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Metabolic and productive adaptive response of beef cows to successive short-nutritional challenges

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ABSTRACT

This study aimed to analyze the response of lactating beef cows to repeated short nutritional challenges with their performance parameters and plasma metabolites. Multiparous lactating beef cows were subjected to three repeated nutritional challenges in the fourth month of lactation. Each challenge consisted of a 4-d feed restriction (55% of their average energy and protein requirements), followed by a 3-d refeeding period (100% requirements). Cows were classified into two groups differing in their performance (milk yield) and metabolic adaptation [non esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHB**)] to diet changes (metabolic response, **MR**): High and Low MR cows, where the High MR cows showed a faster and larger response to diet changes than the Low MR cows ($P < 0.001$). The loss in milk yield during restriction was the smallest in challenge 1 ($P < 0.001$). Milk urea increased during restriction in challenges 1 and 2 ($P < 0.001$). The High MR cows had greater **NEFA** concentrations than their Low MR counterparts during restrictions, and greater **BHB** concentrations during the restriction of challenge 2 ($P < 0.001$). Restriction increased NEFA, BHB (only in the High MR cows) and urea ($P < 0.01$). During refeeding, both milk yield and plasma metabolites recovered basal values ($P > 0.05$). These results highlight the ability of beef cows to respond to and recover from successive short-term nutrient restrictions, and that despite a certain degree of sensitization of milk yield may have occurred, there were only minimal changes in the metabolic strategies triggered to cope with repeated underfeeding.

1. Introduction

Feed shortage caused by either climate change or high feed prices due to current global energy scarcity (Benoit and Mottet, 2023) may lead to a reduction in the availability and quality of feedstuffs that farmers provide livestock with. Furthermore, the frequency of extreme weather events is predicted to increase (Chang-Fung-Martel et al., 2017), and consequently, livestock could be more frequently exposed to repeated restriction periods of variable length. With restricted nutrient supply, a range of physiological adaptation mechanisms have been described in lactating cows, including body fat and protein mobilization and lower milk yields (Agenäs et al., 2003; Bauman and Currie, 1980; Bell, 1995). Animals' ability to 'bounce back' from relatively short-term disturbance is defined as resilience (Friggens et al., 2022). When the initial state has completely recovered after a challenge, the response is considered elastic, otherwise it is flexible (Blanc et al., 2010). The metabolic response (**MR**) to undernutrition, defined as the homeostatic strategy adopted to cope with the challenge, may differ among individuals depending on their priority for nutrient allocation for the different physiological functions. Therefore, identifying groups of cows with similar response profiles could be interesting for the application of targeted management strategies to different groups within the herd (de Koster et al., 2019).

The MR of beef cows undergoing a short feed restriction has previously been studied to find indicators of robustness (De La Torre et al., 2022) or determine the impact effect of the month of lactation in which the restriction occurred (Orquera-Arguero et al., 2022, 2023b). The response could also be affected by repeated exposure to feed restrictions, because the coping mechanisms depend on the nature, frequency, duration, and intensity of the stressor (Chen et al., 2016). Habituation has been defined as decreased responsiveness to repeated stimuli, whereas sensitization implies increased responsiveness (Blumstein, 2016). Habituation studies have been performed in cattle by applying repeated exposures to stressors, such as regrouping and relocation

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(Veissier et al., 2001), acidosis challenges (Dohme et al., 2008; Nagata et al., 2018) or oscillations in diet quality (Rauch et al., 2021). The responses were reported to decrease (Nagata et al., 2018), not change (Rauch et al., 2021) or be more acute (Dohme et al., 2008) with successive challenges over time. Here we hypothesized that when lactating beef cows were exposed to successive short-term nutrient restrictions, the accumulated effect of repeated exposure would affect their response and reduce the negative impact on milk yield. Therefore, this experiment conducted during the indoor feeding period of suckler cattle aimed to: (1) cluster lactating beef cows according to their MR to three repeated short nutritional challenges and subsequent refeeding; (2) analyze the effect of the MR profile and repeated feeding challenges on the performance parameters and plasma metabolites that are indicative of energy and protein status.

2. Materials and methods

The experimental procedures (protocol no. CEEA-03-2018-01), which follow the guidelines of EU Directive 2010/63 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

The study was performed at La Garcipollera Research Station (Spain, 42°37′ N, 0°30′ W, 945 m a.s.l.) using 31 multiparous lactating Parda de Montaña beef cows [at calving (mean ± SD): 626 ± 47.7 kg body weight (**BW**), 2.8 ± 0.22 body condition score (0 to 5 scale) and 7.5 ± 2.91 years]. One week after calving, cows were randomly assigned to pens (7 or 8 cows/pen, 10 × 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 06:00 h and 14:00 h, according to the traditional management system in mountain farms (Blanco et al., 2008).

Before the start of this study the cows had been subjected to and fully recovered from two 4-d nutritional challenges in the second and third month of lactation, as described in Orquera-Arguero et al. (2022). To analyze the effects of repeated perturbation in the short term, the current experiment started 83 (± 5.4) days post-partum (**dpp**) with a pre-challenge period (−4 to −1 d), followed by three consecutive challenges (1, 2, and 3), each consisting of a 4-d restriction followed by a 3-d refeeding period (Fig. 1). Cows were fed a diet composed of different quantities of permanent grassland 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 (**NE**): 5.4 MJ/kg DM; metabolizable protein (**MP**): 59 g protein digestible in the intestine (**PDI**)/kg DM] and cereal-based concentrate (DM: 915 g/kg; CP: 166 g/kg DM; NDF: 255 g/kg DM; ADF: 119 g/kg DM; NE: 7.6 MJ/kg DM; MP: 120 g PDI/kg DM). The INRA equations (INRA, 2007) were used to calculate diets to meet either 100% (pre-challenge and refeeding periods) or 55% (restriction periods) of the NE and MP requirements for the maintenance and lactation of a standard cow (BW at calving: 615 kg, peak milk yield: 8.5 kg/d).

