

Article

Assessment of Metabolic Adaptations in Periparturient Dairy Cows Provided 3-Nitrooxypropanol and Varying Concentrate Proportions by Using the GreenFeed System for Indirect Calorimetry, Biochemical Blood Parameters and Ultrasonography of Adipose Tissues

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Citation: Schilde, M.; von Soosten, D.; Frahm, J.; Kersten, S.; Meyer, U.; Zeyner, A.; Dänicke, S. Assessment of Metabolic Adaptations in Periparturient Dairy Cows Provided 3-Nitrooxypropanol and Varying Concentrate Proportions by Using the GreenFeed System for Indirect Calorimetry, Biochemical Blood Parameters and Ultrasonography of Adipose Tissues. *Dairy* **2022**, *3*, 100–122. <https://doi.org/10.3390/dairy3010009>

Academic Editor: Andre M. Almeida

Received: 22 September 2021

Accepted: 29 November 2021

Published: 3 February 2022

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Abstract: Methanogenesis in ruminants contributes to both greenhouse gas emissions and feed energy losses whereby the latter becomes specifically important in energy-deficient periparturient cows. It was hypothesized that increased concentrate feed proportions (CFP) and feeding with the methane inhibitor 3-nitrooxypropanol (3-NOP), as well as their potential synergism, improve the energy status of periparturient cows. Periparturient dairy cows were fed low or high dietary CFP either tested without or combined with 3-NOP. The GreenFeed system was used to calculate the metabolic respiration quotient ($RQ_{\text{metabolic}}$) and tissue energy retention (ER_{tissue}) by methods of indirect calorimetry. The calorimetrically estimated ER_{tissue} coincided with a conventionally calculated energy balance except for the antepartal period. Neither CFP nor 3-NOP affected the ultrasonographically assessed lipomobilization in adipose depots. In the group fed 3-NOP and a high concentrate feed proportion, the $RQ_{\text{metabolic}}$ significantly rose over the course of the experiment and the ER_{tissue} was also increased. Serum non-esterified fatty acid concentrations were lower in the 3-NOP groups albeit β -hydroxybutyrate (BHB) remained unaffected. Higher CFP reduced BHB and increased blood glucose levels. In conclusion, 3-NOP and high CFP improved the energy budget of the cows in an interactive manner, which was, however, not apparent in all of the examined parameters. The application of the GreenFeed system for indirect calorimetry is a promising approach, which needs further validation in the future.

Keywords: dairy cows; methane production; 3-nitrooxypropanol; GreenFeed; indirect calorimetry; energy metabolism

1. Introduction

In ruminants, feed is mainly converted to volatile fatty acids (VFA) by the rumen microbiota, thereby yielding hydrogen (H_2) and carbon-dioxide (CO_2), which are redirected to methane (CH_4) formation in methanogenic archaea [1]. 3-nitrooxypropanol (3-NOP), a structural analogue of methyl-coenzyme M, is currently supposed to be one of the most potent CH_4 inhibitors in cattle [2,3]. The CH_4 -mitigating effect of 3-NOP potentially amounts to $39.0 \pm 5.40\%$ in dairy cows [4] but ranges widely from 7 [5] to 60% [6] depending on the provided ration type (neutral-detergent fibre (NDF) content), administration technique (mixing in with the total-mixed ration (TMR), infusion, pulse-dose) and dosage level [4].

Besides the ecological benefits of reducing greenhouse gas emissions from ruminant livestock [7], CH₄ mitigation is assumed to improve feed energy efficiency as up to 12% of the gross energy intake (GEI) can be lost by methanogenesis in the bovine rumen [8]. Furthermore, both increased dietary concentrate feed proportion (CFP) [9] and 3-NOP [4] were observed to shift rumen fermentation to H₂-consuming propionic-metabolic typed pathways [4,9] which could increase the hepatic supply of glucogenic precursors [10], with this being specifically advantageous in periparturient cows. Hence, transitional dairy cows metabolically adapt to the negative energy balance (EB), which is the disparity between energy intake and requirements for maintenance and lactogenesis, by induction of an accelerated lipolysis in adipose tissue (AT) depots [11]. Subsequently, the massive hepatic influx of non-esterified fatty acids (NEFA) risks a metabolic overload of the hepatic capacity for NEFA oxidation, which results in increased ketone body synthesis and predisposition of the cow to hyperketonaemia and hepatic lipidosis [11,12].

Reynolds et al. [5] identified a decreased total EB and increased heat production (HP) when energy metabolism was expressed as a percentage of digested energy in dairy cows supplemented with 2500 mg of 3-NOP per day. In contrast, van Gastelen et al. [13] reported that HP and energy retention in body fat and protein remained unaffected in 16 early-lactation dairy cows supplemented with 51 mg of 3-NOP/kg dry matter (DM). Correspondingly, energy allocation to body weight gain (BWG), representing a positive EB at the tissue level, was observed to be either increased [2,13] or not affected in 3-NOP-fed dairy cows, as reported by Haisan et al. [14] and in the accompanying manuscript [15]. 3-NOP was comprehensively reported to exert no influence on energy expenditure (EE) for milk production [16] even though varying 3-NOP effects on milk composition were published [13,16,17]. However, the effects on milk ingredients were attributed to the aforementioned 3-NOP-induced shift in the rumen fermentation pattern toward a decreased acetate-propionate ratio [18] rather than being associated with alterations in post-ruminal energy metabolism [17]. Accordingly, excessive accumulation of NEFA and β -hydroxybutyrate (BHB) in blood, being indicative of a severe negative EB [19], were not affected in early- [20] and mid-lactating [14] 3-NOP-supplemented cows.

However, there is a gap in the knowledge of the energy turnover at the tissue level and intermediary pools of correspondingly regulated blood metabolites in cows provided varying CFP combined with 3-NOP in their rations during the periparturient period. Gas exchange measurements of CO₂ production and O₂ consumption in dairy cows are commonly measured in the respiration chamber (RC), which is often referred to as the 'gold-standard' technique. These gas measurements allow for an indirect calorimetric estimation of the HP and the total (RQ_{total}) and metabolic (rumen fermentation corrected) respiration quotient (RQ_{metabolic}). The RQ_{total} mirrors the whole animal metabolism including feed nutrient degradation in the rumen, whereby the RQ_{metabolic} rather reflects the intermediary oxidation of specific macronutrients and, therefore, dynamics in physiological and nutritional adaptations [21]. Accordingly, RQ_{metabolic} values of 1.0 mirror a prevailing carbohydrate oxidation whereas those of fat oxidation and deposition amount to 0.71 and above 1.0, respectively. Protein oxidation is associated with RQ_{metabolic} values of 0.81 [22]. However, the costly gas quantification in RC restricts the cow's normal behaviour and only allows the measurement of small animal numbers over short-term periods [23]. Therefore, the present approach aimed to use spot gas flux measurements of CH₄, CO₂ and O₂ from the open-circuit GreenFeed (GF) system (C-Lock Inc., Rapid City, SD, USA) for the indirect calorimetric estimation of EE for maintenance, production and energy retention in body tissues (ER_{tissue}) in periparturient dairy cows provided 3-NOP and varying CFP in the ration. This assessment of cow energetics was accompanied by an ultrasonic-based estimation of lipomobilization from AT depots in concert with frequent blood sampling for analyses of energy-related metabolites.

It was hypothesized that increased glucogenic propionate [15] and energy spared from methanogenesis due to feeding 3-NOP in combination with high CFP caused a surplus of energy being utilized to cope with the negative EB in periparturient dairy cows, which was

reflected by the decreased lipomobilization from AT depots and serum concentrations of NEFA and BHB.

2. Materials and Methods

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) in Brunswick, Germany in accordance with the German Animal Welfare Act and approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany) (33.19-42502-04-15/1858).

2.1. Experimental Design

The presented investigations are part of a comprehensive experiment: the fundamental experimental design, CH₄ and CO₂ emissions, dry matter intake (DMI), rumen VFA, BW measures, EB according to the 'Gesellschaft für Ernährungsphysiologie' (GFE) [24] (EB_{GFE}), milk performance parameters and feed efficiency are presented in Schilde et al. [15]. During the present experiment, 55 pluriparous German Holstein cows were assigned to four groups according to a 2 × 2-factorial design. In this context, treatments of low (LC) or high (HC) CFP were tested without supplements (CONLC (*n* = 14), CONHC (*n* = 15)), or combined with 3-NOP (DSM Nutritional Products AG, Kaiseraugst, Switzerland) (NOPLC, 48.4 mg/kg dry matter (DM) (*n* = 14); NOPHC, 51.2 mg 3-NOP/kg DM (*n* = 12)) in the ration from d 28 ante-partum (a.p.) until d 120 post-partum (p.p.). The whole experimental period (d 28 a.p. until d 120 p.p.) was split into three periods, namely the ante-partum (Per 1: d 28 a.p. until parturition) and post-partum (Per 2: d 1 until d 28 p.p.) phase of the transition period and the early-lactation period (Per 3: d 29 until d 120 p.p.) in order to compare metabolic and respiratory changes assessed by indirect calorimetry with energy expenditures and supply between the different periods. The experimental groups were balanced for calculated date of calving, 4% fat-corrected milk yield (FCM) in their previous lactation (6207 ± 1248 kg; mean ± SD), body condition score six weeks ante-partum (a.p.) (3.3 ± 0.4) and number of lactations (3.0 ± 1.1). The cows were housed in a free stall barn with high bed cubicles and slatted floor. Three cows out of the initial 58 did not complete the trial (two cases of abomasal displacement and one case of necrotising endometritis in the NOPLC group). Ten out of the 55 cows were cannulated (three cows in each of the 3-NOP and two cows in each of the CON groups). During the a.p. period, CFP amounted to 15% for LC and 40% for HC groups. Starting from the day of parturition until d 21 p.p., a gradual increase in CFP from 30 to 55%, where it remained until the end of the experiment, was scheduled in HC groups. In LC groups, CFP was fixed at 30% from the day of parturition until termination of the trial. The target CFP was administered by computerized self-feeding stations.

Cows were offered a partial mixed ration (PMR) for ad libitum intake in weighing troughs (type RIC, Insentec B.V., Marknesse, the Netherlands) which was composed of 70% maize silage, 20% grass silage and 10% of a pelletized concentrate including either 3-NOP or the placebo (DM basis). Further 3-NOP compound was incorporated into pelletized concentrates, which was provided by the concentrate feeders to adjust the aforementioned 3-NOP target concentration. This two-way method of supplementing concentrate pellets, including 3-NOP via mixing with the PMR and the concentrate feeders, facilitated the regulation of 3-NOP target consumption and the 3-NOP supply synchronously to the meal event [15]. The CONLC and CONHC groups received a placebo in the concentrate feed pellets that contained propylene glycol, with SiO₂ also being part of the 3-NOP supplement.

2.2. Sample Collection

The DMI of the PMR and concentrates was continuously monitored by the computerized weighing troughs and concentrate feeders. Concentrates and PMR were sampled once and twice a week and pooled to collective samples of four-week periods, respectively.

Continuously from d 28 a.p. until d 120 p.p., gas samples were collected from the exhaust air pipe to measure gas mass fluxes (g/d) of O₂ consumption and CH₄ and

CO₂ emissions using the GF system (C-Lock Inc., Rapid City, SD, USA) as described previously [15].

