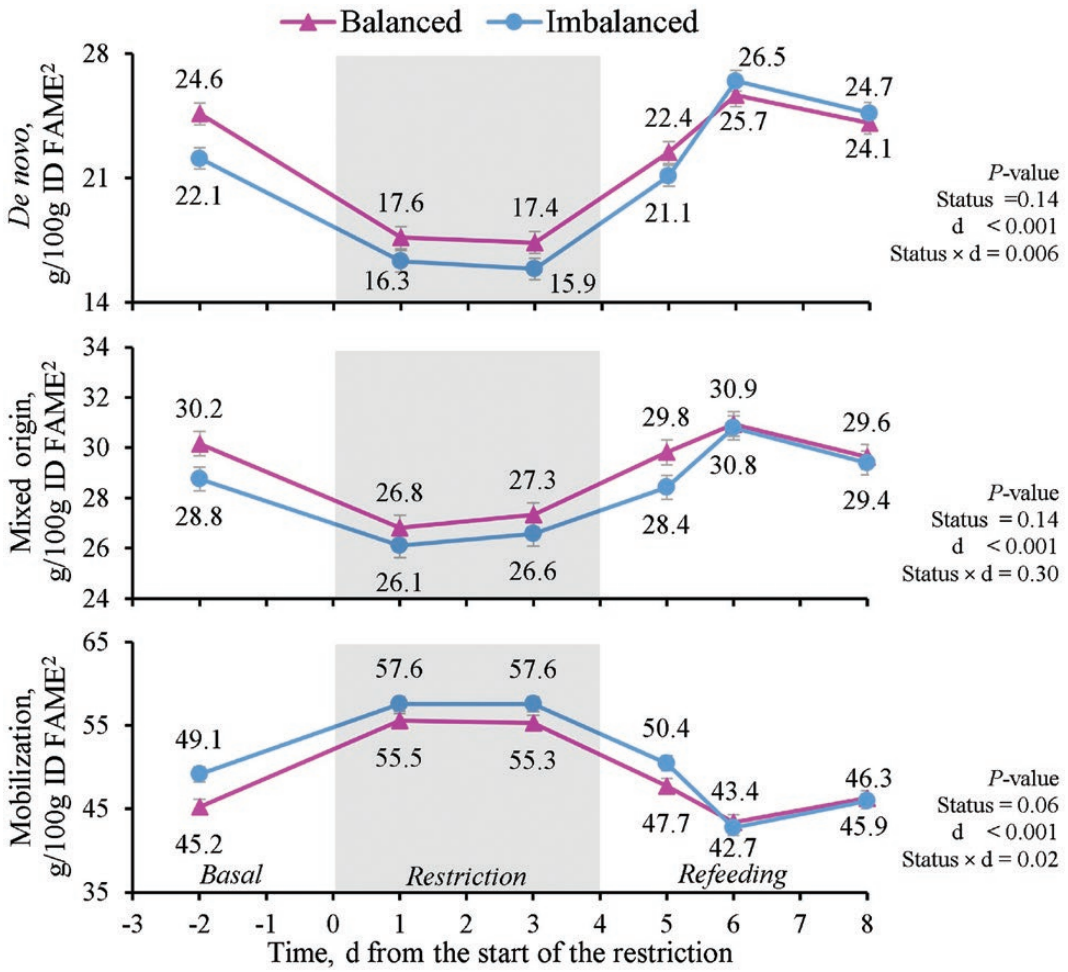


Figure 6. Effect of the status cluster[1] and day (d) on beef cows' milk concentrations of grouped fatty acids (FA) according to their origin: De novo FA (C4:0–C15:1), mixed origin FA (C16:0–C16:1), and mobilization FA (≥ C17:0). The gray area represents the 4 d feed restriction at 55% of cows' energy and metabolizable protein requirements. Vertical bars indicate the standard error. [1] according to the clustering analysis based on pre-challenge cow traits and energy status. [2] identified fatty acid methyl esters.