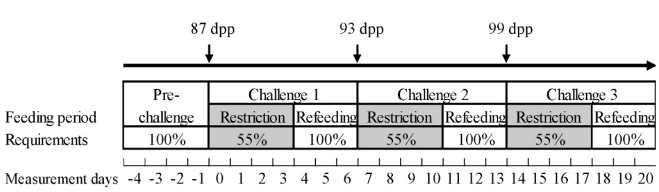

Fig. 1. Schematic representation of the timeline of three repeated short nutritional challenges. Dpp: days post-partum. ↑: sampling days for all traits.

All the cows were fed the same diet in the same amount during each period, irrespectively of their individual requirements. It 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. Hay was offered at 08:00 h as a single meal in individual feeders with cows tied up for approximately 2 h until they finished their ration, and refusals were collected if the cows did not consume the entire diet provided. The individual hay intake was recorded daily. During the basal and refeeding periods, ALPRO feeding stations were programmed to offer cows the established amount of concentrate, and the individual concentrate intake was recorded daily. Cows had free access to water and mineral blocks throughout the experiment.

2.2. Measurements, samplings and chemical analyses

All the measurements and samples of the feedstuffs, milk and blood were collected daily at 07:00 h, before cows had access to diet (Fig. 1). The chemical composition of feedstuffs was analyzed in duplicate following official methods as reported in Orquera-Arguero et al. (2022). These data were used to calculate their nutritional value (INRA, 2007). The daily individual hay and concentrate intakes were calculated on a DM basis.

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 during both suckling periods (morning and afternoon). Immediately after calf removal after the morning suckling period, cows were administered an intramuscular injection of oxytocin (40 UI, Facilpart, Laboratorios Syva, León, Spain) 5 min before manual extraction to facilitate residual milk letdown. The 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 milk composition analysis. 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 coccygeal vein using heparinized and K2 EDTA-containing tubes (BD Vacutainer Becton-Dickenson and Company, Plymouth, UK) and immediately centrifuged at 3500 rpm for 20 min at 4 °C. Plasma was frozen at −20 °C until further analyses. Randox kits (Randox Laboratories Ltd., Country Antrim, UK) were used to determine the plasma concentrations of non esterified fatty acids (NEFA, colorimetric method, sensitivity: 0.072 mmol/L) and β-hydroxybutyrate (BHB, kinetic enzymatic method, sensitivity: 0.100 mmol/L). An automatic analyzer (Gernon, RAL S.A, Barcelona, Spain) measured the plasma urea concentrations (kinetic method, sensitivity: 0.056 mmol/L). The mean intra- and inter-assay coefficients were respectively 4.3% and 4.7% for NEFA, 6.6% and 7.4% for BHB, and 4.0% and 5.1% for urea.

2.3. Calculations and statistical analyses

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

To explore the variability of milk yield, plasma NEFA and BHB, data distribution was represented by challenge and feeding period with violin plots using the ggplot2 package of R (R Development Core Team, 2021). The F-test was employed to test whether the variances from the different challenges and periods were equal using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The response of cows' milk yield, plasma NEFA, and BHB concentration were modelled as spline curves according to Orquera-Arguero et al. (2022) to describe their adaptive strategy to successive nutritional challenges. Briefly, the curve predicted for each trait (milk yield, NEFA, BHB) and animal, measured on a daily basis through the whole experiment, was modelled using natural cubic splines with eight knots with the library splines of R. The following six new variables that summarized animals' response to each challenge were calculated from the fitted curve: **baseline** (values with no feed restriction according to a linear interpolation from values obtained pre challenge and post challenge); **peak** (maximum difference between the actual daily value and the baseline value); **days to peak** (days from the time restriction started to the time the peak values were met); **days to regain** (days from the time restriction started to the time the contents reached the baseline again); **AUC$_{rest}$** (area under the curve (AUC) during restriction, calculated as the estimated total loss/gain of contents during restriction compared to the baseline values); **AUC$_{refeed}$** (the estimated total loss/gain of contents during refeeding until the baseline values were regained).

The curve response variables corresponding to the three nutrient restriction periods (18 variables per animal and parameter) were used to perform a principal component analysis (the **PCA** function in the FactoMineR package of R). Then hierarchical clustering on these principal components (the HCPC function in the FactoMineR package of R) was carried out to group those cows with a similar response pattern into the same MR cluster, the optimum number of which was automatically calculated by the algorithm.

Daily data were averaged within animals and during periods to compare feeding periods. The curve response variables, performance parameters, plasma metabolites, and their percentage of change were analyzed with mixed models for repeated measurements considering the MR cluster, 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 cow as the random effect. The inclusion of cow as random effect accounts for the repeated measurements in the dataset. The variance components structure was selected based on the lowest Akaike and Bayesian information criteria. Degrees of freedom were adjusted with the Kenward-Roger correction. Least square (LS) means and standard errors were obtained, along with multiple comparisons adjusted with the Tukey correction. The normal distribution of variables was tested from residuals of the models using a Q-Q plot. Normality could not be confirmed for NEFA, therefore the values were log-transformed to perform the statistical analysis and then the model estimates were transformed into the original scale for reporting the results. The mean value of each cow within diet (pre-challenge and refeeding periods; restriction; $n = 62$ per trait) was used to explore the associations among the performance parameters and plasma metabolites by Pearson's rank correlations (r) using the CORRPLOT procedure of R. For all the 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 feeding period is shown in Fig. 2. A large range of responses was observed in NEFA and BHB concentrations during the restriction periods, whereas the milk yield response to nutrient restriction was less variable among cows. For milk yield, the pre-challenge variance was greater than those observed during the restrictions in challenges 2 and 3 ($P < 0.05$). On the opposite, for 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 during the pre-challenge period and the refeeding periods of the three challenges.