Rectal temperature was measured each time before blood sampling. Blood samples were taken by puncturing a Vena jugularis externa at d 28, 14, 7, 3 a.p. and d 1, 3, 7, 14, 21, 28, 35, 49, 73, 98, and 120 p.p. after morning milking using heparinized sample syringes (Werfen GmbH, Kirchheim, Germany) and serum tubes. Serum tubes were allowed to clot for 30 min at 303 K, were subsequently centrifuged (Heraeus Varifuge 3.0R Heraeus Instruments GmbH, Hanau, Germany; 2123 × g, 288 K, 15 min), and separated serum was stored at −80 °C until further analyses were conducted.

According to Raschka et al. [25], ultrasonic measurements (UM) of fat layer thickness in millimetres were conducted in duplicate at the seven topographic points on the right side of the cow at d 3 and 28 p.p. with the use of a Mindray M5 Vet (Mindray, Shenzhen, China) diagnostic ultrasound system equipped with a linear (6 MHz, Mindray 6LE5Vs) and a convex probe (3 MHz, Mindray 3C5s). The description of the seven topographic measurement points was detailed in Schäfers et al. [26].

2.3. Analyses

Samples of concentrates and PMR components were dried for 72 h at 55 °C, ground to pass a 1-mm screen (SM 1, Retsch GmbH, Haan, Germany) and analysed for chemical composition according to the standard methods published by the Association of German Agricultural Analysis and Research Centers [27]. The chemical composition of the experimental diets is illustrated in Table 1. The 3-NOP contents in concentrate feed samples were analysed by DSM Nutritional Products AG, Kaiseraugst, Switzerland.

Table 1. Chemical composition, peNDF and energy (means) of the total rations offered during the experimental period from d 28 ante-partum until d 120 post-partum (reproduced from and with permission from Schilde et al. [15] at Taylor & Francis Group <https://www.tandfonline.com/>) © 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group (<https://www.tandfonline.com/>) under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

Item	CON [†]		3-NOP [§]	
	LC	HC	LC	HC
DM ⁺ (g/kg)	467	582	467	597
Nutrient (g/kg of DM)				
Crude ash	63	61	63	61
Crude protein	130	138	129	140
Utilizable crude protein	142	150	142	151
Ether extract	32	35	32	36
aNDF _{om} [¶]	402	344	404	337
Acid detergent fibre _{om}	226	191	227	187
peNDF _{>8mm} [#]	268	269	274	273
Starch	249	303	246	307
Sugar	17	25	17	27
Energy [§] (MJ/kg of DM)				
GE	18.4	18.5	18.4	18.4
ME	11.0	11.5	11.0	11.5
NE _L	6.6	7.0	6.6	7.1

[†] Control (CON) groups were provided a placebo and low (LC) or high (HC) concentrate feed proportion in the ration. [§] 3-nitrooxypropanol (3-NOP) groups were supplemented with 48.4 and 51.2 mg of 3-NOP/kg of DM and LC and HC in the ration. ⁺ DM, dry matter. [¶] aNDF_{om}, α-amylase treated neutral detergent fibre without residual ash. [#] peNDF_{>8mm}, physically effective NDF in the partial mixed ration defined as the proportion of DM retained by a 8-mm screen multiplied by the dietary NDF content [28]. [§] Calculations for concentrates based on table values according to DLG [29], silages according to VDLUFA [27] analyses and GE calculated according to GE [30].

Gas samples and background gases were analysed by the already installed GF sensors. CH₄ and CO₂ concentrations were analysed by non-dispersive infrared absorption sensors and O₂ was analysed using a paramagnetic sensor. Sensor calibration was performed automatically on a daily basis using a zero (O₂ = 200,000 mg/kg, N₂ = 800,000 mg/kg) and a span gas (CH₄ = 1004 mg/kg, CO₂ = 10,000 mg/kg, O₂ = 210,000 mg/kg, H₂ = 9.50 mg/kg, H₂S = 9.80 mg/kg, while the remainder of the gas was nitrogen). The air velocity in the pipe was measured by an anemometer to determine total mass flow of all gases. CO₂ recovery tests were conducted once a month (recovery rate ± SD: 101% ± 5.7). The amount of bait feed delivered per feed drop was calibrated on a weekly basis.

Serum samples were photometrically analysed (Indiko Plus, Thermo Scientific GmbH, Dreieich, Germany) for concentrations of BHB, NEFA, triacylglycerides (TAG) and glucose. An automated blood gas and electrolyte analyser (GEM Premier 4000, Werfen GmbH, Kirchheim, Germany) was used to determine the temperature-corrected pH, hydrogen carbonate ions, haemoglobin and lactate concentrations immediately after sample collection.

2.4. Calculation of Energy Metabolism Parameters by Indirect Calorimetry and Ultrasonography

Processing and validation of gas exchange data were conducted by C-Lock Inc. Gas measurements were converted from g/d to L/d according to the gas density of 0.717 kg/m³ for CH₄, 1.977 kg/m³ for CO₂ and 1.729 g/m³ for O₂ under standard conditions (1013.25 hPa). The cow's visiting time and head position in the GF were used to check for data plausibility [31]. Daily means of GF data were averaged to weekly means using the previously described arithmetic averaging method [32]. Due to technical reasons, O₂ consumption in CON groups was estimated from weekly means of CO₂ production and DMI using the following regression equation:

$$\text{O}_2 \text{ (g/d)} = 2056 - 72.5 \times \text{dry matter intake (kg/d)} + 0.62 \times \text{CO}_2 \text{ (g/d)} \quad (1)$$

with R² = 0.90 and a residual standard error (RSE) of 371 g/d on 337 degrees of freedom.

In ruminants, total CO₂ production (VCO₂) is the sum of fermentative (VCO_{2fermentative}) and metabolic (VCO_{2metabolic}) CO₂ derived from microbial fermentation in the rumen and the intermediary metabolism, respectively [33]. A differentiation between the two is essential in order to refer to the intermediary substrate oxidation [34]. As proposed by Chwalibog et al. [33], VCO_{2fermentative} was calculated by applying the stoichiometrically derived factor of 1.7, which was confirmed to be applicable for a variety of ration compositions [35].

$$\text{VCO}_{2\text{fermentative}} \text{ (L/d)} = 1.7 \times \text{VCH}_4 \text{ (L/d)} \quad (2)$$

Then, VCO_{2fermentative} was subtracted from VCO₂ to obtain VCO_{2metabolic}, which was used to calculate the RQ_{metabolic} mirroring the intermediary oxidation of the macronutrients of carbohydrates, fat and protein [36]:

$$\text{RQ}_{\text{metabolic}} = \text{VCO}_{2\text{metabolic}} \text{ (L/d)} \div \text{VO}_2 \text{ (L/d)} \quad (3)$$

The total RQ (RQ_{total} = total VCO₂ production (L/d) ÷ VO₂ consumption (L/d)) reflected the cow's nutritional plane. Gross energy (GE) content of the feedstuffs was calculated according to GfE [24]. The metabolizable energy (ME) content of the concentrates was derived from tabular values according to DLG [29] and that of silages was derived according to VDLUFA [27] analyses.

HP was quantified according to the Brouwer [22] formula:

$$\text{HP (kJ)} = 16.18 \times \text{VO}_2 \text{ (L/d)} + 5.02 \times \text{VCO}_2 \text{ (L/d)} - 2.17 \times \text{VCH}_4 \text{ (L/d)} - 5.99 \times \text{N}_U \text{ (g/d)}, \quad (4)$$

whereby urinary nitrogen excretion (N_U) was set to 50 g/d [37] even though the real N_U in dairy cows varies between 75 and 150 g/d [38]. However, the N_U contribution to HP is negligible and an error of about 0.3% in the absolute HP values was accepted [39].

Methane energy (CH_4E ; MJ/d) was derived from the multiplication of the energy equivalent value of 39.54 kJ/L of CH_4 [22] and the daily CH_4 production (L/d).

The partitioning of EE for energy retention (ER) was computed as follows:

$$\text{ER in body tissues and milk (ER}_{\text{total}}) \text{ (MJ/d)} = \text{ME intake} - \text{HP} \quad (5)$$

$$\text{ER in body tissues (ER}_{\text{tissue}}) \text{ (MJ/d)} = \text{ME intake} - \text{HP} - \text{ME}_E - \text{NE}_P \quad (6)$$

Analyses and calculations of milk energy excretion (ME_E ; MJ/d) according to GfE [24] were used from Schilde et al. [15]. Net energy demand for pregnancy (NE_P ; MJ/d) was averaged for period 1 according to constants proposed by GfE [24] with 13 MJ NE_L /d for week 4 a.p. and 18 MJ NE_L /d for week 3 a.p. until calving resulting in an average of 17 MJ NE_L /d for period 1. The EB_{GFE} data were extracted from Schilde et al. [15] in which EB_{GFE} was calculated according to GfE [24].

The residual ER in body protein and intramuscular fat was assessed as:

$$\text{ER}_{\text{residual}} \text{ (MJ/d)} = \text{ER}_{\text{tissue}} - \text{ER}_{\text{fat depot}} \quad (7)$$

$\text{ER}_{\text{fat depot}}$ was calculated from UM as described in the following:

$$\text{ER}_{\text{fat depot}} \text{ (MJ/d)} = \text{daily mobilized fat depot masses (kg/d)} \times 39.8 \text{ (MJ/kg)} \times 0.84 \quad (8)$$

The daily mobilization of fat depot masses from each AT depot was described by the difference in AT masses between d 3 p.p. and d 28 p.p. divided by the number of days. The energy release from mobilized fat depots being used for milk production was calculated based on the assumption that 1 g of body fat corresponds to 39.8 kJ of GE [22], whereby 16% is lost as heat energy when body tissue energy is used for milk synthesis [40].

Depot masses of each AT, namely the retroperitoneal (RAT), mesenteric (MAT) and omental (OAT), collectively referred as the visceral AT (VAT), and the subcutaneous AT (SAT) were estimated in kg from ultrasonographically measured distances of the different sites as described in Schäfers et al. [26] according to the following regression equations established by Raschka et al. [25]:

$$\text{SAT} = -6.66 + 0.72 \times \text{R12} + 0.31 \times \text{AW3c} \quad (9)$$

$$\text{RAT} = -9.55 + 0.62 \times \text{R12} + 0.06 \times \text{KD3b} \quad (10)$$

$$\text{OAT} = -2.32 + 0.55 \times \text{BFT} + 0.37 \times \text{AW3b} \quad (11)$$

$$\text{MAT} = -12.8 + 0.38 \times \text{AW1b} + 1.73 \times \text{AW3b} - 1.45 \times \text{AW3c} + 0.07 \times \text{KD2c} \quad (12)$$

$$\text{VAT} = \text{RAT} + \text{OAT} + \text{MAT} \quad (13)$$

The efficiency of utilization of ME for lactation (k_1) was calculated according to AFRC [41]:

$$k_1 = (\text{ME}_E + a \times \text{ER}_{\text{tissue}}) / (\text{ME intake} - \text{ME}_m), \quad (14)$$

where ME_E is adjusted to zero energy balance with a coefficient of $a = 0.84$ for negative $\text{ER}_{\text{tissue}}$ or $a = 1/0.95$ for positive $\text{ER}_{\text{tissue}}$. $\text{ER}_{\text{tissue}}$ is the energy balance obtained by indirect calorimetry using the GreenFeed system. The maintenance requirement (ME_m) was estimated using the equation from GfE [24]:

$$\text{ME}_m \text{ (MJ/d)} = 0.488 \times \text{BW}^{0.75} \text{ (kg)}, \quad (15)$$

where $\text{BW}^{0.75}$ is the metabolic body weight.