Imbalanced than Balanced cows, plus the positive correlation between BHB and milk yield, suggest increased NEFA oxidation to provide energy substrates for milk production (Wathes et al., 2007). The BHB concentrations did not differ among feeding periods, as observed in dairy and beef cows at mid-lactation with a similar feed restriction period lasting 4 d (Carlson et al., 2006; Bjerre-Harpøth et al., 2012; De La Torre et al., 2022). These results imply that NEFA mobilization did not exceed the liver's metabolizing capacity and provided sufficient energy supply for nutrient-restricted cows. However, a peak occurred at the end of the restriction phase, with a delayed response to energy intake compared to NEFA, as observed by Gross et al. (2011a) in dairy cows at mid-lactation. The extent of this delay can be influenced by the lactation stage and restriction duration (Carlson et al., 2006; Orquera-Arguero et al., 2022). Apparently, feed restriction length did not suffice here to have a prolonged effect on BHB. Plasma BHB can be used as an indirect marker of a negative EB in dairy cows, but has been shown to be a poor indicator in beef cattle (De La Torre et al., 2022; Orquera-Arguero et al., 2022), as observed here. Hyperketonemia, defined when BHB exceeds a critical threshold of 1.2 mmol/L, is associated with increased risk of disease, milk yield losses, and impaired reproductive performance in dairy cows (Jorjong et al., 2015). In our study, both NEFA and BHB concentrations were below the above-mentioned thresholds because our beef cows had a less severe negative EB due to their lower milk yields.

Lack of differences in these metabolites between status clusters was not expected. De Koster et al. (2019) observed that plasma glucose was greater and NEFA and BHB were lesser in balanced than in imbalanced dairy cows. Vossebeld et al. (2022) clustered cows according to their postpartum EB profile. They found that those with a more negative EB had greater plasma NEFA and BHB concentrations. However, differences in EB between the dairy cow groups in both studies, and associated with their different DMI, BW, and milk yield, were much larger than those herein recorded. Our similar results for both cow groups in different EB could be partly ascribed to wide individual variation in cows' metabolic adaptive capacity, as pointed out by Kessel et al. (2008), or to the lower milk yield and associated metabolic load in beef cows.

Circulating urea in lactating ruminants originates from either dietary protein intake or the catabolism of body protein reserves when energy intake is restricted and the AA stored in skeletal muscle are mobilized (Bell, 1995). Given their similar protein intake, the greater plasma urea concentrations in

Imbalanced cows indicate greater body protein turnover to support gluconeogenesis and to cope with their more negative EB. These differences observed in plasma were probably not large enough to be reflected in milk urea contents, despite them being significantly correlated, as observed by Kessler et al. (2020). The minor differences among days, which decreased at the end of the restriction and had risen by the end of the refeeding period, showed a delayed response to diet changes, which falls in line with Bjerre-Harpøth et al. (2012).

Oxidative stress occurs during periods of high metabolic demand, when the production of free oxidant radicals cannot be counteracted by the natural anti-oxidant system. Castillo et al. (2006) found increased lipid peroxidation only at very early postpartum, with wide individual variation. Bernabucci et al. (2005) reported that dairy cows with greater BCS loss, and greater BHB and NEFA concentrations, also had greater concentration of reactive oxygen metabolites, which agrees with Schuh et al. (2019), plus lesser concentrations of antioxidants. In our study, Imbalanced cows tended to have greater MDA concentrations, which mirrored the trend observed for BHB concentrations. This finding also reflects fat mobilization and oxidation, and is associated with hepatic stress. This positive correlation between MDA and BHB agrees with those observed by Li et al. (2016) in dairy cows, who also report a positive association with NEFA, but it was not observed in our experiment. This supports the lack of differences in oxidative status among feeding periods, where the increased NEFA and the decreased milk yield allowed cows to cope with metabolic stress without further lipid oxidation. In line with our results, Urh et al. (2019) found that diets that included different amounts of concentrate affected NEFA concentrations, but neither BHB nor the oxidative status of dairy cows, which they associated with relatively small differences in cows' energy intake, as we observed here with a flat-rate feeding regime.