The PCA performed on the curve response variables for milk yield, NEFA, and BHB explained 48% of total variance with the first three principal components (Dim) (25%, 14%, and 9% in Dim 1, Dim 2, and

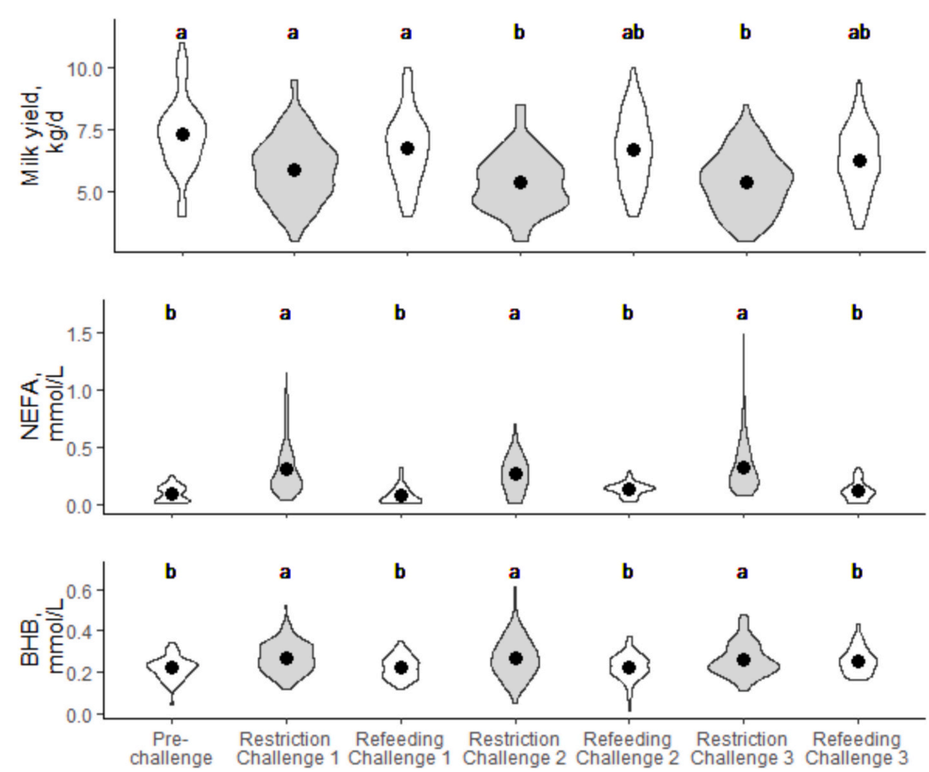

Fig. 2. Frequency distribution of milk yield, non esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB).
The gray area represents the 55% nutritional restriction of cows' energy and metabolizable protein (MP) requirements. The black dot indicates the mean value. Within a parameter, different letters (a,b) indicate differences in variance among feeding periods ($P < 0.05$).

Dim 3, respectively). Dim 1 was related to the milk yield curve variables, Dim 2 to the NEFA curve variables, and Dim 3 to the milk yield and BHB curve variables in the recovery phase (Supplemental Fig. S1). The clustering analysis generated two groups of cows with different MRs, hereafter denoted as the High MR ($n = 15$) and Low MR ($n = 16$) cows (Supplemental Fig. S2). The mean values for the curve response variables of milk yield, NEFA and BHB plasma concentration are available in the Supplemental Material (Tables S1, S2, and S3, respectively). All the curve response variables for milk yield differed between MR clusters ($P \leq 0.02$; Table S1). The cows in the High MR cluster had a greater baseline, greater peak and AUC, and were faster at reaching the peak and slower for regaining the baseline. Regarding the metabolites curve response variables, the High MR cluster cows had greater NEFA baseline values, and greater NEFA and BHB peaks and greater BHB AUC during restriction than their counterparts ($P \leq 0.004$; Table S2 and S3).

3.1. Performance parameters

Cow BW and milk yield according to MR cluster and feeding period are presented in Table 1. Cow BW was affected by the MR cluster-feeding period interaction ($P = 0.04$). Restriction reduced cows' BW as compared to the pre-challenge values in both MR clusters, but to different extents in the three challenges. After refeeding, BW only recovered the pre-challenge values in challenge 1 in the High MR cows ($P > 0.82$). Milk yield was affected by both MR cluster and feeding period ($P < 0.001$; Table 1), and only tended to be affected by their interaction ($P = 0.09$). The High MR cows had a greater milk yield than the Low MR cows ($P < 0.001$). Milk yield lowered with restriction and increased with refeeding in all three challenges ($P < 0.001$), but to different extents. Compared to the pre-challenge values, the milk yield percentage loss was smaller in challenge 1 (−19%, $P < 0.001$) than in challenges 2 and 3 (−27% and − 26%, respectively, $P \leq 0.008$). Daily milk yield throughout the experiment according to the MR cluster is plotted in Fig. 3. The daily analyses showed that milk yield decreased on the first day of restriction for all three challenges in both MR clusters, except for the Low MR cows in challenge 1, when it lowered on the second day ($P < 0.001$, Fig. 3). In the three challenges, the pre-challenge milk yield recovered on the first day of refeeding for the High MR cows, but on the second day for the Low MR cows ($P < 0.004$, Fig. 3).