2.5. Statistical Analyses

Prior to statistical evaluation, means were calculated per cow and week for variables used in indirect calorimetry. A.p. blood samples were retrospectively assigned to the actual

day relative to parturition by tolerating a deviation of 24 h for the d −3 sample and a deviation of 2 days for the d −7 and d −14 samples. Due to gas leakage through the fistula, cannulated cows were excluded from statistics except for blood and ultrasonic variables. The statistical analyses were conducted using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA) and a repeated measures mixed model (PROC MIXED) fitted by a restricted maximum likelihood [42]. The sequence of day, week of sampling or period (PER) was a repeated measure. 3-NOP, CFP, time relative to parturition and the interaction between them were set as fixed effects and each cow within treatment was set as a random effect. Data on indirect calorimetry and gas measurements were evaluated according to periods fixed at d 28 a.p. until parturition (period 1), d 1 until d 28 p.p. (period 2) and d 29 until d 120 p.p. (period 3). For clinical chemistry parameters, the autoregressive variance–covariance structure was selected based on the best fit according to the lowest Akaike Information Criterion and the result of the first measurement at d 28 before 3-NOP supplementation was regarded as a covariate. Parameters of indirect calorimetry and ultrasonic measurements were tested using a compound symmetry structure. Effects were regarded as statistically significant at p -values ≤ 0.05 and a trend was implied at p -values between 0.05 and 0.10. Multiple t -tests (PROC PDIF) with Tukey-adjusted p -values were computed to evaluate significant means.

The R software package (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) was used to calculate Pearson correlation coefficients and to perform linear regression of EB_{GFE} and ER_{tissue} data. Further, R was applied to estimate the O_2 consumption in CON groups in a linear regression model, whereby significant independent variables and related regression coefficients were estimated in a forward stepwise manner.

The degree of agreement between both methods to estimate the energy balance (ER_{tissue} and EB_{GFE}) was assessed according to Bland and Altman [43]. The differences between EB_{GFE} and ER_{tissue} were plotted against the arithmetic mean of these pairs at each cow and period. The bias as the mean difference including a 95% confidence interval and the standard deviation (SD) of the differences were calculated. The lower and upper limits of agreement (LoA) were calculated (bias \pm 1.96 SD) and used to define the range within which 95% of the differences lay. A regression line was plotted through the differences to detect changes in the bias depending on the magnitudes of the measurements themselves. The normality of differences was tested using the Shapiro–Wilk test in R.

3. Results

3.1. Methane Emission and Respiratory Gas Exchange Measured with the GreenFeed SYSTEM

Data on the emitted fermentation gases and metabolic respiratory gas exchange are presented in Table 2 while detailed dynamics of VCH_4 and VCO_2 emissions on a weekly basis are presented in Schilde et al. [15]. The $VCO_{2metabolic}$ expressed as a percentage of total VCO_2 decreased after parturition until week 4 p.p. in the LC groups, as depicted in Figure 1A. $VCO_{2metabolic}$ in NOPLC continued to decline over the course of the experiment (Figure 1A; 3-NOP \times CFP \times PER; $p < 0.001$). The mean VCH_4 over the three periods was reduced by 24.2% in NOPLC and 29% in NOPHC when compared to the respective CON group (Table 2). VCH_4 increased from an average of 315 L/d in period 2 by 27.7% to 438 L/d in NOPLC and by 8% to 341 L/d in NOPHC in period 3 and, therefore, to a greater extent in the NOPLC than in the NOPHC group. VCH_4 (L/d) positively correlated to VCO_2 (L/d) ($r = 0.67$; $p < 0.001$; $N = 917$). Both VCH_4 and VCO_2 production (total and metabolic VCO_2) decreased from the a.p. period to parturition but increased thereafter over the p.p. period, which was also the case for VO_2 consumption (Table 2). In period 3, VCO_2 and VCH_4 emissions were affected by 3-NOP and the high CFP (Table 2; 3-NOP \times CFP \times PER; $p < 0.001$). Hence, VCO_2 was significantly higher in CONHC, whereas VCH_4 decreased, which was most apparent for NOPHC. VCO_2 production was positively correlated to VO_2 consumption ($r = 0.92$; $p < 0.001$; $N = 915$). VO_2 consumption decreased in the 3-NOP groups over the course of the experiment (Table 2; 3-NOP \times PER; $p < 0.001$), whereas CFP did not exert an influence. Both the RQ_{total} and $RQ_{metabolic}$ were affected by the

3-NOP \times CFP \times TIME interaction (Table 3; $p < 0.001$). The $RQ_{\text{metabolic}}$ markedly dropped from approximately 0.92 ± 0.03 during the a.p. period to its lowest point of 0.90 ± 0.007 in week 1 p.p. (Figure 1B; Table 2). Afterwards, the $RQ_{\text{metabolic}}$ increased to 0.99 in NOPLC and 0.94 in CONLC until week 4 p.p. and remained more or less constant. In contrast, the $RQ_{\text{metabolic}}$ continued to slightly increase to 1.01 in NOPHC and 0.98 in CONHC until week 9 p.p., respectively (Table 2; Figure 1B; 3-NOP \times CFP \times TIME; $p = 0.024$).

Table 2. Fermentation and respiration gases (L/d; measured using the GreenFeed system), total (RQ_{total}) and metabolic respiration quotient ($RQ_{\text{metabolic}}$) of the experimental groups during period (Per) 1 (d 28 ante-partum until day of calving), 2 (d 1 until d 28 post-partum (p.p.)) and 3 (d 29 until d 120 p.p.).

Item	Treatments [†]				SEM	<i>p</i> -Values [§]				
	CONHC (<i>n</i> = 12)	CONLC (<i>n</i> = 13)	NOPHC (<i>n</i> = 11)	NOPLC (<i>n</i> = 9)		3-NOP	CFP	3-NOP \times PER	CFP \times PER	3-NOP \times CFP \times PER
VCH ₄ production										
Per 1	0490	0495	0381	0397	20	<0.001	<0.156	<0.001	<0.001	<0.001
Per 2	0440	0475	0314	0316						
Per 3	0535	0542	0341	0438						
Total VCO ₂ production										
Per 1	6632	6378	6662	6369	160	<0.064	<0.132	<0.001	<0.282	<0.001
Per 2	6368	6289	6002	5823						
Per 3	7278	6719	6557	6553						
VCO _{2metabolic} ⁺										
Per 1	5798	5537	6014	5695	140	<0.653	<0.041	<0.001	<0.043	<0.003
Per 2	5621	5481	5468	5285						
Per 3	6368	5797	5978	5809						
VO ₂ consumption										
Per 1	6348	6190	6267	6205	138	<0.073	<0.436	<0.001	<0.342	<0.166
Per 2	5988	5933	5709	5693						
Per 3	6480	6167	5910	5911						
$RQ_{\text{metabolic}}$ [¶]										
Per 1	0.91	0.90	0.96	0.92	0.007	<0.001	<0.001	<0.001	<0.014	<0.001
Per 2	0.94	0.92	0.95	0.93						
Per 3	0.98	0.94	1.01	0.99						
RQ_{total} [#]										
Per 1	1.04	1.03	1.06	1.03	0.008	0.638	0.004	0.001	0.602	<0.001
Per 2	1.06	1.06	1.05	1.02						
Per 3	1.12	1.09	1.11	1.11						

[†] Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), time period relative to parturition (PER), and interactions between them; effect of PER with $p < 0.001$ and 3-NOP \times CFP with $p > 0.1$ for all variables. ⁺ $VCO_{2\text{metabolic}}$ (L/d) = Total VCO₂ production (L/d) – $VCO_{2\text{fermentative}}$ (L/d), whereby $VCO_{2\text{fermentative}}$ (L/d) = $1.7 \times VCH_4$ production (L/d). [¶] $RQ_{\text{metabolic}}$ = $VCO_{2\text{metabolic}}$ (L/d) \div VO_2 (L/d), corrected for fermentative VCO_2 . [#] RQ_{total} = VCO_2 (L/d) \div VO_2 (L/d).

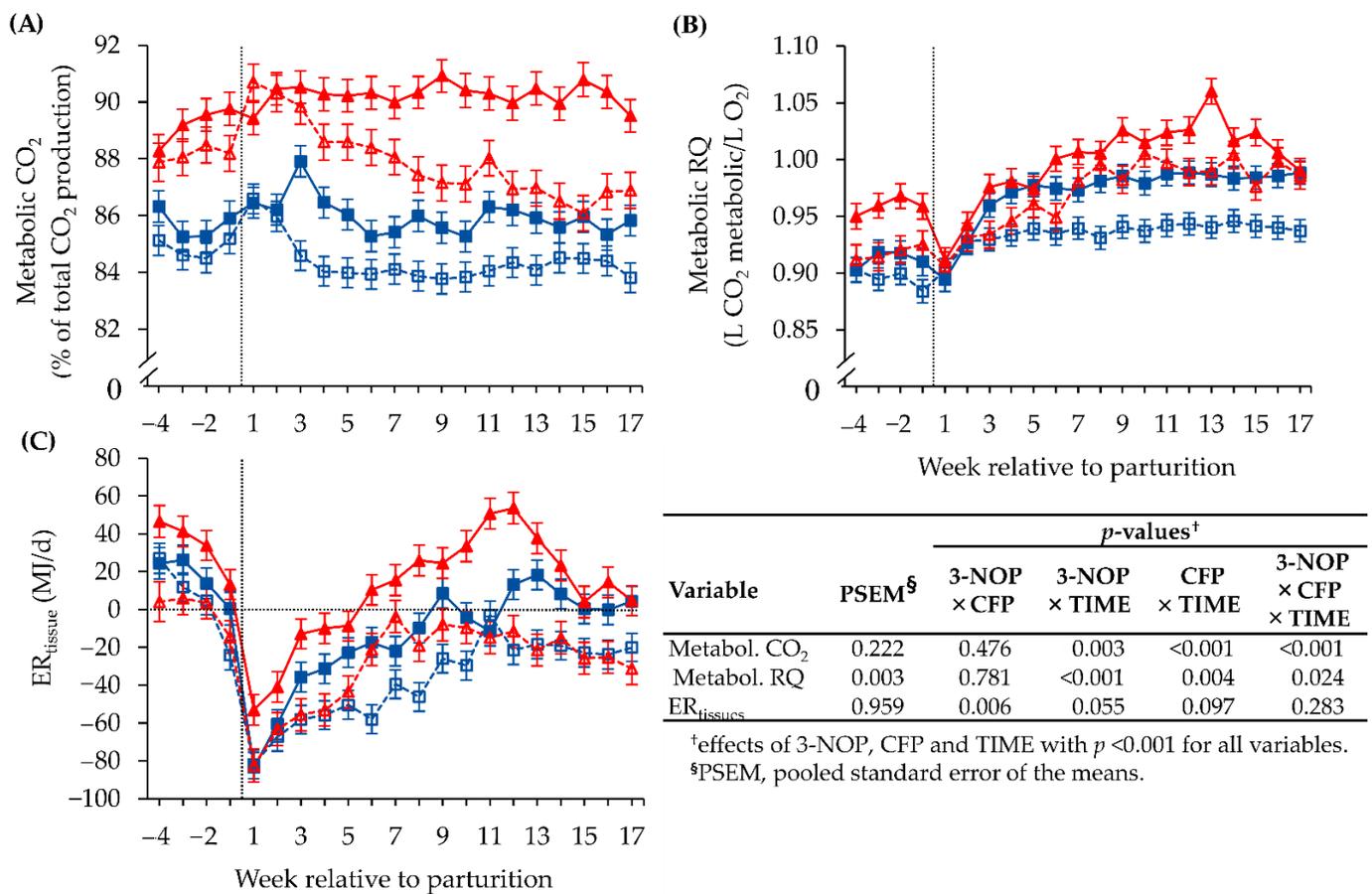


Figure 1. Effects of 3-nitrooxypropanol (3-NOP), dietary concentrate feed proportion (CFP) and time relative to parturition (TIME) on (A) metabolic CO₂ production, (B) metabolic respiration quotient (Metabolic RQ) and (C) energy retention in body tissues (ER_{tissue}) in peripartal dairy cows. ■, solid line = control high CFP (CONHC, n = 12); □, dashed line = control low CFP (CONLC, n = 13); ▲, solid line = 3-NOP high CFP (NOPHC, n = 11); △, dashed line = 3-NOP low CFP (NOPLC, n = 9). Values are presented as LS-means.