Diet FA intake and milk FA content

The total FA intake decreased by −62% due to the restriction, whereas the extent of the decrease in individual FA intake varied, with a greater reduction (−81% to −89%) for those that were more abundant in the concentrate (C18:2 n-6 and C18:1 cis-9) than for those that were predominant in hay (C16:0 and C18:0). These differences in relative individual FA intake reflected both the reduction in DMI and the change in diet among periods. Diet composition affects the milk FA profile because short- and medium-chain milk FA derive from de novo synthesis from acetate and the transformations of butyrate that occur during the ruminal fermentation of carbohydrates (Bauman and Griinari, 2003), both of which increase when the forage proportion in diet increases. However, the milk FA profile does not exactly mirror the relative intake of the different FA because they can be modified by ruminal biohydrogenation and mammary lipogenic and Δ-9 desaturation pathways (Chilliard et al., 2007).

Research into the relation between energy intake and EB with the milk FA profile is extensive in dairy cows, but literature on milk FA composition of beef cows is scarce. To the best of our knowledge, this is the first study to report changes in beef cows' milk FA contents in response to feed restriction. As in the case of milk yield and circulating metabolites, the response patterns of milk FA in beef cows follow the trends observed in dairy cows although the changes are of a lesser magnitude. Here, we observed that energy status had a marked effect in both the long (differences between status clusters, e.g. C14:0 and C16:0 tended to be greater and C18:1 cis-9 lesser in Balanced vs. Imbalanced cows) and short terms (differences among feeding periods, e.g., lowest C14:0 and C16:0 and highest C18:1 cis-9 during the restriction) on milk contents of major FA and different FA proportions according to both their degree of saturation and origin. When a negative EB induces body fat mobilization, the major FA in subcutaneous and abdominal depots (C16:0, C18:0, and C18:1 cis-9) are released to plasma, where they constitute a high proportion of circulating NEFA, and where C18:1 cis-9 is the most abundant FA in both dairy (Hostens et al., 2012) and beef (Lake et al., 2007) cows. These NEFA are taken up by the mammary gland and directly used for milk fat synthesis (Bauman and Griinari, 2003). Consequently, their relative proportions in milk fat should reflect this lipid mobilization in response to EB. Furthermore, when these long-chain FA are released into plasma, de novo synthesis of short-chain FA by the mammary gland is inhibited (Chilliard et al., 2007). Gross et al. (2011b) described how the milk FA profile responds quickly to dietary energy changes, with significant reductions in most FA of ≤C16:0 and increments of preformed FA of > C16:0 within 1 wk of feed restriction, and the basal values recover within 1 wk of refeeding. This pattern was confirmed in our experiment, even on the first day after diet change. As we noted, C14:0 milk contents were positively associated with EB, and increased with improved energy status with advancing dairy cows' lactation (Craninx et al., 2008). On C16:0, literature offers conflicting results, which are explained by its mixed origin (Chilliard et al., 2000). C16:0 contents increased with either a negative EB (Stoop et al., 2009) or feed restriction (Abdelatty et al., 2017), but the decrease herein observed during the restriction period agrees with the patterns reported by Gross et al. (2011b) and Billa et al. (2020), which suggests that despite its mixed origin, here it reflects the reduced de novo FA synthesis. Regarding long-chain FA, milk C18:0 did not increase during the restriction, unlike previous reports (Gross et al., 2011b; Billa et al., 2020), but decreased with refeeding as a result of less fat mobilization, which agrees with the aforementioned studies. Finally, milk oleic acid contents (C18:1 cis-9) have been associated with a negative EB and high plasma NEFA concentrations (Stoop et al., 2009; Jorjong et al., 2014; Dórea et al., 2017), which agrees with our results. It has even been proposed as an early predictor of subclinical ketosis in dairy cows (Van Haelst et al., 2008), and as a better indicator of a negative EB than actual plasma NEFA and BHB concentrations (Churakov et al., 2021), which can vary diurnally depending on the time that elapses between feeding and blood sampling (Mäntysaari et al., 2019). This was confirmed herein by the stronger correlation of EB with milk C18:1 cis-9 contents than with these plasma metabolites. This relation also explains the greater milk contents of C18:1 cis-9 in Imbalanced cows, and the rise that occurred during the restriction period in association with a more negative EB in both cases.