Table 1
Effect of the metabolic response (MR) cluster and feeding period (FP) on body weight (BW), milk yield, and milk protein content of beef cows to a repeated 4-d restriction and a 3-d refeeding challenge.

	BW, kg		Milk yield, kg/d		Milk protein, g/100 g	
	High MR	Low MR	High MR	Low MR	High MR	Low MR
Pre-challenge	591^{a}	579^{a}	8.1^{a,x}	6.6^{a,y}	2.91^{a}	3.02
Challenge 1						
Restriction	584^{bc}	566^{b}	6.7^{c,x}	5.2^{cd,y}	2.82^{b}	3.04
Refeeding	587^{ab}	567^{b}	7.7^{ab,x}	5.8^{b,y}	2.82^{ab}	3.00
Challenge 2						
Restriction	575^{d}	556^{c}	6.0^{d,x}	4.8^{d,y}	2.83^{ab}	3.01
Refeeding	578^{d}	560^{c}	7.5^{ab,x}	5.9^{b,y}	2.91^{a}	3.02
Challenge 3						
Restriction	571^{e}	555^{c}	6.1^{d,x}	4.7^{d,y}	2.83^{ab,y}	3.08^{x}
Refeeding	582^{cd}	563^{b}	7.2^{bc,x}	5.4^{bc,y}	2.84^{ab}	3.00
RSD[1]	7.6		0.79		0.112	
P-values						
MR cluster	0.24		<0.001		0.009	
FP	<0.001		<0.001		0.006	
MR cluster × FP	0.04		0.09		<0.001	

Within a column, different superscripts (^{a,b,c,d,e}) indicate differences among feeding periods ($P < 0.05$). Within a parameter and row, different superscripts ^{x,y} indicate differences between MR clusters ($P < 0.05$).
[1] Residual standard deviation.

Milk composition according to MR cluster and feeding period is presented in Table 1 and Fig.4. Milk protein content was affected by the MR cluster-feeding period interaction ($P < 0.001$, Table 1). Milk protein contents were similar between MR clusters, except for a tendency to differ during restriction in challenge 1 (2.82 *vs.* 3.04 g/100 g in the High MR and the Low MR group, respectively, $P = 0.06$, respectively). Moreover, a significant difference was noted during restriction in challenge 3 ($P = 0.02$). The milk fat, lactose, and urea contents were affected only by feeding period ($P < 0.001$, Fig. 4). The milk fat contents in challenge 1 were greater than in challenge 3 ($P = 0.03$). Lactose decreased with restriction and increased to the pre-challenge contents during refeeding in challenges 1 and 2 ($P < 0.001$). Restriction increased the milk urea content in challenge 1 (+37%, $P < 0.001$) and in challenge 2 (+9%, $P = 0.01$). It lowered during refeeding to the pre-challenge values in challenge 1, and further lowered in challenges 2 and 3 ($P < 0.05$).

3.2. Plasma metabolites

The plasma NEFA, BHB and urea concentrations according to the feeding period are presented in Table 2 and the daily plasma concentrations throughout the experiment are depicted in Fig. 5. The plasma NEFA were affected by both MR cluster and feeding period ($P < 0.001$; Table 2) and tended to be affected by their interaction ($P = 0.051$). The High MR cluster cows had a greater NEFA concentration than those in the Low MR cluster ($P < 0.001$). Restriction increased NEFA by 3-fold ($P < 0.01$). Regarding the daily data (Fig. 5), during restriction, the NEFA concentrations were greater than their pre-challenge values ($P < 0.05$) during the four days in challenge 1, but not on day 1 in challenge 2 (for both MR clusters) and 3 (only in the High MR cluster). The pre-challenge values were recovered on the first day of refeeding in the three challenges for both MR clusters ($P > 0.05$), except for the Low MR cows in challenge 2, which recovered on the second day of refeeding ($P > 0.05$).

The plasma BHB concentrations were affected by the MR cluster-feeding period interaction ($P = 0.005$), since they significantly changed in the High MR cows, but remained stable in the Low MR cows (Table 2). The BHB concentrations of High MR cows were higher than those of the Low MR cows during restriction in challenge 2 ($P < 0.001$) but the difference did not reach significance in challenges 1 and 3 ($P = 0.10$ and $P = 0.27$, respectively). The daily analyses showed that BHB increased in the High MR cows on the second day of restriction in all the challenges ($P \leq 0.01$) and the pre-challenge concentrations were recovered on the first day of refeeding ($P > 0.05$; Fig. 5).

The plasma urea concentrations were only affected by feeding period ($P < 0.001$). As compared to their pre-challenge values, their concentrations increased during restriction ($P \leq 0.01$) and dropped below the pre-challenge concentrations during refeeding ($P < 0.05$). Regarding daily evolution, concentrations were only greater than those from the pre-challenge period on day 1 and 3 of restriction in challenge 1, and on day 1 in challenges 2 and 3 ($P < 0.05$). During refeeding, urea concentrations decreased below basal values in the three challenges ($P < 0.05$).

The correlations among the performance parameters and plasma metabolites are shown in Fig. 6. The EB correlated strongly and negatively with milk urea and plasma NEFA, BHB, and urea ($P < 0.001$). The milk yield correlated negatively with milk protein ($P < 0.001$) and also with milk urea and plasma BHB and NEFA ($P < 0.05$). There were strong positive correlations between milk urea and plasma urea and NEFA ($P < 0.001$), and moderate positive correlations with plasma BHB ($P < 0.01$).

