RESPONSE OF DAIRY COWS TO THE PARTIAL REPLACEMENT OF SOYBEAN MEAL WITH FLEXYPRO

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MATERIALS AND METHODS

Location, Cows, and Experimental Design

The experiment was conducted from December 27th, 2017 to February 5th, 2018 in an open-walled, sand-bedded tie-stall barn with fans and high-pressure sprinklers at the Better Nature Research Center (http://www.holandesflamma.com.br/), located at Ijaci, Minas Gerais, Brazil. The research center is located at 846 m above sea level, 21º 09' 52.41" latitude south, and 44º 55' 52.40" longitude west. Environmental temperature and relative humidity at the center of the barn were measured at 30-min intervals with a digital thermometer (EasyLog-USB-2-LCD. Lascar Electronics, Salisbury, United Kingdom) located 2.5 m from the floor. The temperature-humidity index (THI) was calculated according to Yousef (1985): THI = T + 0.36 x DP + 41.2; where T = temperature (ºC) and DP = dew point (ºC).

Thirty-two Holstein cows (233 ± 75 DIM at the beginning of the experiment), 8 primiparous, were individually fed the same TMR for a 2-wk standardization period. Milk yield, solids yield, body weight, and body condition score obtained on the last four days of standardization period were used as covariate in the statistical model. Cows were paired blocked based on parity, body weight, and milk yield and assigned to one of two treatments for a 56-d comparison period, in a covariate adjusted randomized block design with repeated measures over time. Cows were divided on 16 blocks with one cow per treatment. One cow leaved the trial at the first week of comparison, due to severe mastitis, and her data were excluded from analysis. Treatments were: Control diet (CTL) with 17,8% (of DM) of soybean meal and FlexyPro diet (FXP) with 11,6% (of DM) of soybean meal and 6,3% of FlexyPro. Offered diets and consumed nutrients are on Table 1.

Feed Management, Measurements, and Analytical Procedures

The two TMR were prepared 2 x/d in a vertical stationary vertical mixer (Unimix 1200. Casale, São Carlos, Brazil) and cows had access to new feed at 0700 and 1300 h daily. The silage DM concentration was monitored weekly with an electric moisture tester (Koster Crop Tester, Strongsville, OH) and diets were adjusted if necessary. Individual cow intake was assessed daily by recording the amount of feed offered and orts (as fed basis). The feed was offered in sufficient quantity to obtain at least 10% of the offered as daily refusals.

Samples of dietary ingredients were collected daily and composite samples made per week. Ort samples were collected daily and composited per cow per week. Composite samples were dried in forced-air oven at 55 ^{o}C for 72 h and ground through a 1-mm mesh screen (Wiley mill, Thomas Scientific, Swedesboro, NJ). The DM concentration was determined by drying at 100 ^{o}C for 24 h. The composition of the consumed diet in nutrients was calculated for each cow based on the composition of offered ingredients and orts on a DM basis. The CP concentration was determined with a micro Kjeldahl apparatus (AOAC International, 1990), ash by incineration at 550 ^{o}C for 8 h, and the ash-free NDF by filtration in porous crucibles with heat stable alpha-amylase and sodium sulfite (Van Soest et al., 1991). The ether extract was analyzed as in AOAC International (1990) and starch was enzymatically determined according to Hall (2009). The NFC fraction was calculated: 100 – (CP + EE + ash + NDF).

Cows were milked 3 x/d in an adjacent herringbone parlor starting at 0500, 1300, and 19:30 h with milk yield recorded daily. Proportional milk samples from each milking were obtained on d 11 to 14 of the standardization period and on d 6 and 7 of each week of the comparison period. Milk solids, SCC, and MUN were analyzed by mid-infrared analysis (Bentley Instruments Inc., Chaska, MN) in a commercial laboratory (Laboratory of the Paraná State Holstein Breeders Association, Curitiba, Brazil). Milk energy secretion (Milk E; Mcal/d) was calculated as: [(0.0929 × % fat) + (0.0547 × % protein) + (0.0395 × % lactose)] × kg of milk (NRC, 2001). The secretion of energy corrected milk (kg/d) was calculated as: Milk E/0.70 (assumes 0.70 Mcal/kg of milk with 3.7% fat, 3.2% protein, and 4.6% lactose). The 4% fat corrected milk (kg/d) was calculated with the Gaines equation (NRC, 2001): 0.4 × kg of milk + 15 × kg of fat. The BCS was the mean of the same 3 independent evaluators (1 to 5 scale. Wildman et al., 1982) on day 14 of the standardization period and on days 14, 28, 42, and 56 of the comparison period. The BW was measured after the morning milking on d 13 and 14 of the standardization period and d 13, 14, 27, 28, 41, 42, 55, and 56 of the comparison period (mean value was used per week).