According to their degree of saturation, the differences between status clusters and feeding periods followed the differences in major FA and in other less abundant ones. During the feed restriction, SFA decreased by −14% whereas MUFA and PUFA increased by +26% and +24%, respectively. This agrees with the results of Gross et al. (2011b) except for their stable PUFA concentrations, but contrasts with those of Stoop et al. (2009), who found greater proportions of SFA, mainly C16:0 and C18:0 from body fat, in those cows with a greater energy imbalance. The reduction in SFA during the

restriction and the lesser concentration in SFA in Balanced cows in our study seemed to be driven by the predominant behavior of C16:0 as a de novo synthesized FA, and also by the minimal response of C18:0 to EB, as observed by Abdelatty et al. (2017). Regarding the origin of milk FA, Grummer (1991) suggests that almost all the C4:0 to C14:0, and about half the C16:0 in milk, are synthesized de novo in the mammary gland, whereas the rest of the C16:0 and all long-chain FA derive from mammary uptake of circulating triacylglycerol and NEFA. Unless diet composition significantly varies (Khiaosa-ard et al., 2020), the relative proportions of de novo synthesized and preformed FA mainly reflect changes in the EB (Gross et al., 2011b). Accordingly in our study, milk de novo FA content was significantly greater and that of mobilization FA was lesser in Balanced vs. Imbalanced cows. In dairy cows that underwent a 6 d 50% energy restriction, Billa et al. (2020) reported that milk contents C10:0 to C15:0 decreased by −37%, and those of C16:0 by −23%, while FA > C16:0 rose by almost +60%, and basal contents were recovered within a week of refeeding. Here with a similar but shorter feed restriction in beef cows, the relative changes were less intense, i.e., both de novo and mixed origin FA decreased (by −28% and −10%), while mobilization FA increased by +20%, and the basal values were also regained during the refeeding period in response to the improved EB. These changes are consistent with the strong correlations of the FA of different origins with EB and NEFA contents, as also described by Khiaosa-ard et al. (2020), who also found correlations with BHB contents that were not herein observed.

Several ratios between milk FA of different origins (mostly long-chain vs. short- and medium-chain FA or linear and branched FA) have been proposed as indicators related to cow diet or energy status (Craninx et al., 2008; Dórea et al., 2017). Of them, Jorjong et al. (2015) established that the C18:1 cis-9 to C15:0 ratio was the most discriminating factor for early hyperketonemia diagnosis (BHB ≥ 1.2 mmol/L), for which they proposed a threshold of between 34 and 45. Dórea et al. (2017) indicated that it could also be used to accurately predict plasma NEFA and that when this ratio exceeded 62, the cows would be at risk of developing metabolic disorders. In our experiment, the C18:1 cis-9 to C15:0 ratio differed between the status clusters and feeding periods by following the differences observed in EB and plasma NEFA contents, with which it correlated, and could therefore be used as a biomarker of the energy status of cows. However, our values were far from the above-mentioned thresholds described for dairy cows.

Conclusions

A short-term feed restriction and refeeding induced a transient negative EB in beef cows, to which they responded with lower milk yield and changes in plasma metabolites and milk composition, which are associated with the mobilization of body reserves. Despite some of these traits differing between Balanced and Imbalanced cows, with different BW, milk yields and EB before the challenge, they responded similarly to dietary changes by showing a consistent pattern across several individual nutritional statuses. The milk FA profile, which has been rarely studied in beef cows for practical purposes, also differed between Balanced and Imbalanced cows. In particular, the milk C18:1 cis-9 to C15:0 ratio proved to be an accurate indicator of metabolic status, which supports its use in experimental models.

Acknowledgments

Special thanks are expressed to the technical staff at CITA-La Garcipollera Research Station for their technical support, to M. Joy for laboratory coordination and to M.A. Legua for laboratory assistance. We also thank D. Villalba (University of Lleida) for his contribution to data analyses. Research funded by the European Union's Horizon 2020 Research and Innovation program (GenTORE, grant agreement No. 727213), INIA-ERDF (RZP2015-01), and the Government of Aragón (Grant Research Group Funds A14-20R, pre-doctoral contract of Karina G. Orquera-Arguero).

Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

Abdelatty, A. M., M. E. Iwaniuk, M. Garcia, K. M. Moyes, B. B. Teter, P. Delmonte, A. K. G. Kadegowda, M. A. Tony, F. F. Mohamad, and R. A. Erdman. 2017. Effect of short-term feed restriction on temporal changes in milk components and mammary lipogenic gene expression in mid-lactation Holstein dairy cows. *J. Dairy Sci.* 100:4000–4013. doi:10.3168/jds.2016-11130

Agenäs, S., K. Dahlborn, and K. Holtenius. 2003. Changes in metabolism and milk production during and after feed deprivation in primiparous cows selected for different milk fat content. *Livest. Prod. Sci.* 83:153–164. doi:10.1016/s0301-6226(03)00096-4

AOAC. 2000. *Official methods of analysis*. Arlington (VA): Association of Official Analytical Chemist.

Bauman, D., and B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. *J. Dairy Sci.* 63:1514–1529. doi:10.3168/jds.S0022-0302(80)83111-0

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. *Annu. Rev. Nutr.* 23:203–227. doi:10.1146/annurev.nutr.23.011702.073408

Baumgard, L. H., R. J. Collier, and D. E. Bauman. 2017. A 100-year review: regulation of nutrient partitioning to support lactation. *J. Dairy Sci.* 100:10353–10366. doi:10.3168/jds.2017-13242

Bell, A. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *J. Anim. Sci.* 73:2804–2819. doi:10.2527/1995.7392804x

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *J. Dairy Sci.* 88:2017–2026. doi:10.3168/jds.S0022-0302(05)72878-2

Bertolín, J. R., M. Joy, and M. Blanco. 2019. Malondialdehyde determination in raw and processed meat products by UPLC-DAD and UPLC-FLD. *Food Chem.* 298:125009. doi:10.1016/j.foodchem.2019.125009

Billa, P. A., Y. Faulconnier, T. Larsen, C. Leroux, and J. A. A. Pires. 2020. Milk metabolites as noninvasive indicators of nutritional status of mid-lactation Holstein and Montbéliarde cows. *J. Dairy Sci.* 103:3133–3146. doi:10.3168/jds.2019-17466

Bittante, G. 2022. Effects of breed, farm intensiveness, and cow productivity on infrared predicted milk urea. *J. Dairy Sci.* 105:5084–5096. doi:10.3168/jds.2021-21105

Bjerre-Harpøth, V., N. C. Friggens, V. M. Thorup, T. Larsen, B. M. Damgaard, K. L. Ingvartsen, and K. M. Moyes. 2012. Metabolic and production profiles of dairy cows in response to decreased nutrient density to increase physiological imbalance at different

stages of lactation. *J. Dairy Sci.* 95:2362–2380. doi:10.3168/jds.2011-4419

Bocquier, F., and E. González-García. 2010. Sustainability of ruminant agriculture in the new context: feeding strategies and features of animal adaptability into the necessary holistic approach. *Animal*. 4:1258–1273. doi:10.1017/S1751731110001023

Broderick, G. A. 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. *J. Dairy Sci.* 86:1370–1381. doi:10.3168/jds.S0022-0302(03)73721-7

Carlson, D. B., N. B. Litherland, H. M. Dann, J. C. Woodworth, and J. K. Drackley. 2006. Metabolic effects of abomasal L-carnitine infusion and feed restriction in lactating holstein cows. *J. Dairy Sci.* 89:4819–4834. doi:10.3168/jds.S0022-0302(06)72531-0

Castillo, C., J. Hernández, I. Valverde, V. Pereira, J. Sotillo, M. Löpez Alonso, and J. L. Benedito. 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. *Res. Vet. Sci.* 80:133–139. doi:10.1016/j.rvsc.2005.06.003

Chilliard, Y., A. Ferlay, R. M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. *Anim. Res.* 49:181–205. doi:10.1051/animres:2000117

Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid Sci. Technol.* 109:828–855. doi:10.1002/ejlt.200700080