Table 3. Energy intake, expenditure and retention of the experimental groups during period (Per) 1 (d 28 ante-partum until day of calving), Per 2 (d 1 until d 28 post-partum (p.p.)) and Per 3 (d 29 until d 120 p.p.) estimated according to gas measurements presented in Table 4.

Item	Treatments [†]				SEM	p-Values [§]					
	CONHC (n = 12)	CONLC (n = 13)	NOPHC (n = 11)	NOPLC (n = 9)		3-NOP	CFP	3-NOP × CFP	3-NOP × PER	CFP × PER	3-NOP × CFP × PER
Metabolic body weight (kg BW ^{0.75})											
Per 1	146	144	144	146	2.9	0.780	0.868	0.517	0.227	<0.001	0.417
Per 2	132	131	129	131							
Per 3	131	127	128	128							

Table 3. Cont.

Item	Treatments [†]				SEM	<i>p</i> -Values [§]					
	CONHC (<i>n</i> = 12)	CONLC (<i>n</i> = 13)	NOPHC (<i>n</i> = 11)	NOPLC (<i>n</i> = 9)		3-NOP	CFP	3-NOP × CFP	3-NOP × PER	CFP × PER	3-NOP × CFP × PER
Energy intake (kJ/kg BW^{0.75} and d)											
Gross energy intake (GEI)											
Per 1	1963	1812	2022	1738	64	0.546	<0.001	0.210	0.146	<0.001	0.092
Per 2	2468	2434	2620	2335							
Per 3	3272	2900	3379	2961							
Metabolizable energy intake (MEI)											
Per 1	1193	1076	1288	1031	40	0.325	<0.001	0.110	0.379	<0.001	0.084
Per 2	1529	1458	1631	1402							
Per 3	2032	1734	2111	1769							
Energy expenditures (kJ/kg BW^{0.75} and d)											
Net energy demand for pregnancy (NE _P) ⁺											
Per 1	115	117	118	116	2.9	0.702	0.951	0.575			
Milk energy excretion (ME _E) [¶]											
Per 2	978	991	945	960	25	0.195	0.492	0.476	0.852	<0.001	0.056
Per 3	1010	931	948	935							
Heat production (HP) [#]											
Per 1	916	905	930	896	17	0.028	0.211	0.738	<0.001	0.750	0.267
Per 2	967	966	940	918							
Per 3	1071	1044	1001	987							
Methane energy (CH ₄ E) [‡]											
Per 1	134	137	106	106	5.3	<0.001	0.099	0.913	<0.001	<0.001	<0.001
Per 2	132	144	96	95							
Per 3	161	170	106	134							
Energy retention (kJ/kg BW^{0.75} and d)											
Energy retention in body tissues and milk (ER _{total}) [◇]											
Per 1	295	183	372	125	41	0.084	<0.001	0.137	0.002	<0.001	0.176
Per 2	562	492	697	485							
Per 3	961	690	1103	783							
Energy retention in body tissues (ER _{tissue}) [◆]											
Per 1	170	65	254	6	45	0.036	<0.001	0.101	0.003	0.022	0.715
Per 2	−396	−499	−222	−474							
Per 3	−23	−230	167	−139							
Energy balance calculated according to GfE [24] (EB _{GFE}) [*]											
Per 1	323	245	379	216	28	0.104	<0.001	0.077	0.066	0.059	0.982
Per 2	−323	−397	−229	−398							
Per 3	−58	−171	54	−154							
Energy retention in fat depots (ER _{fat depot})											
Per 2	−185	−175	−169	−181	24	0.841	0.974	0.637			
Residual energy retention (ER _{residual}) ^Π											
Per 2	−211	−324	−55	−292	48	0.058	0.001	0.205			

[†] Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), time period relative to parturition (PER), and interactions between them; effect of PER with *p* < 0.001 for all variables. ⁺ NE_P (MJ/kg) = mean of 13 MJ of NE_L/d in week 4 a.p. and 18 MJ of NE_L/d during week 3 until parturition according to GfE [24]. [¶] ME_E (MJ/kg) data from Schilde et al. [15]. [#] HP (MJ/d) = 16.18 × O₂ (L/d) + 5.02 × CO₂ (L/d) − 2.17 × CH₄ (L/d) − 5.99 × 50 (g of urine nitrogen excretion/d) [22], gas volumes used from Table 4. [‡] CH₄E (MJ/kg) = CH₄ (L/d) × 0.03954 (MJ/L) [22]. [◇] ER_{total} (MJ/d) = MEI − HP. [◆] ER_{tissue} = MEI − HP − NE_P and ME_E, resp. ^{*} EB_{GFE} (MJ/d) = energy balance data from Schilde et al. [15] and calculated according to GfE [24] (footnote Figure 2). ^{||} ER_{fat depot} (MJ/d) = loss of fat depot masses (kg/d) from d 3 until d 28 p.p. from ultrasonic measurements × 39.8 (MJ/kg) × 0.84, 1 kg of body fat corresponds to 39.8 MJ of GE [22], whereby 16% is lost as heat when body tissue energy is converted into milk [40], 1 kg of body fat corresponds to 39.8 MJ of GE [22]. ^Π ER_{residual} (MJ/d) = ER_{tissue} − ER_{fat depot}.

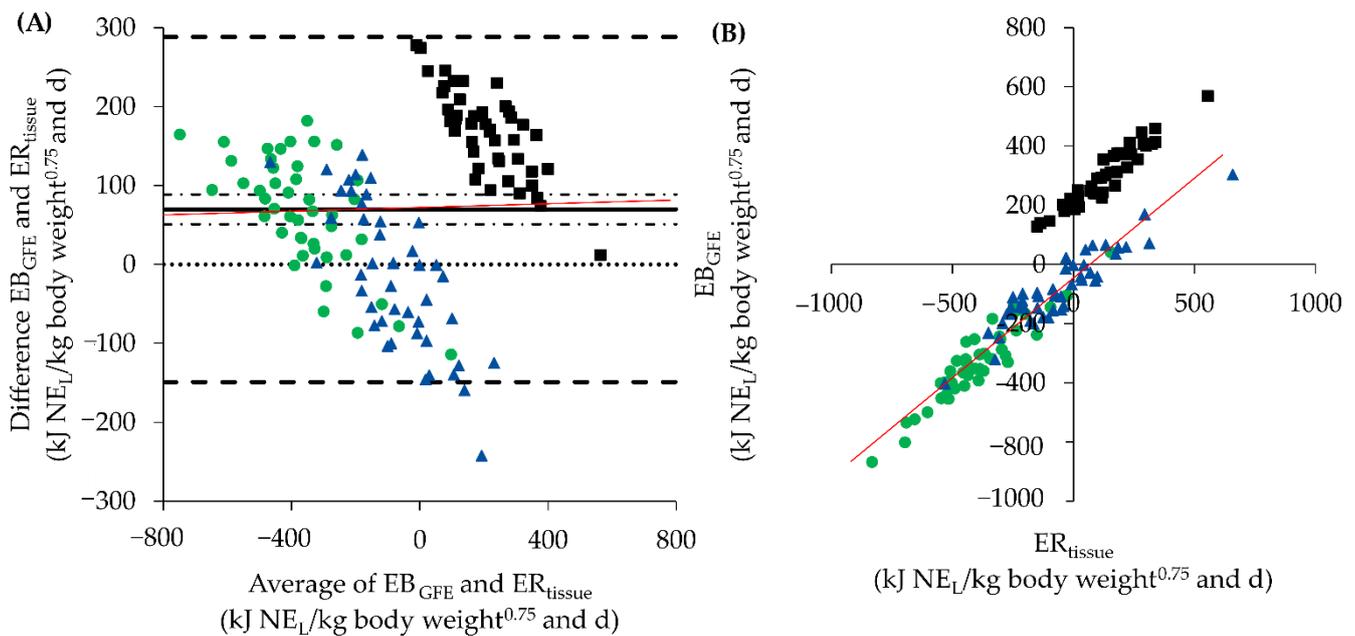


Figure 2. Bland–Altman plot (A) and linear relationship (B) of energy balance (EB_{GFE}) and energy retention in body tissues (ER_{tissue}) during ■ = period 1 (d 28 ante-partum – d 1 post-partum (p.p.)), ● = period 2 (d 1 p.p. – d 28 p.p.) and ▲ = period 3 (d 28 p.p. – d 120 p.p.). ER_{tissue} was calculated by indirect calorimetry using the GreenFeed system and EB_{GFE} according to GfE [24]. **Formula EB_{GFE}:** EB (kJ NE_L/kg body weight^{0.75} (BW^{0.75}) and d) = net energy intake – net energy requirements for maintenance (NE_M) – net energy for pregnancy (NE_P) – net energy for lactation (NEL) with NE_M (kJ NE_L/d) = 0.293 × BW^{0.75} (kg), milk energy (kJ NE_L/d) = 0.3 × milk fat % + 0.21 × milk protein % + 0.95, and NEL (kJ NE_L/d) = milk energy (kJ NE_L/d) + 0.1 × milk yield (kg/d). **Formula ER_{tissue}:** ER in body tissues (kJ NE_L/kg BW^{0.75} and d) = metabolizable energy intake – heat production – NEL – NE_P (period 1). **Statistics 2A:** Bias (—): 70 kJ NE_L/kg BW^{0.75} and d; $p < 0.001$ with confidence interval (---); lower limits of agreement (LoA) (---): -149 kJ NE_L/kg BW^{0.75} and d; upper LoA: 288 kJ NE_L/kg BW^{0.75} and d; regression line (—): $y = 0.01x + 71$ (RSE = 112 kJ/kg BW^{0.75} and d on 133 degrees of freedom, $R^2 = 0.0007$, $p = 0.756$). **Statistics 2B:** regression line (—; period 2 and 3): $y = 0.67_{(0.02)}x - 51_{(7)}$; (RSE = 51 kJ NE_L/kg BW^{0.75} and d on 88 degrees of freedom, $R^2 = 0.92$, $p < 0.001$).