Rectal and skin temperatures and respiration rate were measured on d 4 and 7 of each week of the comparison period starting at 0900, 1500, and 2200 h. Measurements were made randomly within block. Rectal temperature was measured with digital thermometers (G-Tech TH186 Onbo Eletronics, Shenzhen, China. Accuracy of 0.1 ^{o}C). The respiration rate (breaths/min) was the mean of 3 consecutive 30 s flank movement countings. The skin temperature of shaved areas of the shoulder and flank were measured with an infrared thermometer (HighMed 88E, São Paulo, Brazil. Accuracy of 0.1 ^{o}C), manually pointed at 20 cm from the skin.

The sweating rate was evaluated on d 4 and 7 of each week at 1500 h, randomly within block. The colorimetric technique used paper discs impregnated with cobalt chloride (Schleger and Turner, 1965). Filter paper (Whatman n^{o} 1, 11 μm porosity) was immersed in a 10% cobalt chloride solution and then oven dried at 105 ^{o}C for 12 h. Paper discs (0.5 cm diameter) were cut and dried for 2 min. Three discs were placed on histological slides and fixed with transparent adhesive tape. The slides with disks were immediately placed in a sealed glass containing silica. A 3 × 10 cm rectangular area was shaved on the left flank of each cow, 10 cm below the lumbosacral vertebrae, 1 d before the measurement. The tape with discs was fixed over the shaved area. The time for each disc to change color from blue violet to light pink was recorded and the mean value was used to calculate the sweating rate (g/m^2/h) as: 38,446.6/T, where T is time in seconds (Schleger and Turner, 1965). The estimated body surface area (m^2) was: kg BW$^{0.56}$ × 0.15 m^2/kg BW$^{0.56}$ (McLean, 1963).

Jugular blood acid-base balance was analyzed on d 51 of comparison period at 6 h after morning feeding in a clinical commercial laboratory (Laboratório Santa Cecília, Lavras, Brazil). Samples were delivered at laboratory for analysis within less than 1 h after sampling. Blood samples were collected in heparinized vacuum tubes, within a 30 min period at random within block, and were kept on ice from sampling to analysis.

The total tract apparent digestibility of DM, OM, NDF, non-NDF OM, and starch were determined on d 54 to 56 by total collection of feces in buckets. Feces were collected concurrent to defecation during three 8-h sampling periods and weighed. The second and third sampling periods were each delayed by 8 h, thus representing a 24-h collection. Fecal aliquots (proportion to each amount defecated, 1% of fresh weight) were taken and immediately frozen along the collection period and a composite sample was formed per cow. Composite fecal samples were dried for 72 h at 55 ^{o}C in a forced air oven and ground through a 1-mm mesh screen (Wiley mill, Thomas Scientific, Swedesboro, NJ). The DM concentration was determined by drying at 100 ^{o}C for 24 h.

Concentrations of NDF, ash, and starch were determined as described previously. The daily digestible OM intake (DOMI) on d 54 to 56 of the experimental period was calculated.

Total urinary output was collected in buckets, simultaneously to fecal sampling, to estimate the relative ruminal microbial synthesis based on purine derivative excretion (mmol/d). A 20% sulfuric acid solution (200 mL) was added to 20 L buckets and urine was added to it during the collection period. At the end of 3-d collection period, composite urine samples were diluted 1:5 with a 4% sulfuric acid solution and frozen at -20 ºC. Allantoin was determined according to Chen and Gomes (1992).

Blood samples were also obtained on d 53 to determine the concentration of plasma urea-N (PUN). Samples were obtained immediately before the first daily feeding and at 2, 4, 8, 12, 16, and 20 h (± 20 min) after feeding. The PUN was analyzed with a laboratory kit (Urea 500, Doles Reagentes para Laboratórios, Goiânia, Brazil). Plasma glucose concentration was measured on d 11, 25, 39, and 53 on blood samples obtained 12 h after morning feeding with vacuum tubes containing EDTA and potassium fluoride. Glucose was analyzed with a laboratory kit (Glicose Enzimática Líquida, Doles Reagentes para Laboratórios, Goiânia, Brazil).