Churakov, M., J. Karlsson, A. Edvardsson Rasmussen, and K. Holtenius. 2021. Milk fatty acids as indicators of negative energy balance of dairy cows in early lactation. *Animal*. 15:100253. doi:10.1016/j.animal.2021.100253

Craninx, M., A. Steen, H. Van Laar, T. Van Nespen, J. Martin-Tereso, B. De Baets, and V. Fievez. 2008. Effect of lactation stage on the odd- and branched-chain milk fatty acids of dairy cattle under grazing and indoor conditions. *J. Dairy Sci.* 91:2662–2677. doi:10.3168/jds.2007-0656

De la Fuente, M. A., V. Rodríguez-Pino, and M. Juárez. 2015. Use of an extremely polar 100-m column in combination with a cyanoalkyl polysiloxane column to complement the study of milk fats with different fatty acid profiles. *Int. Dairy J.* 47:52–63. doi:10.1016/j.idairyj.2015.02.010

De Koster, J., M. Salavati, C. Grelet, M. A. Crowe, E. Matthews, R. O'Flaherty, G. Opsomer, L. Foldager, and M. Hostens; GplusE. 2019. Prediction of metabolic clusters in early-lactation dairy cows using models based on milk biomarkers. *J. Dairy Sci.* 102:2631–2644. doi:10.3168/jds.2018-15533

De La Torre, A., L. Barreto-Mendes, J. A. A. Pires, I. Cassar-Malek, I. Ortigues-Marty, and F. Blanc. 2022. Exploration of robustness indicators using adaptive responses to short-term feed restriction in suckling primiparous beef cows. *Animal*. 16:100556. doi:10.1016/j.animal.2022.100556

Dórea, J. R. R., E. A. French, and L. E. Armentano. 2017. Use of milk fatty acids to estimate plasma nonesterified fatty acid concentrations as an indicator of animal energy balance. *J. Dairy Sci.* 100:6164–6176. doi:10.3168/jds.2016-12466

EU. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. 276:33–79.

Fiems, L. O., J. L. De Boever, B. Ampe, J. M. Vanacker, S. De Campeneere, and B. Sonck. 2015. Effect of energy restriction and dietary protein level during the re-alimentation period on feed efficiency in double-muscled Belgian Blue cows. *Livest. Sci.* 174:46–52. doi:10.1016/j.livsci.2015.01.017

Gross, J. J., and R. M. Bruckmaier. 2019. Metabolic challenges in lactating dairy cows and their assessment via established and novel indicators in milk. *Animal*. 13:s75–s81. doi:10.1017/S175173111800349X

Gross, J. J., H. A. van Dorland, R. M. Bruckmaier, and F. J. Schwarz. 2011a. Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. *J. Dairy Sci.* 94:1820–1830. doi:10.3168/jds.2010-3707

Gross, J. J., H. A. Van Dorland, R. M. Bruckmaier, and F. J. Schwarz. 2011b. Milk fatty acid profile related to energy balance in dairy cows. *J. Dairy Res.* 78:479–488. doi:10.1017/S0022029911000550

Grummer, R. R. 1991. Effect of feed on the composition of milk fat. *J. Dairy Sci.* 74:3244–3257. doi:10.3168/jds.S0022-0302(91)78510-X

Henriksen, J. C. S., M. R. Weisbjerg, P. Løvendahl, T. Kristensen, and L. Munksgaard. 2019. Effects of an individual cow concentrate strategy on production and behavior. *J. Dairy Sci.* 102:2155–2172. doi:10.3168/jds.2018-15477

Hostens, M., V. Fievez, J. L. M. R. Leroy, J. Van Ranst, B. Vlaeminck, and G. Opsomer. 2012. The fatty acid profile of subcutaneous and abdominal fat in dairy cows with left displacement of the abomasum. *J. Dairy Sci.* 95:3756–3765. doi:10.3168/jds.2011-5092

INRA. 2007. *Alimentation des bovins, ovins et caprins. Besoins des Animaux-Valeur des aliments*. Paris, France: Quae Versailles.

ISO. 2015. Animal and vegetable fats and oils. Gas chromatography of fatty acid methyl esters. Part 4: Determination by capillary gas chromatography. Vernier, Switzerland: ISO Copyright Office.