3.2. Energy Turnover Estimated by Indirect Calorimetric and Ultrasonic Methods

The parameters of BW^{0.75}, GEI, metabolizable energy intake (MEI), EE and ER are presented in Table 3. GEI and MEI significantly increased in the HC groups by an average of 28% from period 1 to period 2 and by 31% up to period 3, whereas in the LC groups, GEI and MEI increased, on average, by 35% from period 1 to period 2 and by 23% up to period 3 (CFP × PER; $p < 0.001$). The experimentally intended gradual increase in energy intake resulted, during period 3, in significantly higher daily GE and ME uptakes in the HC groups, by about 0.4 MJ and 0.32 MJ/kg BW^{0.75} and d, respectively, when compared to the LC groups.

The 3-NOP × PER interaction ($p < 0.001$) of HP was driven by a decreasing HP from 3-NOP when compared to the CON groups in period 3 (Table 3). HP was positively correlated with MEI, which was not different between treatment groups ($r = 0.37$; $p < 0.001$; $N = 895$). During the course of the experiment, ME_E decreased in the LC groups, whereas that of the HC groups increased (CFP × PER; $p < 0.001$). With regard to period 3, CH₄E was lowest in NOPHC, in contrast with the NOPLC and the CON groups (3-NOP × CFP × PER; $p < 0.001$).

Table 4. Changes in fat layer thickness (mm/d) and adipose tissue (AT) depot mass (kg/d) estimated from ultrasonic measurements of the experimental cows from d 3 until d 28 post-partum.

Item	Treatments [†]				SEM	<i>p</i> -Values [§]		
	CONHC (<i>n</i> = 14)	CONLC (<i>n</i> = 15)	NOPHC (<i>n</i> = 14)	NOPLC (<i>n</i> = 12)		3-NOP	CFP	3-NOP × CFP
Change in fat layer thickness								
Back fat thickness	−0.15	−0.15	−0.14	−0.12	0.03	0.709	0.847	0.786
Rib fat thickness	−0.16	−0.15	−0.17	−0.13	0.03	0.814	0.493	0.699
Change in AT depot mass								
Mesenteric	−0.26	−0.22	−0.20	−0.31	0.05	0.775	0.479	0.136
Omental	−0.18	−0.18	−0.16	−0.15	0.02	0.285	0.956	0.952
Retroperitoneal	−0.12	−0.10	−0.13	−0.11	0.02	0.780	0.367	0.894
Subcutaneous	−0.17	−0.18	−0.17	−0.14	0.02	0.410	0.616	0.399
Visceral [†]	−0.56	−0.50	−0.48	−0.57	0.07	0.998	0.996	0.330
Visceral and subcutaneous	−0.73	−0.68	−0.65	−0.70	0.08	0.758	0.994	0.627

[†] Values are presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), and interactions between them. [†] PSEM, pooled standard error of the means. [†] Visceral AT depot mass (kg/d) = mesenteric + omental + retroperitoneal AT depot mass (kg/d).

During the course of the experiment ER_{total} and ER_{tissue} increased with elevated CFP in the diet (Table 3; CFP × PER; $p < 0.05$). ER_{tissue} was more positive in the NOPHC group over the experimental periods (Table 3; Figure 1C: 3-NOP × CFP; $p = 0.006$). ER_{tissue} is shown on a weekly basis in Figure 1C and a sharp drop can be seen in ER_{tissue} starting from the initiation of the trial until week 1 p.p., when the tissue energy balance was the most negative, independent of the experimental group (Figure 1C; TIME; $p < 0.001$). In all of the treatment groups, a continuous rise in ER_{tissue} was observed from week 1 p.p. onwards, with this being the most distinctive in the HC groups (CFP; $p < 0.001$). In the NOPHC group, ER_{tissue} reached a positive range in week 4 p.p. which was earlier when compared to CONHC (positive ER_{tissue} from week 8 p.p.). In contrast, ER_{tissue} in the LC groups remained in a negative range until termination of the trial.

The described group differences concerning the extent of energy retained in body tissues were, however, not recovered in the $ER_{fat\ depot}$ (Table 3; 3-NOP × CFP; $p = 0.637$) which was estimated ultrasonographically during period 2. The effect of time (Table A1; TIME; $p < 0.001$) was reflected by a decrease in each AT depot (Table 4). Irrespective of treatment group, the average lipomobilization from the visceral and subcutaneous AT of 0.69 kg of fat depot masses per day contributed to a daily energy release of about 177.5 kJ/kg $BW^{0.75}$ and d being potentially utilizable for milk synthesis (Table 3). Correspondingly, back fat and rib fat thickness decreased, on average, by 0.14 and 0.15 cm/d, respectively (Table 4). In addition, the visceral fat deposit was mobilized to a larger extent when compared to the subcutaneous one (0.53 kg/d vs. 0.17 kg/d; Table 4).

Due to the described differences in ER_{tissue} between groups but the missing effects of 3-NOP and CFP on depot fat mobilization from ultrasonic measurements, $ER_{residual}$ was higher in the 3-NOP and HC groups (Table 3; 3-NOP; $p = 0.058$; CFP; $p = 0.001$).

3.3. Validation of the ER_{tissue} Outcome of the GreenFeed Indirect Calorimetry Method

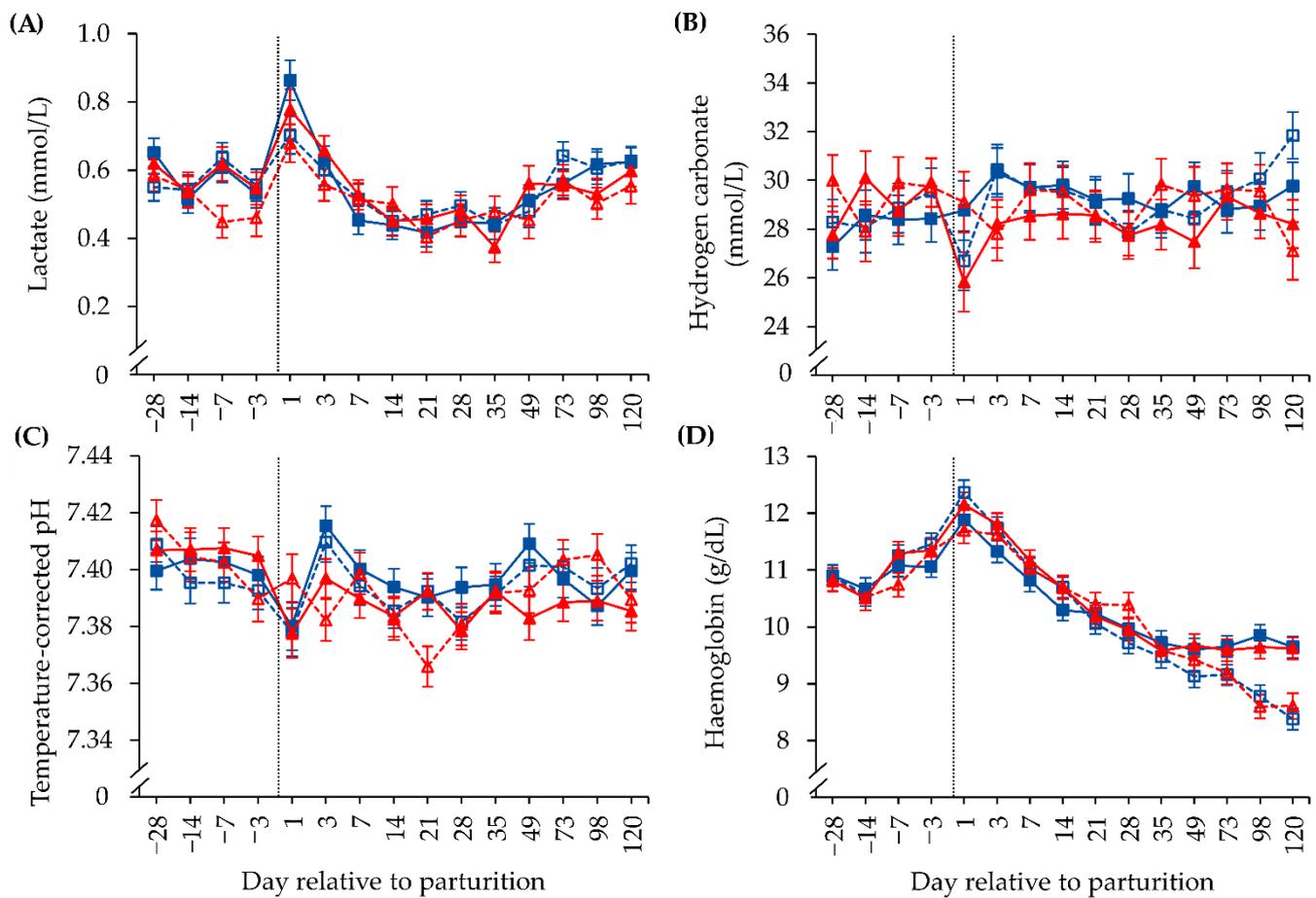
The EB_{GFE} varied between experimental periods, which was similar to ER_{tissue} (Table 3; PER; $p < 0.001$). In contrast to ER_{tissue} , the EB_{GFE} of the NOPHC group was more positive

during each of the experimental periods when compared to the other treatment groups (Table 3; 3-NOP \times CFP; $p = 0.082$). The Bland–Altman analysis (Figure 2A; mean bias of 70 kJ NE_L/kg BW^{0.75} and d; $p < 0.001$ over all of the experimental periods) and the slope of the regression line of the linear relationship indicated that the EB_{GFE} was estimated to be approximately 33% (Figure 2B) higher when compared to ER_{tissue}. The slope of the regression line through the data points of differences was not significant ($p = 0.756$), indicating a constant bias over the experimental periods (Figure 2A). Nevertheless, greater differences between both methods with increasing magnitude of a positive energy balance can be visually identified regarding the a.p. period 1 (Figure 2A,B). Furthermore, the agreement between the EB_{GFE} and the calorimetrically obtained ER_{tissue} was most accurate concerning period 2 and 3 (Figure 2A,B). Hence, a non-significant mean bias of 21 kJ NE_L/kg BW^{0.75} and d ($p = 0.051$) was calculated for the agreement between both methods for period 2 and 3. In contrast, greater differences between EB_{GFE} and ER_{tissue} were found in period 1, with a mean bias of 167 kJ NE_L/kg BW^{0.75} and d ($p < 0.001$). The average k_1 over the experimental groups and periods totalled 0.61 (data not shown).

3.4. Biochemical Blood Parameters

Lactate peaked on the day of calving (Figure 3A; TIME; $p < 0.001$). Hydrogen carbonate and the temperature-corrected blood pH marginally fluctuated around their mean of 28.9 mmol/L (TIME; $p = 0.208$; Figure 3B) and 7.39, respectively. However, a slight drop in blood pH values was observed at d 1 p.p. (TIME; $p < 0.001$; Figure 3C). Antepartal haemoglobin levels slightly increased, on average, from 10.6 to 12.0 g/dL on the day of parturition but continuously decreased by approximately 24% afterwards. From d 49 until termination of the experiment, haemoglobin diverged to constant levels of 9.5 g/dL in the HC groups but still decreased to approximately 8.5 g/dL in the LC groups (Figure 3D; CFP \times TIME; $p < 0.001$).