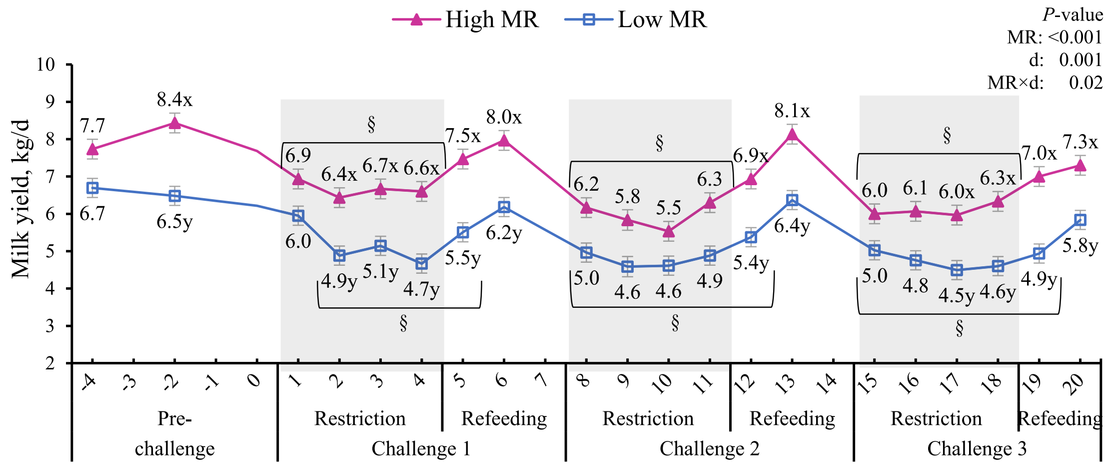

Fig. 3. Daily milk yield according to the metabolic response (MR) cluster throughout the experimental period.
The gray area represents the 55% nutritional restriction of cows' energy and metabolizable protein (MP) requirements. LS Means are presented. Vertical bars indicate standard error.
Within a day, different letters (x,y) denote differences between MR clusters ($P < 0.05$).
§ Denotes differences between a day and the pre-challenge values ($P < 0.05$).

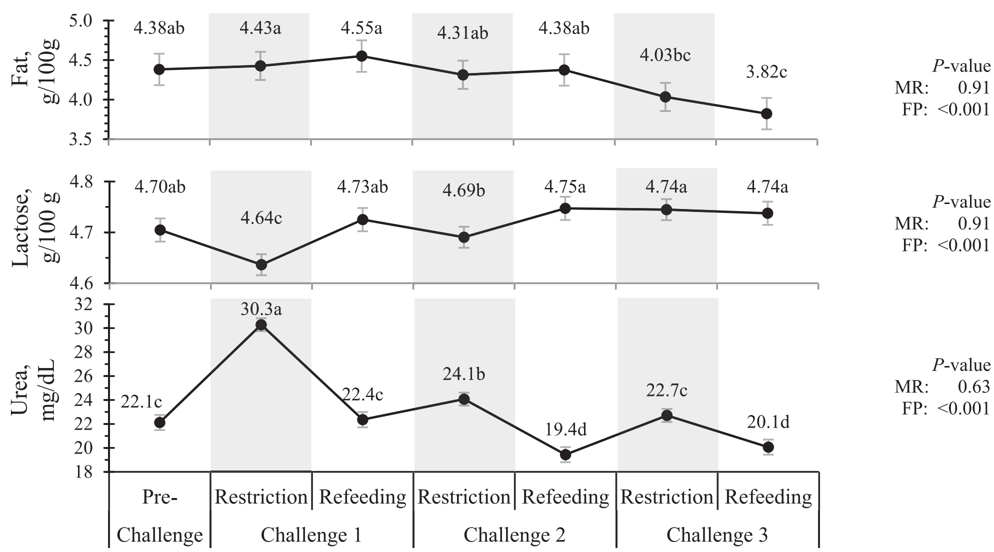

Fig. 4. Milk fat, lactose, and urea contents during the experiment.
The gray area represents the 55% nutritional restriction of cows' energy and metabolizable protein (MP) requirements. LS Means are presented. Vertical bars indicate standard error.
Within a parameter, different letters (a, b, c) indicate differences between feeding periods (FP) ($P < 0.05$). The interactions between FP and metabolic response (MR) cluster were not significant.

4. Discussion

This study of the individual variability in milk yield, plasma NEFA and BHB in response to different diets showed that during restriction periods, milk production variance lowered, while those of the NEFA and BHB concentrations increased. Accordingly with previous research on the individual response of beef cows to short-term nutrient restriction (De La Torre et al., 2022; Orquera-Arguero et al., 2022), the cows reacted with large differences in the mobilization capacity of their fat reserves. This alleviated the negative impact of 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 both response and recovery from a challenge can be used to identify animal types with different adaptive capacities (Friggens et al., 2016). For this purpose, we modelled the response curves of milk yield, NEFA, and BHB with repeated feed challenges by quantifying the gap between the potential and perturbed curve as an indicator of animals' resilience (Barreto-Mendes et al., 2022; Ben Abdelkrim et al., 2021; Poppe et al., 2020). The response variables allowed us to discriminate two distinct groups of cows which differed in their adaptation strategies to repeated challenges. This clustering analysis has proven useful for providing a decision-making basis at the herd level (de Koster et al., 2019; Tremblay et al., 2018) because it identifies distinct aggregated response patterns and provides more relevant information than differentiating cows only by a single trait. Another previous study clustered the same cows according to their response to short nutritional perturbations in different lactation months (Orquera-Arguero et al., 2022). Most of them (27 out of 31) were classified into the same groups according to their MR as in the present experiment. This suggests that, irrespective of the timing and frequency of feed challenges, there are inherent differences in the metabolism of beef cows, at least in part determined by genetics, which influence how nutrients are partitioned towards various biological

Table 2
Effect of the metabolic response (MR) cluster and feeding period (FP) on the plasma metabolites of beef cows to a repeated 4-d restriction and a 3-d refeeding challenge.