Jorjong, S., A. T. M. van Knegsel, J. Verwaeren, R. M. Bruckmaier, B. De Baets, B. Kemp, and V. Fievez. 2015. Milk fatty acids as possible biomarkers to diagnose hyperketonemia in early lactation. *J. Dairy Sci.* 98:5211–5221. doi:10.3168/jds.2014-8728

Jorjong, S., A. T. M. van Knegsel, J. Verwaeren, M. V. Lahoz, R. M. Bruckmaier, B. De Baets, B. Kemp, and V. Fievez. 2014. Milk fatty acids as possible biomarkers to early diagnose elevated concentrations of blood plasma nonesterified fatty acids in dairy cows. *J. Dairy Sci.* 97:7054–7064. doi:10.3168/jds.2014-8039

Kessel, S., M. Stroehl, H. H. D. Meyer, S. Hiss-Pesch, H. Sauerwein, F. Schwarz, and R. M. Bruckmaier. 2008. Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. *J. Anim. Sci.* 86:2903–2912. doi:10.2527/jas.2008-1016

Kessler, E. C., R. M. Bruckmaier, and J. J. Gross. 2020. Milk urea nitrogen concentration is higher in Brown Swiss than in Holstein dairy cows despite identical feeding. *J. Anim. Physiol. Anim. Nutr. (Berl)*. 104:1671–1677. doi:10.1111/jpn.13408

Khiaosa-ard, R., M. T. Kleefisch, Q. Zebeli, and F. Klevenhusen. 2020. Milk fatty acid composition reflects metabolic adaptation of early lactation cows fed hay rich in water-soluble carbohydrates with or without concentrates. *Anim. Feed Sci. Technol.* 264:1144701–1144714. doi:10.1016/j.anifeedsci.2020.114470

Kramer, J. K. G., V. Fellner, M. E. R. Dugan, F. D. Sauer, M. M. Mossoba, and M. P. Yurawecz. 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids*. 32:1219–1228. doi:10.1007/s11745-997-0156-3

Lake, S. L., T. R. Weston, E. J. Scholljegerdes, C. M. Murrieta, B. M. Alexander, D. C. Rule, G. E. Moss, and B. W. Hess. 2007. Effects of postpartum dietary fat and body condition score at parturition on plasma, adipose tissue, and milk fatty acid composition of lactating beef cows. *J. Anim. Sci.* 85:717–730. doi:10.2527/jas.2006-353

Lawrence, D., M. O'Donovan, T. M. Boland, E. Lewis, and E. Kennedy. 2016. An examination of two concentrate allocation strategies which are based on the early lactation milk yield of autumn calving Holstein Friesian cows. *Animal*. 10:796–804. doi:10.1017/S1751731115002724

Le Neindre, P., and H. Dubroeucq. 1973. Observations sur l'estimation de la production laitière des vaches allaitantes per la peseè du veau avant et aprés la tétée. *Ann. Zootech.* 22:413–422. doi:10.1051/animres:19730402

Leduc, A., S. Souchet, M. Gelé, F. Le Provost, and M. Boutinaud. 2021. Effect of feed restriction on dairy cow milk production: a review. *J. Anim. Sci.* 99:1–12. doi:10.1093/jas/skab130

Li, Y., H. Y. Ding, X. C. Wang, S. B. Feng, X. B. Li, Z. Wang, G. W. Liu, and X. W. Li. 2016. An association between the level of oxidative stress and the concentrations of NEFA and BHBA in the plasma of ketotic dairy cows. *J. Anim. Physiol. Anim. Nutr. (Berl)* 100:844–851. doi:10.1111/jpn.12454

Lowman, B. G., N. A. Scott, and S. H. Somerville. 1976. *Condition Scoring of Cattle*. Edinburgh School of Agriculture. Bulletin no. 6: 1-31.