Blood serum concentrations of BHB, NEFA, TAG and glucose are presented in Figure 4. During the transitional period, the characteristic changes of BHB, NEFA, TAG and glucose were observed in all treatment groups (TIME; $p < 0.001$; Figure 4). In all experimental groups, TAG and glucose decreased by 68% from d 3 a.p. until d 3 p.p. and by 11% from d 1 p.p. to d 7 p.p., respectively. Starting from an initial value of 0.205 mmol/L, the NEFA concentration peaked to 0.856 mmol/L at d 1 p.p. followed by a decline to the a.p. baseline level until d 98 p.p. BHB increased from 0.63 mmol/L at d 3 a.p. to 1.11 mmol/L at d 7 p.p. in the CON groups, whereas a numerically lower peak of 0.88 mmol/L was observed in the 3-NOP groups. 3-NOP treatment did not impact the BHB, TAG and glucose concentrations but lowered that of NEFA by approximately 19.5% in the 3-NOP compared to the CON groups (3-NOP; $p < 0.001$). CFP affected neither NEFA nor TAG but did affect BHB (CFP \times TIME; $p = 0.009$). Thus, a more pronounced decrease in BHB serum concentrations was observed in the HC compared to the LC groups from d 7 p.p. until termination of the experiment. Elevated blood glucose levels in the HC groups were considered significant from d 21 p.p. until d 73 p.p., in contrast with the LC groups (CFP \times TIME; $p = 0.073$; CFP; $p = 0.006$). NEFA concentration was correlated with TAG after parturition ($r = 0.47$; $p < 0.001$; $N = 350$). Blood glucose was positively related to TAG ($r = 0.49$; $p < 0.001$; $N = 532$) and HP ($r = 0.24$; $p < 0.001$; $N = 512$) but negatively associated with both serum NEFA ($r = -0.28$; $p < 0.001$; $N = 532$) and BHB ($r = -0.47$; $p < 0.001$; $N = 532$). NEFA and BHB were significantly interrelated ($r = 0.42$; $p < 0.001$; $N = 532$) and decreased with elevated ER_{tissue} ($r = -0.52$; $p < 0.001$ for NEFA and $r = -0.29$; $p < 0.001$ for BHB; $N = 511$) and MEI ($r = -0.29$; $p < 0.001$; $N = 525$ for NEFA and $r = -0.18$; $p < 0.001$; $N = 514$ for BHB). Accordingly, increased MEI went along with increased ER_{tissue} ($r = 0.39$; $p < 0.001$; $N = 914$) and CO₂ yield (g CO₂/kg DMI) ($r = 0.41$; $p < 0.001$; $N = 914$). CO₂ yield had a strongly positive correlation with TAG ($r = 0.56$; $p < 0.001$; $N = 511$) and postpartal NEFA levels ($r = 0.44$; $p = 0.001$; $N = 350$) but had a negative relationship with ER_{tissue} ($r = -0.35$; $p < 0.001$; $N = 914$).



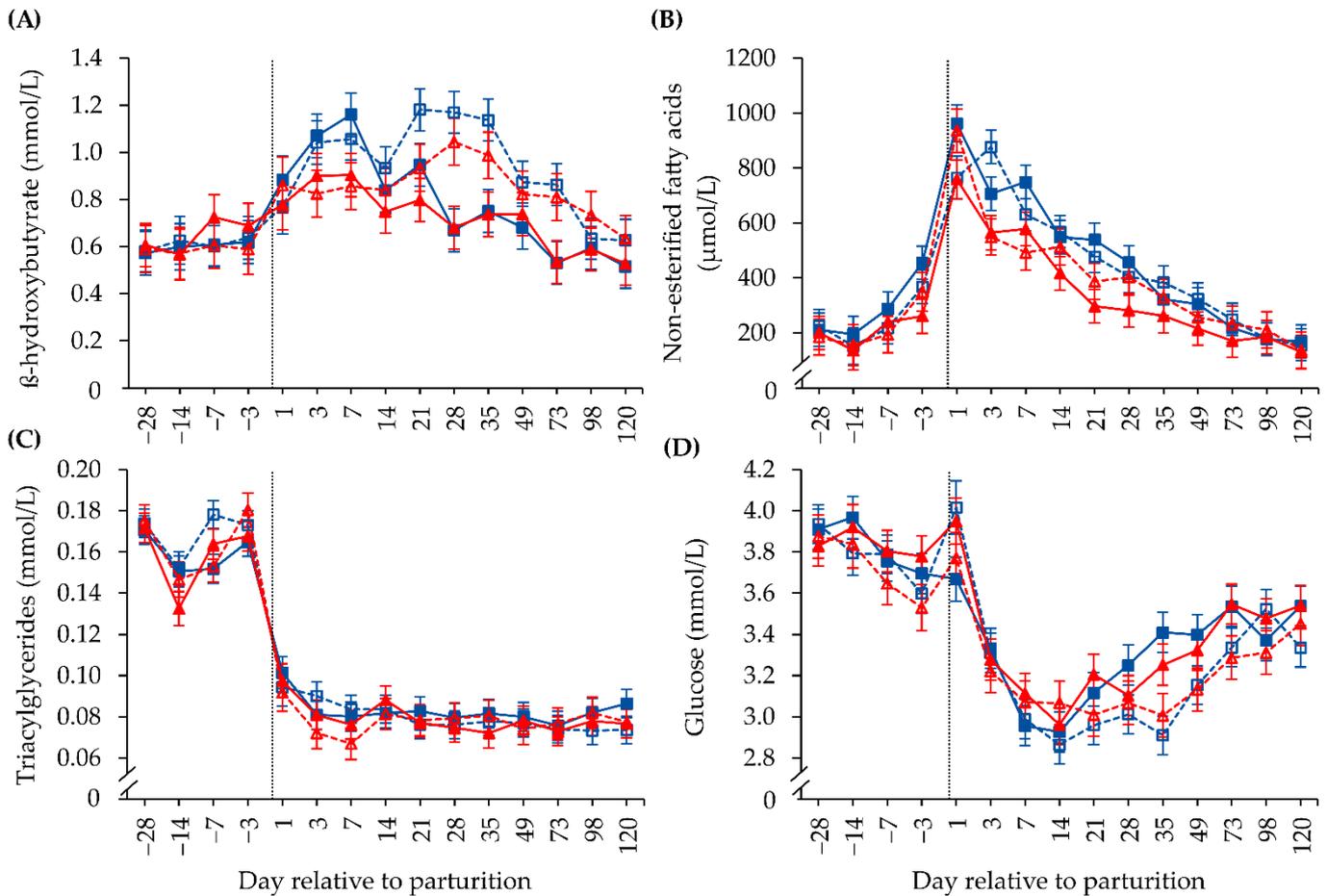
Variable	SEM	<i>p</i> -Values						
		3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP×CFP ×TIME
Lactate	0.012	0.077	0.102	<0.001	0.102	0.714	0.261	0.699
Hydrogen carbonate	0.53	0.587	0.315	0.208	0.398	0.062	0.906	0.424
Temperature-corrected pH	0.002	0.191	0.726	<0.001	0.263	0.014	0.334	0.611
Haemoglobin	0.10	0.513	0.083	<0.001	0.700	0.399	<0.001	0.257

Figure 3. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion in the ration (CFP) and time relative to parturition (TIME) on blood chemical parameters of (A) lactate, (B) hydrogen carbonate, (C) temperature-corrected pH, and (D) haemoglobin in peripartal dairy cows. ■, solid line = control high CFP (CONHC, $n = 14$); □, dashed line = control low CFP (CONLC, $n = 15$); ▲, solid line = 3-NOP high CFP (NOPHC, $n = 14$); △, dashed line = 3-NOP low CFP (NOPLC, $n = 12$). Values are presented as LS-means. SEM, standard error of the means. Statistics with first measured value as covariate.

3.5. Interrelations between Metabolic RQ, Energy Metabolism and Methane Emission

Figure 5A shows that $RQ_{\text{metabolic}}$ was positively correlated with ER_{tissue} ($r = 0.37$; $p < 0.001$; $N = 912$) and negatively with serum NEFA ($r = -0.29$; $p < 0.001$; $N = 510$) (multiple $R^2 = 0.37$; $p = 0.015$). ER_{tissue} and serum NEFA levels were adversely interrelated. Lower serum NEFA concentrations could be identified for 3-NOP groups (Figure 5A) and the NOPHC group showed increased ER_{tissue} (Figure 5A). Figure 5B shows that CH_4 yield was negatively related to molar propionate proportions in rumen fluid ($r = -0.46$; $p < 0.001$;

N = 165; data from Schilde et al. [15]), whereby the opposite holds true concerning p.p. NEFA concentration (multiple $R^2 = 0.59$, $p < 0.001$). Serum NEFA and propionate were inversely related ($r = -0.22$; $p = 0.007$; N = 165) and affected CH_4 yield in an interactive manner (Figure 5B). Approximately, 59% of the variation of the CH_4 yield can be explained by the explanatory variables (multiple $R^2 = 0.59$, $p < 0.001$). The CH_4 yield was decreased by 3-NOP supplementation (Figure 5B).



Variable	PSEM	<i>p</i> -Values						
		3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
β-hydroxybutyrate	0.075	0.111	0.003	<0.001	0.595	0.634	0.009	0.998
Non-esterified fatty acids	8.091	<0.001	0.633	<0.001	0.138	0.334	0.703	0.437
Triacylglycerides	0.001	0.254	0.842	<0.001	0.991	0.932	0.914	0.344
Glucose	0.086	0.989	0.006	<0.001	0.665	0.949	0.073	0.395

Figure 4. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion in the ration (CFP) and time relative to parturition (TIME) on energy-related biochemical blood parameters of (A) β-hydroxybutyrate, (B) non-esterified fatty acids, (C) triacylglycerides, and (D) glucose in peripartal dairy cows. ■, solid line = control high CFP (CONHC, n = 14); □, dashed line = control low CFP (CONLC, n = 15); ▲, solid line = 3-NOP high CFP (NOPHC, n = 14); △, dashed line = 3-NOP low CFP (NOPLC, n = 12). Values are presented as LS-means. PSEM, pooled standard error of the means. Statistics with first measured value as covariate.