	NEFA[1], mmol/L		BHB[2], mmol/L		Urea[3], mmol/L
	High MR	Low MR	High MR	Low MR	
Pre-challenge	0.11^{bc,x}	0.05^{d,y}	0.22^{c}	0.22	3.92^{c}
Challenge 1					
Restriction	0.34^{a,x}	0.18^{ab,y}	0.30^{a}	0.25	4.60^{a}
Refeeding	0.06^{c}	0.05^{d}	0.23^{c}	0.21	3.33^{d}
Challenge 2					
Restriction	0.26^{a,x}	0.16^{ab,y}	0.31^{a,x}	0.23^{y}	4.31^{b}
Refeeding	0.13^{b}	0.11^{bc}	0.24^{bc}	0.21	2.79^{e}
Challenge 3					
Restriction	0.33^{a}	0.23^{a}	0.28^{ab}	0.24	4.34^{ab}
Refeeding	0.12^{b}	0.07^{cd}	0.27^{abc}	0.24	3.50^{d}
RSD[4]	0.15		0.06		0.71
P-values					
MR cluster	<0.001		0.008		0.65
FP	<0.001		<0.001		<0.001
MR cluster × FP	0.05		0.005		0.47

Within a column, different superscripts (^{a,b,c}) indicate differences among feeding periods ($P < 0.05$). Within a parameter and row, different superscripts ^{x,y} indicate differences between MR clusters ($P < 0.05$).
[1] non esterified fatty acids. Statistical analysis performed with log-transformed data. Results of LS means of log (NEFA) converted to the original scale.
[2] β-hydroxybutyrate.
[3] LS means of both MR clusters are presented together because the interaction was not significant.
[4] Residual standard deviation.

functions (Friggens and Newbold, 2007).

4.1. Performance parameters

Lactating cows very much depend on supply of nutrients to the udder to support milk synthesis (Agenäs et al., 2003). Despite the array of physiological mechanisms which come into play to maintain homeostasis (Bauman and Currie, 1980; Baumgard et al., 2017), rapidly declining milk yield can be expected during a feed restriction period, as observed in cows with different basal EB (Orquera-Arguero et al., 2023a). Nutrient restriction herein reduced milk yield to a lesser extent in the first than in the subsequent challenges. This finding implies that the severity of impact increased until the second challenge, but not thereafter, and therefore sensitization was limited. In general, the High MR cows had greater milk yields and showed a faster response to diet changes than the Low MR cows, which needed another day of refeeding to recover the pre-challenge yields. This could partly be associated to a different nutritional status, given that all the cows received the same diet during each period irrespectively of individual differences in performance. It could also be related to differences in nutrient partitioning, these findings are similar to those reported by Baumgard et al. (2017) between high- and low-yielding dairy cows because the former show more marked priority for diverting absorbed nutrients to mammary glands to ensure milk synthesis, as would be the case for the High MR cows in the current experiment. Despite this difference, the daily values show that both MR groups were able to regain their pre-challenge yield by the second day of refeeding in all the challenges, as observed previously in beef cows (De La Torre et al., 2022; Orquera-Arguero et al., 2022) and regardless of repeated exposure, which indicates that beef cows are resilient under these conditions and even with this relatively short recovery phase. Therefore in beef cows, milk yield can be considered susceptible to sensitization after repeated underfeeding events, but also a trait with elastic properties, because deformation is reversible and can return to its original state (Blanc et al., 2010).

The repeated nutritional challenges produced minor changes in milk components. Milk fat content only dropped during the refeeding of challenge 3, which suggests that it was affected only by the cumulative effect of the three challenges. The milk fat content in beef cows does not seem to be largely affected by short feed restrictions. Nonetheless, Orquera-Arguero et al. (2023a) reported a significant effect of diet changes on the fine fatty acid composition of milk fat. Regarding lactose, the reduction during restriction and recovery during refeeding agrees with previous observations in dairy (Bjerre-Harpøth et al., 2012) and beef cows (Orquera-Arguero et al., 2023a), but the response was less intense after successive challenges, which suggests habituation.

Milk urea changes are more evident because, due to the diffusion from blood to milk (Spek et al., 2013), they quickly reflect changes in the balance of dietary protein and energy supply for ruminal microbial metabolism (Kessler et al., 2020). This was confirmed by the negative correlation of milk urea with EB herein observed. Conflicting results have been obtained for the response of milk urea to feed restriction in dairy cows. Those results range from an increase (Carlson et al., 2006) attributed to amino acid catabolism for energy supply, to a decrease (Abdelatty et al., 2017; Kvidera et al., 2017) associated with a smaller supply of amino acids from intestine absorption (Billa et al., 2020). Our findings support the first hypothesis and suggest greater protein catabolism in the first challenge, as reflected by the greater milk urea content, which would decrease in challenges 2 and 3 as corroborated by the strongly correlated plasma urea concentrations. Overall, the milk yield results suggest that the impact of the repeated restriction increased only with the second challenge, but further exposure to a third challenge did not elicit a more acute response than that observed in the second one. Regarding milk composition, the cumulative effect of repeated exposure led to sensitization in milk fat, while milk lactose and urea exhibited habituation by the third challenge.