Mann, S., D. V. Nydam, A. L. Lock, T. R. Overton, and J. A. A. McArt. 2016. Short communication: association of milk fatty acids with early lactation hyperketonemia and elevated concentration of nonesterified fatty acids. *J. Dairy Sci.* 99:5851–5857. doi:10.3168/jds.2016-10920

Manninen, M., K. Saarijärvi, H. Huhta, L. Jauhiainen, and P. Aspila. 2004. Effects of winter feeding strategies with alternative feeds on the performance of mature suckler cows and their progeny. *Agric. Food Sci.* 13:348–362. doi:10.2137/1239099043633323

Mäntysaari, P., E. A. Mäntysaari, T. Kokkonen, T. Mehtiö, S. Kajava, C. Grelet, P. Lidauer, and M. H. Lidauer. 2019. Body and milk traits as indicators of dairy cow energy status in early lactation. *J. Dairy Sci.* 102:7904–7916. doi:10.3168/jds.2018-15792.

Orquera-Arguero, K. G., D. Villalba, M. Blanco, J. Ferrer, and I. Casasús. 2022. Modelling beef cows' individual response to short nutrient restriction in different lactation stages. *Animal*. 16:100619. doi:10.1016/j.animal.2022.100619

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: critical thresholds for prediction of clinical diseases. *J. Dairy Sci.* 93:546–554. doi:10.3168/jds.2009-2277

Palmquist, D. L. 2009. Milk fat: origin of fatty acids and influence of nutritional factors thereon. In: Fox, P. F., and P. L. H. McSweeney, editors, *Advanced dairy chemistry*. Vol. 2. New York: Springer Science; p. 43–92.

R Core Team. 2021. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Schuh, K., H. Sadri, S. Häussler, L. A. Webb, C. Urh, M. Wagner, C. Koch, J. Frahm, S. Dänicke, G. Dusel, et al. 2019. Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated v. normal body condition at dry-off. *Animal*. 13:1478–1488. doi:10.1017/S1751731118003385

Shingfield, K. J., S. Ahvenjärvi, V. Toivonen, A. Ärölä, K. V. V. Nurmela, P. Huhtanen, and J. M. Griinari. 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. *Anim. Sci.* 77:165–179. doi:10.1017/s1357729800053765

Stoop, W. M., H. Bovenhuis, J. M. L. Heck, and J. A. M. van Arendonk. 2009. Effect of lactation stage and energy status on milk fat composition of Holstein-Friesian cows. *J. Dairy Sci.* 92:1469–1478. doi:10.3168/jds.2008-1468

Sukhija, P. S., and D. L. Palmquist. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J. Agric. Food Chem.* 36:1202–1206. doi:10.1021/jf00084a019

Urh, C., J. Denißen, E. Gerster, N. Kraus, E. Stamer, B. Heitkönig, H. Spiekers, and H. Sauerwein. 2019. Pro- and antioxidative indicators in serum of dairy cows during late pregnancy and early lactation: testing the effects of parity, different dietary energy levels, and farm. *J. Dairy Sci.* 102:6672–6678. doi:10.3168/jds.2019-16248

Van Haelst, Y. N. T., A. Beeckman, A. T. M. Van Knegsel, and V. Fievez. 2008. Short communication: elevated concentrations of oleic acid and long-chain fatty acids in milk fat of multiparous subclinical ketotic cows. *J. Dairy Sci.* 91:4683–4686. doi:10.3168/jds.2008-1375

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2

Vossebeld, F., A. T. M. van Knegsel, and E. Saccenti. 2022. Phenotyping metabolic status of dairy cows using clustering of time profiles of energy balance peripartum. *J. Dairy Sci.* 105:4565–4580. doi:10.3168/jds.2021-21518

Wathes, D. C., Z. Cheng, N. Bourne, V. J. Taylor, M. P. Coffey, and S. Brotherstone. 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. *Domest Anim. Endocrinol.* 33:203–225. doi:10.1016/j.domaniend.2006.05.004

Xu, W., A. T. M. van Knegsel, J. J. M. Vervoort, R. M. Bruckmaier, R. J. van Hoeij, B. Kemp, and E. Saccenti. 2019. Prediction of metabolic status of dairy cows in early lactation with on-farm cow data and machine learning algorithms. *J. Dairy Sci.* 102:10186–10201. doi:10.3168/jds.2018-15791