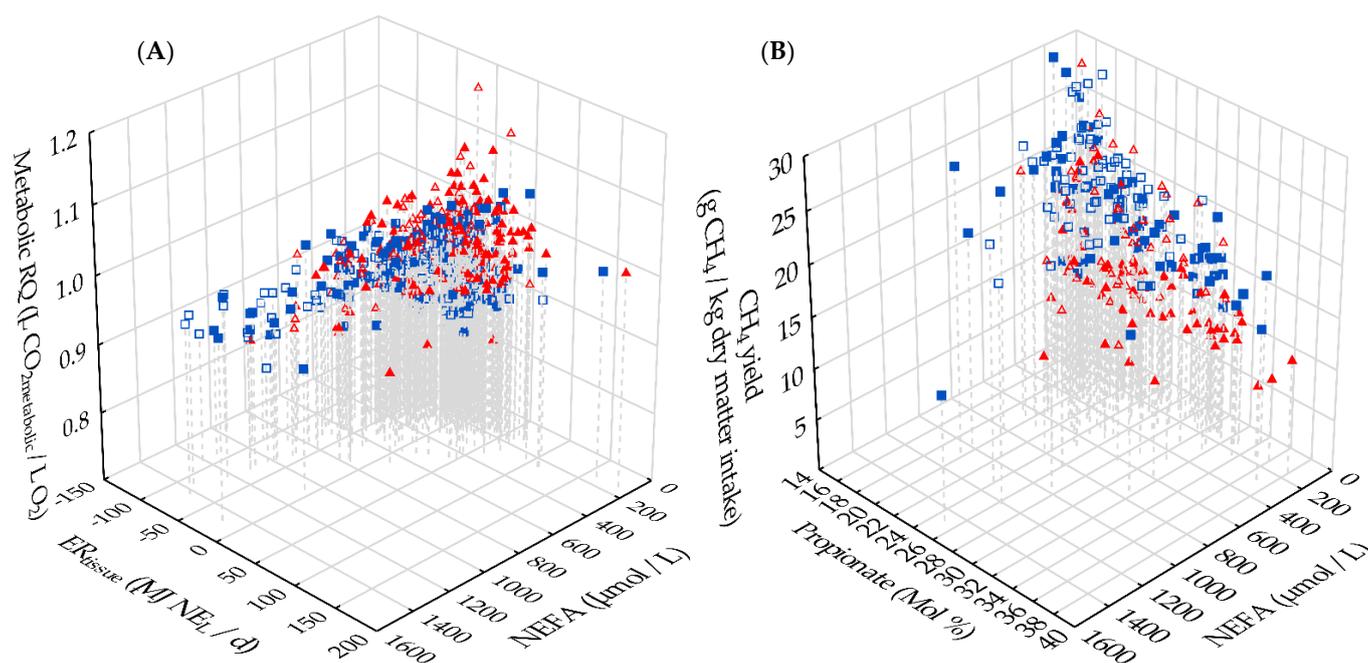


Figure 5. (A) Relationship between (A) metabolic respiratory quotient (metabolic RQ), energy retention in body tissues (ER_{tissue} MJ/d) and non-esterified fatty acids (NEFA ($\mu\text{mol/L}$)) as well as (B) Relationship between CH_4 yield (g CH_4 /kg dry matter intake), molar propionate proportion in rumen fluid (Mol %) (data are shown in Schilde et al. [15] © 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group (<https://www.tandfonline.com/>) under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way) and NEFA ($\mu\text{mol/L}$) from d 28 ante-partum until d 120 post-partum in experimental dairy cows supplied with 3-nitrooxypropanol (3-NOP) and varying concentrate feed proportion (CFP) in the ration. ■ = control high CFP (CONHC); □ = control low CFP (CONLC); ▲ = 3-NOP high CFP (NOPHC); △ = 3-NOP low CFP (NOPLC). **Statistics (A):** multiple $R^2 = 0.38$, $p < 0.001$. **Statistics (B):** multiple $R^2 = 0.59$, $p < 0.001$.

4. Discussion

4.1. Limitations of the GreenFeed Technology for Its Use in Indirect Calorimetry

The accurate indirect calorimetric calculation of the HP and RQ depends on precise gas respiration measurements [44]. Due to technical reasons, the VO_2 consumption of CON groups needed to be regressively predicted from VCO_2 and DMI. Even though slightly increased VO_2 consumption rates were temporarily observed in the CON groups, the highly predictive performance of the applied model ($R^2 = 0.90$; $\text{RSE} = 371$ g/d) confirmed its validity. In contrast to RC, a reliable within-day gas exchange pattern could not be obtained from GF measurements, which precluded investigations on intraday HP and RQ kinetics [23] and potentially explained some of the variations, as shown for $\text{RQ}_{\text{metabolic}}$ and HP. Over the present trial period, the coefficients of variation for the within-day GF spot measurements of VCH_4 , VO_2 and VCO_2 were, on a weekly average, 22.1, 10.0 and 11.1%, respectively. Correspondingly, this could have resulted in deviations of HP of approximately ± 1.3 MJ (1.02% of total HP), ± 9.7 MJ (7.65% of total HP) and ± 3.5 MJ (2.76% of total HP), respectively, indicating that HP estimation is most sensitive towards variations in O_2 consumption. In conclusion, the overall variability of the present GF gas mass flux measurements was stated to be low. This was related to an accurate data acquisition, which was realized by a high-sampling frequency being evenly distributed throughout the day. In addition, GF data were averaged over seven days and validated for visiting time and head positioning of the cow in the GF hood. Hence, the measurement

procedure applied herein (detailed in Schilde et al. [15]) was previously noted to produce comparable results to those obtained from RC [23,32]. In particular, both RC and GF used the same equations and sensor types for O₂ (para-magnetic), CH₄ and CO₂ (non-dispersive infrared) respiration measurements. However, in particular, further validation of the GF algorithm principles is needed as O₂ sensor validation data from RC measurements are lacking. In the present study, the VCO_{2metabolic} was differentiated from the fermentative VCO₂ to calculate RQ_{metabolic} at the intermediary level. Indeed, this fractionation can be visually conducted for each cow visit from the VCO₂ gas-measurement trajectory depicted in the GF graphical online interface. In this way, a “baseline” CO₂ level reflects the amount of expired lung-derived CO₂ (VCO_{2metabolic}) that needs to be corrected for background CO₂ gas concentration. The “baseline” CO₂ level is temporarily interrupted by CO₂ eructation peaks (VCO_{2fermentative}) [45]. However, this visual evaluation is impractical for large datasets and, therefore, algorithms for an automatized graphical assessment should be developed in the future. As a consequence, the commonly applied factor of 1.7 [34,36] was used, resulting in VCO_{2fermentative} proportions of 12 ± 0.5% in CONHC, 9 ± 0.5% in NOPHC and, more incrementally, 13 ± 0.4% in CONLC and 10 ± 1.1% in NOPLC (mean ± SD) (Figure 1A; Table 2). Comparatively, Caetano et al. [45] visually estimated the VCO_{2fermentative} from the GF online interface to be between 6 and 20% of the total VCO₂ production in beef cattle offered diets of varying energy density for ad libitum and restricted intake.

4.2. Validation of the Energy Partitioning Estimated by Indirect Calorimetry and Ultrasonography

The present GF method of indirect calorimetry resulted in ER_{tissue} values that strongly corresponded to the EB_{GFE} values measured for period 2 and 3. However, both methods significantly differed with regard to the a.p. period (period 1; Figure 2A; compare ER_{tissue} and EB_{GFE} in Table 3). Erdmann et al. [39] compared the EB_{GFE} with that calculated from indirect calorimetric RC measurements over the same antepartal period as the present period 1 and also reported higher EB_{GFE} values (by about 33 MJ/d) when compared to the RC energy balance. The higher EB_{GFE} could have been a result of an underestimation of EE during the ante-partum period 1 when compared to the calorimetrically derived ER_{tissue}. Thus, the dynamically increasing antepartal energy requirements for the onset of lactogenesis and foetal growth could have been captured more accurately by continuous calorimetric measurements in contrast with the constants applied in EB_{GFE} calculations. Furthermore, the impact of maintenance requirements on the EB outcome was proportionally higher during the dry period when compared to the lactation period. The factors applied in the German NE system for calculating ME_m were derived from 40-year-old data. Meanwhile, the breeding of higher genetic merit cows resulted in generally increased body sizes of cows and a greater proportion of liveweight as body protein mass while back fat thickness decreased [46]. As a consequence, the increased feed intake resulted in greater digestive loads and blood flow-rates in the total splanchnic tissues being paralleled by increased metabolic rates, internal organ masses and O₂ consumption [47]. These metabolic changes are related to higher energy demands for maintenance metabolism, which implies an underestimation of maintenance energy requirements in the German NE feeding system and a further explanation of the higher EB_{GFE} when compared to the ER_{tissue} values.

The mean k_1 value of 0.61 is within the range of k_1 values (0.60 to 0.67) summarized in a literature review by Agnew and Yan [47] and close to the k_1 value of 0.60 reported by Van Es [48], which confirms the suitability of the GreenFeed system as an indirect calorimeter.

The estimated energy released from the ultrasonographically assessed lipolysis in AT depots (ER_{fat depot}; Equation (8)) was subtracted from the negative ER_{tissue} in period 2 (Table 3) yielding the remaining fraction of glycogen, triglycerides and proteins deposited in skeletal muscles and organs (ER_{residual}). It was supposed that protein and lipid breakdown in skeletal muscles around parturition partially compensated for the observed negative ER_{tissue} (Figure 1C; Table 3) and contributed to the decreased RQ_{metabolic} (Figure 1B) [11]. Thus, gluconeogenesis from the oxidation of alanine, one of the most important glucogenic

amino acids (AA) [11], and intramuscular lipids result in very low $RQ_{\text{metabolic}}$ values of 0.13 and 0.7, respectively. Tamminga et al. [49] estimated the fractional rate of skeletal muscle protein breakdown in dairy cows to be 0.38, 0.22, 0.04 and 0.02 kg per day in week 1, 2, 3 and 4 p.p. From a rough calculation, this would correspond to a total of 4.6 kg mobilized body protein (92 MJ NE_L) during the complete period 2 and an energy equivalent of 3.3 MJ NE_L/d (92 MJ NE_L from proteolysis divided by 28 days in period 2 = 3.3 MJ NE_L/d ; 1 g of body protein = 23.8 kJ [22]; energy efficiency of 84% [50]). Comparatively, von Soosten et al. [51] reported a lower energy yield from body protein mobilization, which amounted to an average of 2.1 MJ/d over the period from d 1 until d 42 p.p. in primiparous cows measured by the comparative slaughter technique. However, those results are not directly comparable to the present periparturient pluriparous dairy cows and observation period (d 1 until d 28). Von Soosten et al. [51] assumed protein accretion in the growing primiparous cows (BW of approximately 490 kg) and protein mobilization is generally supposed to change to repletion from d 35 p.p. onwards [52]. The non-explained remainder of the difference between ER_{residual} and the estimated energy supply from skeletal muscle proteolysis can be partially assigned to energy mobilized from inter- and intramuscular and organ tissues. Furthermore, the corresponding models estimating the fat depot masses are, to some extent, prone to error. Although ER_{tissue} was more positive in the HC groups (Figure 1C), the ultrasonographically assessed lipolysis from AT and serum NEFA levels (indicative for negative EB) were not different between the HC and LC groups (Table 3; Figure 4B). Raschka et al. [25] validated the ultrasonographic-based multiple regression model for the predicted weights of the SAT and VAT depots as highly accurate with R^2 values of 0.88 and 0.94 and root mean square errors of 3.4 and 6.1 kg, respectively. In the present experiment, an assumed $\pm 10\%$ variation between the predicted and actual daily changes in SAT and VAT would result in $ER_{\text{fat depot}}$ variations of approximately ± 2.3 MJ NE_L/d .

4.3. Effects of 3-NOP, CFP and Parturition on Energy Metabolism Parameters

Both RQ and HP notably depend on the magnitude of MEI ($r = 0.69$ and $r = 0.22$ resp.; $p < 0.001$; $N = 914$), its utilization for maintenance and productive purposes [53], whether substrates are either deposited or mobilized in tissues (Figure 5A) and, finally, on the type of the metabolized substrate itself [22,34]. In contrast to LC groups, the higher $RQ_{\text{metabolic}}$ (Figure 1B) and ER_{tissue} (Figure 1C) in HC groups reflected their increased GEI:EE ratio (Table 3) and dietary content of non-fibre carbohydrates (Table 1) being microbially degraded into gluconeogenic substrates. Correspondingly, increased blood glucose (Figure 4C) and reduced BHB (Figure 4A) concentrations were observed in the HC groups.