4.2. Plasma metabolites

The effect of short-term dietary restrictions and subsequent refeeding on the plasma indicators of metabolic status has been documented in both 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). The literature reports greater NEFA release from adipose tissue to be either used for milk fat synthesis by mammary glands or oxidized in the liver into ketone bodies, such as BHB, acetoacetate or acetone, which can be used as energy fuel to support milk production (Bell, 1995; Puppel and Kuczyńska, 2016). Here the rise in the NEFA concentrations in response to feed restriction in all the challenges showed that the cows of both MR groups underwent lipid mobilization, with greater peak values in the High MR cows. Concentrations were lower than those observed in beef cows in earlier lactation stages, when metabolic demand and priority for milk production are greater and then diminish after the peak milk yield is reached in the second month of lactation (Orquera-Arguero et al., 2023b). Changes in the NEFA contents were concomitant with a rise in the BHB plasma concentration, but only in the High MR cows, where greater metabolic demand provoked greater lipolysis to support greater milk yields. Apparently in the Low MR cows, the slighter NEFA increases were insufficient to trigger ketogenesis, which resulted in no change in the BHB plasma concentration (McArt et al., 2013).

The daily analysis provided further insights into the effects of repeated exposure on dynamic response patterns according to MR clusters. A rise in NEFA for at least one day of the 4-day restriction periods occurred in both groups. This increment reached greater values in the High MR cluster, but peak contents were always below the threshold

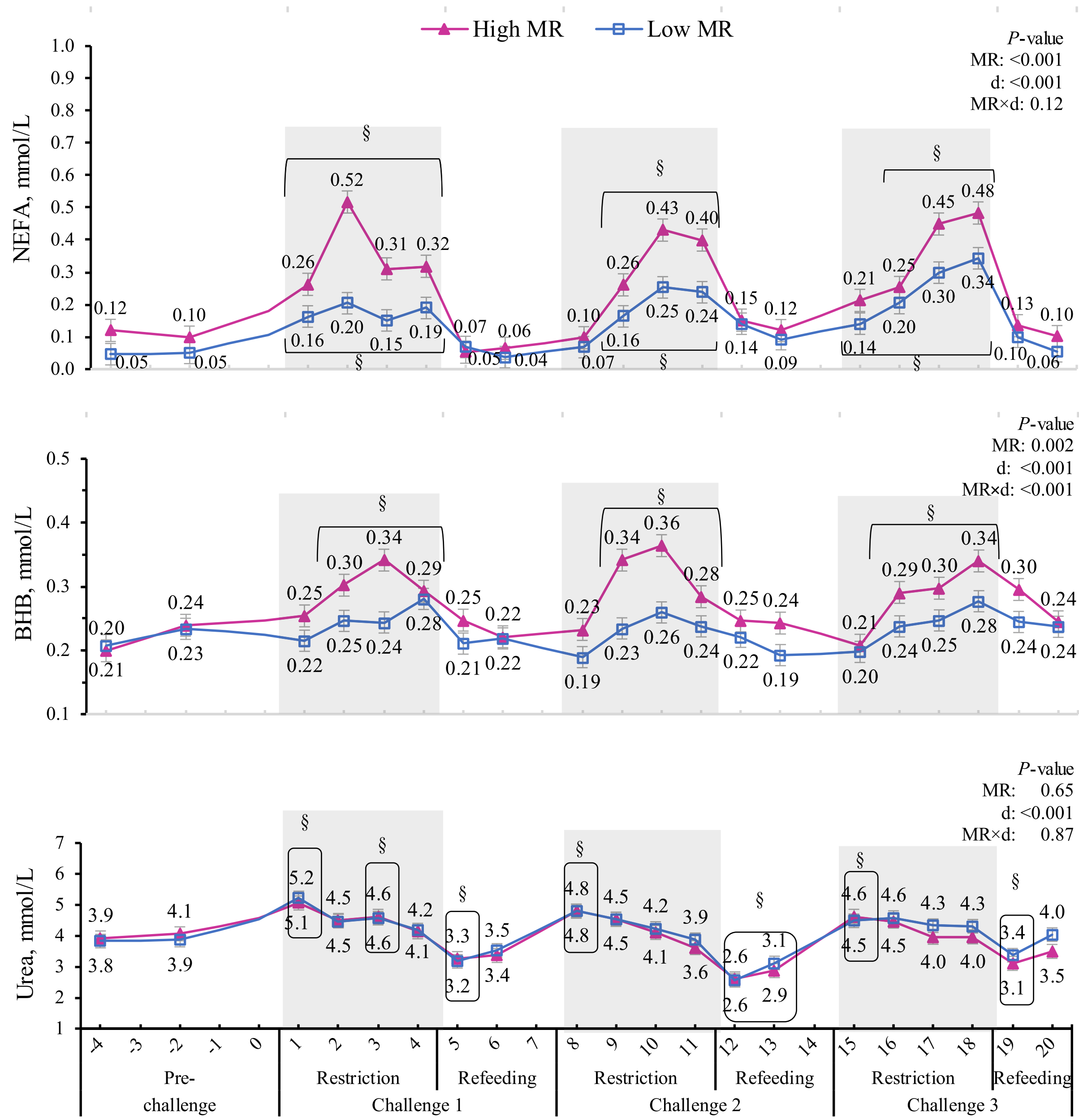

Fig. 5. Daily plasma concentrations of non esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), and urea according to the metabolic response (MR) cluster throughout the experimental period. The NEFA statistical analysis was performed with log-transformed data. Results of LS means of log(NEFA) converted to the original scale.

The gray area represents the 55% nutritional restriction of cows' energy and metabolizable protein (MP) requirements. LS Means are presented. Vertical bars indicate standard error.

Within a parameter and day, different letters (x,y) denote differences between MR clusters ($P < 0.05$).

§ Denotes differences between a day and the pre-challenge values ($P < 0.05$). For urea concentration, the difference is between the daily LS Means of both clusters because the interaction is not significant.

of 0.60 mmol/L proposed as an indicator of risk of metabolic disease in dairy cows (Ospina et al., 2010). The NEFA response to feed restriction tended to be delayed in the High MR cows by at least one day and less intense after the first exposure. Even if limited, this decreased responsiveness would partly explain the higher impact of the successive underfeeding events on milk yield, since the extent of body fat mobilization was reduced and less efficient to cope with the reduced nutrient supply. However, this hypothesis was not confirmed in the Low MR cows.

The BHB plasma concentrations responded slower to restriction than NEFA in both groups, which agrees with Puppel and Kuczyńska (2016), whereas the BHB threshold for the risk of ketosis (1.2 mmol/L, McArt et al., 2013) was not reached in either group. The similar values among challenges imply that BHB was not involved in the different response of milk yield to repeated underfeeding bouts. Despite the differences observed between MR clusters during restrictions, in the refeeding phases both groups had similar NEFA and BHB plasma concentrations as in the pre-challenge period, showing an elastic response. Accordingly, Ferraretto et al. (2014) reported that after dairy cows had received 25% or 50% feed restriction, circulating NEFA returned to the basal concentrations one day after dairy cows returned to their normal intake.

Plasma urea concentrations are influenced by dietary protein intake, but also by the catabolism of the labile protein reserves from muscle tissue and visceral organs such as the liver, kidney and intestinal *epitheliae* under energy deficit (Spek et al., 2013), when glucogenic amino

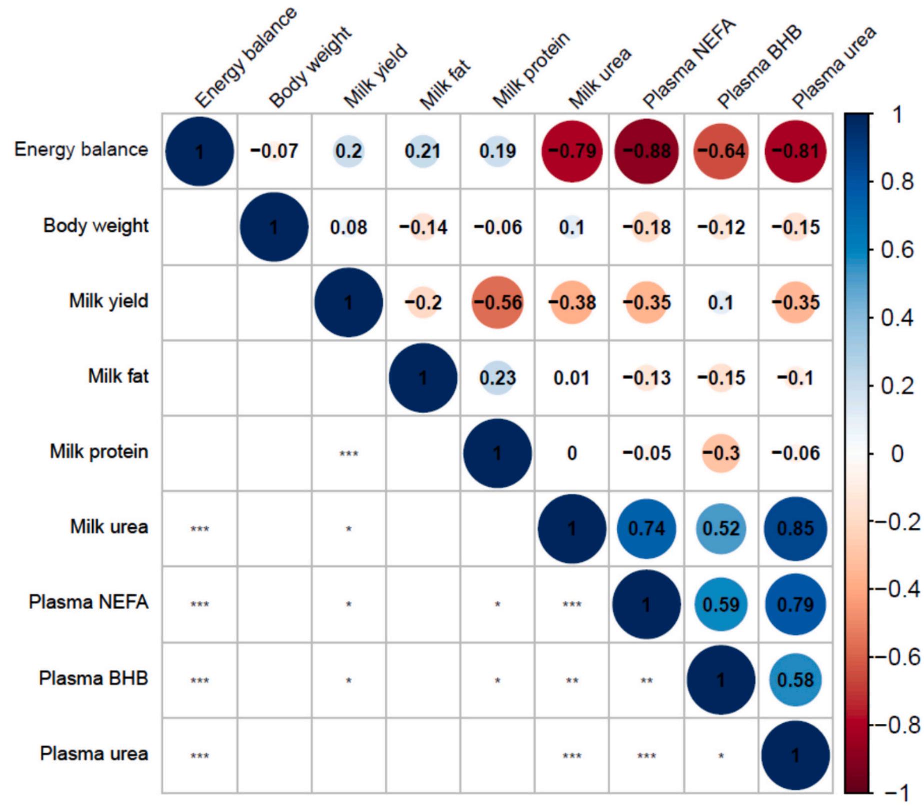

Fig. 6. Pearson's rank correlations (above the diagonal) among performance parameters and plasma metabolites, and their *p*-values (below the diagonal). ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$. NEFA: non esterified fatty acids, BHB: β-hydroxybutyrate. The statistical analysis was performed with log-tranformed data for NEFA.

acids are mobilized to supply glucose and urea is generated during the process (Agenäs et al., 2003; Bell, 1995; Burgos et al., 2001). Some studies conducted in dairy cows have reported how plasma urea decreased (Bjerre-Harpøth et al., 2012) or remained unchanged with feed restriction (Carlson et al., 2006), whereas Horn et al. (2014) described increased urea in underfed cows. Here we observed increments in the mean concentrations during the feed restriction which decreased even below the pre-challenge values with refeeding. However, the small differences observed in the daily values during the experiment suggests that the metabolism of body protein played a minor role compared to that of lipolysis in the response of beef cows to short underfeeding periods, or in their potential habituation or sensitization to their repeated occurrence.

5. Conclusions

Repeated short-term feed restriction and refeeding challenges had effects of different magnitudes on the productive and metabolic traits of lactating beef cows. The cows with distinct MR profiles reacted differently in terms of milk yield and plasma NEFA and BHB concentrations, all of which returned to the basal values after short refeeding. Milk loss in response to restriction worsened after the first challenge, but the lipid metabolism was only minimally affected by the repeated exposure to underfeeding.

CRediT authorship contribution statement

K.G. Orquera-Arguero: Writing – original draft, Methodology, Investigation, Formal analysis. **I. Casasús:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **D. Villalba:** Writing – review & editing, Formal analysis, Conceptualization. **J. Ferrer:** Investigation. **M. Blanco:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105414.

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