During the a.p. period, the pro-lipogenic effect of the dietetically designed energetic oversupply was manifested in the positive ER_{tissue} (Figure 1C) and $RQ_{\text{metabolic}}$ values of 0.92 (Figure 1B). In principle, lipid deposition in AT would result in $RQ_{\text{metabolic}}$ values above 1.0 [44] but $RQ_{\text{metabolic}}$ reflects the net oxidation rates of a mixture of substrates irrespective of the metabolic interconversions of the substrate [44]. Hence, flowing transitions between oxidation and de novo synthesis of lipids were assumed, which became apparent in the steady decrease in ER_{tissue} since the beginning of the trial in spite of the energetic oversupply (Figure 1C). The present energy-deficient transition from gestation to lactation was accompanied by significant metabolic adaptations (Figure 4). The decreased $RQ_{\text{metabolic}}$ corresponded to the negative ER_{tissue} and accumulation of serum NEFA (Figure 5A), collectively indicating excessive fat oxidation from AT resulting in increased O_2 consumption (NEFA vs. O_2 consumption (g/d); $r = 0.18$; $p < 0.001$; $N = 511$) and ketogenesis from acetyl-CoA and NEFA (NEFA vs. BHB; $r = 0.42$; $p < 0.001$; $N = 532$). The observed increased circulating BHB (Figure 4A) likely originated from an oxaloacetate deficiency [10] and a concomitant hepatic overload to completely oxidize the excessively flooding NEFA (Figure 4B), released by lipolysis in AT (Table A1 and Table 4), into ATP and CO_2 [11]. It can be summarized that the decrease in $RQ_{\text{metabolic}}$ could be partially explained by the increased O_2 consumption due to lipolysis in AT, whereby CO_2 did not increase

due to the aforementioned incomplete metabolization of NEFA into BHB but not into CO_2 . Correspondingly, the $\text{RQ}_{\text{metabolic}}$ did not behave in the same manner as the RQ_{total} because the latter also reflected CO_2 production arising from rumen fermentation. Hence, in the early-lactation period, increased fermentative CO_2 production from high-forage diets led to higher RQ_{total} values, whereby $\text{RQ}_{\text{metabolic}}$ decreased due to the abovementioned increased but incomplete fat oxidation resulting in less intermediary CO_2 formation when NEFA were converted to BHB, rather than CO_2 and ATP. Accordingly, as previously published for the present experiment, the CO_2 yield (g CO_2 production/kg DMI) was significantly higher in LC when compared to the HC groups, but the opposite was the case when it came to total CO_2 production (g CO_2 /d) over the complete experimental period [15].

In the present experiment, the tendency for an increased ER_{total} in the NOPHC group (Table 3; Figure 1C) confirmed similar results reported by van Gastelen et al. [13]. The increased $\text{ER}_{\text{tissue}}$, $\text{ER}_{\text{residual}}$ and $\text{RQ}_{\text{metabolic}}$ of 1.01 in the NOPHC group (Figure 1B; Table 2) could be explained by an improved energy budget in that group. Hence, decreased NEFA levels were associated with increased ruminal propionate concentrations, and both were inversely related to CH_4 production (Figure 5B). Recently, it was observed that supplementing 3-NOP combined with high CFP in the ration shifted rumen fermentation pathways to hydrogen-consuming glucogenic propionate and decreased loss of CH_4 energy in a synergistic manner (Tables 2 and 3) (details in Schilde et al. [15]). Correspondingly, lower serum NEFA concentrations were observed in the 3-NOP cows (Figure 4B) although neither 3-NOP nor CFP affected $\text{ER}_{\text{fat depot}}$ and lipomobilization in AT depots (Tables 3 and 4). This could indicate that the increased glucogenic propionate proportions in the 3-NOP groups improved the intramitochondrial oxaloacetate availability and, therefore, the hepatic capacity for NEFA oxidation. Interestingly, neither blood glucose (NEFA oxidation and conversion of elevated propionate levels to glucose and CO_2) nor TAG (re-esterification of NEFA) and BHB (reduced incomplete NEFA oxidation) were affected by 3-NOP, which confirms previous findings [20]. This opens the question as to whether the direct extrapolation of NEFA concentrations to circulating BHB levels is appropriate in the present CH_4 mitigation experiment. Accordingly, in the companion study, Schilde et al. [15] observed that butyrate formation was preferred to that of acetate in the 3-NOP-treated cows, which can be explained by the reduced hydrogen release when carbohydrates are degraded into butyrate and not into acetate [54]. Butyrate also serves as a carbon source for ketone body synthesis in the rumen epithelium [55]; therefore, increased circulating BHB originating from enhanced intraepithelial ketogenesis could have masked the assumed causal relationship that decreased serum NEFA concentrations in the 3-NOP-treated cows, which would necessarily have led to reduced BHB in the blood stream. Besides the intraepithelial butyrate metabolization, propionate can be metabolized to lactate in the rumen epithelium, which could also have reduced the propionate flux to the liver, thereby eliminating the energetic advantage of the 3-NOP-mediated increased propionate formation in the rumen. The observed accumulation of ketoacids (BHB) and the blood lactate peak at d 1 p.p. (Figure 3A) could have increased the risk for metabolic acidosis [44]. Indeed, blood pH was observed to slightly drop from 7.41 to 7.38 at parturition contemporaneously to the lactate peak at d 1 p.p. (Figure 3C; TIME; $p < 0.001$). In this context, the temporal decrease in the 3-NOP groups (Figure 3C; 3-NOP \times TIME; $p = 0.014$) is, however, difficult to explain as blood lactate and BHB were not affected by 3-NOP treatment. The pH decrease at d 1 p.p. possibly caused buffering reactions via the largest CO_2 body pool, hydrogen carbonate, which could have led to an overestimation of HP and $\text{RQ}_{\text{metabolic}}$ [44]. Indirect calorimetry is stated to be accurate as long as body pool sizes of energy-related metabolites (ketone bodies, lactate) and intermediary products (O_2 and CO_2 , N_U) remain stable [44]. However, the potential effects of intermediary pool sizes on HP from nutrient oxidation and $\text{RQ}_{\text{metabolic}}$ were considered negligible because the bicarbonate and pH values remained within their physiological area [56,57] (Figure 3B,C). In general, the CO_2 pool size is supposed to be subjected to greater fluctuations when compared to the O_2 body pool [44]. Accordingly, blood concentrations of haemoglobin, the main O_2 body pool, remained stable within the

physiological range [57] although a slight divergence was observed between the LC and HC groups at the end of the experiment (Figure 3D; CFP \times TIME; $p < 0.001$).

5. Conclusions

The present study revealed that using the GF system as an indirect calorimetry chamber for the assessment of cows' energy metabolisms is a promising approach, although further validations of the O₂ sensor and algorithm principles are needed. The ER_{tissue} determined by indirect calorimetry coincided with that calculated from GfE [24], except for the antepartal period. The hypothesis that feeding 3-NOP in combination with high CFP synergistically improves the cows' energy budgets was partially confirmed because effects were not apparent in all of the examined parameters. 3-NOP combined with high CFP increased RQ_{metabolic} and ER_{tissue} and decreased serum NEFA. In contrast, lipomobilization from fat depots and blood lactate were neither affected by 3-NOP nor CFP and 3-NOP did not affect blood glucose, TAG and BHB levels. Blood pH and bicarbonate remained within their physiological range and metabolic adaptations to energy-related changes via the CO₂ body pool were not observed. High CFP decreased BHB but increased blood glucose and, at the end of the trial, haemoglobin levels, which possibly indicates that the cows adapted differently to metabolic changes. Future research will be focused on the relationship between the 3-NOP-induced changes in the rumen VFA profile and gene expression in the liver.

Author Contributions: Conceptualization, M.S. and S.D.; Data curation, M.S., J.F. and S.K.; Formal analysis, M.S.; Funding acquisition, D.v.S., S.K. and U.M.; Investigation, M.S. and J.F.; Methodology, M.S. and S.D.; Project administration, D.v.S. and U.M.; Resources, D.v.S., J.F., S.K., U.M. and S.D.; Supervision, A.Z. and S.D.; Validation, M.S., A.Z. and S.D.; Visualization, M.S.; Writing—original draft, M.S.; Writing—review and editing, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: The project is supported by funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme (grant number 281B101416).

Institutional Review Board Statement: The experiment was conducted according to the guidelines of the German Animal Welfare Act, and approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany) (approval number: 33.19-42502-04-15/1858).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the present article and in the previously published manuscript of the comprehensive experiment by Schilde et al. [15] (article number: 1877986).

Acknowledgments: The authors thank the staff of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) and the co-workers of its experimental station for taking care of the experimental animals and supporting the sample collection and analysis. The authors also thank DSM Nutritional Products AG (Kaiseraugst, Switzerland) for their support in providing 3-NOP and 3-NOP analyses in the feedstuffs.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Thickness and absolute masses of adipose tissue (AT) depots of the experimental groups at d 3 and d 28 post-partum (p.p.).

Item	Treatments [†]				SEM	<i>p</i> -Values [§]					
	CONHC (<i>n</i> = 14)	CONLC (<i>n</i> = 15)	NOPHC (<i>n</i> = 14)	NOPLC (<i>n</i> = 12)		3-NOP	CFP	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP×TIME
Back fat thickness (cm)											
d1 3 p.p.	1.49	1.34	1.43	1.49	0.08	0.341	0.538	0.152	0.551	0.938	0.924
d 28 p.p.	1.16	1.01	1.15	1.21							
Rib fat thickness (cm)											
d1 3 p.p.	1.6	1.5	1.6	1.6	0.08	0.424	0.671	0.395	0.739	0.395	0.355
d 28 p.p.	1.2	1.1	1.2	1.3							
Absolute masses of AT depot (kg)											
Mesenteric AT											
d1 3 p.p.	13.5	11.7	13.9	13.8	0.84	0.197	0.300	0.415	0.452	0.551	0.486
d 28 p.p.	7.3	7.0	7.6	7.4							
Omental AT											
d1 3 p.p.	14.8	13.3	14.4	14.3	0.66	0.395	0.320	0.305	0.353	0.588	0.806
d 28 p.p.	10.4	9.3	10.5	10.6							
Retroperitoneal AT											
d1 3 p.p.	9.4	8.8	9.7	9.5	0.53	0.422	0.837	0.539	0.603	0.184	0.552
d 28 p.p.	6.8	6.6	6.6	7.3							
Subcutaneous AT											
d1 3 p.p.	13.6	12.8	13.9	13.3	0.73	0.453	0.499	0.594	0.559	0.468	0.336
d 28 p.p.	9.6	8.7	9.6	10.0							
Visceral AT [†]											
d1 3 p.p.	37.7	33.8	37.9	37.5	1.72	0.276	0.387	0.359	0.683	0.324	0.679
d 28 p.p.	24.5	22.9	24.8	25.3							

[†] Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), time relative to parturition (TIME), and interactions between them; effect of TIME with *p* < 0.001 for all variables. [†] Visceral AT = mesenteric + omental + retroperitoneal AT.

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