On d 56 ruminal fluid samples were collected with a flexible oro-gastric tube. Samples were obtained 10 h (± 50 min) after the morning feeding, at random within block. Ruminal fluid pH was measured immediately (Hanna pH 21 pH/mV meter, Hanna Instruments, Woodsocket, RI). A sample was mixed to a 36% formaldehyde solution for protozoa counting. The sample for protozoa enumeration was stained and evaluated with an optical microscope in a Neubauer chamber according to Dehority (1984) and Warner (1962).

Chewing activity was measured on d 52 and feed sorting behavior was evaluated at d 24. Chewing activity was evaluated by visual observation of the buccal activities of each cow at 5-min intervals continuously for three consecutive 24-h periods. Daily values were averaged per cow. Buccal activities evaluated were: feed ingestion, water ingestion, rumination, and idleness. Ingestion and rumination per unit of DMI were calculated using the intake of the day in which chewing activity was evaluated. A meal was defined by at least two consecutive 5-min ingestion events following at least 10 min of idleness or rumination (Mullins et al., 2012). The intermeal interval was calculated by time not ingesting (rumination + idleness) divided by the number of meals/d minus 1. The duration of the first daily meal (conditioned meal) was measured with a chronometer. Five evaluators observed the behavior of all cows after offering feed at 0700 h until the last cow finished

its first meal. The proportions of daily TMR intake between 0700 and 1300 h, 1300 and 1900 h, and 1900 and 0700 h were measured.

Feed sorting behavior was evaluated according to Leonardi and Armentano (2003). The proportion of particles above the 19 mm mesh diameter screen and above and below the 8 mm mesh screen of the Penn State Particle Separator was evaluated at 0600 and 1300 h for the offered TMR and at 1200, 1900, and 0600 h for refusals. Feed refusals at 1200 h were mixed with feed offered at 1300 h for measurement of the offered TMR particle size of each cow. The predicted intake (as-fed basis) of particles on each screen was: % TMR retained on screen × kg of TMR consumed. The observed intake of particles was: % TMR retained on screen × kg of TMR offered - % orts retained on screen × kg of orts. The selection index was: 100 × (observed intake/predicted intake). Sorting values below 100% represent selective refusal, above 100% represent preferential intake, and equal to 100% represent no selection.

Statistical Analysis

The data were analyzed as repeated measures with the Mixed procedure of SAS statistical software with the model: $Y_{ijk} = \mu + CV + B_i + D_j + T_k + D*T_{jk} + e_{ijk}$. Where: μ = overall mean, CV = covariate (measure of the same variable at the end of the standardization period), B_i = random block effect (i = 1 to 16), D_j = fixed treatment effect (j = CTL or FXP), T_k = fixed time effect (k = days or weeks), $D*T_{jk}$ = interaction between treatment and time, and e_{ijk} = residual error. For variables measured only during the comparison period, the same model was used, but without the covariate adjustment. The mean square for the effect of cow nested within treatment was used as the error to test the treatment effect. The best covariance structure was defined by the Akaike's Information Criterion among first order autoregressive or compound symmetry. The frequency of distribution of rectal temperature $\geq$ 39.2 ^{o}C was analyzed with the GENMOD procedure using logistic regression for binomial data. Significance was declared at $P \leq 0.05$ and trends at $P \leq 0.15$.

Table 1. Ingredient composition (% of DM) and particle size of the offered TMR and nutrients of consumed TMR (% of DM) of Control and FlexyPro diets

	Control	FlexyPro
Corn silage	40.8	40.5
Oat hay	4.7	4.8
Soybean meal	17.8	11.6
FlexyPro		6.3
Whole cottonseed	8.3	8.4
Finely ground corn rehydrated and ensiled	14.7	14.7
Citrus pulp	11.0	11.0
Limestone	0.8	0.8
Sodium bicarbonate	0.9	0.9
Magnesium oxide	0.2	0.2
Salt	0.2	0.2
Minerals vitamins[1]	0.6	0.6
CP	16.6	16.7
CP from FlexyPro		3.3
NDF	29.2	31.4
Forage NDF	26.0	25.9
Starch	26.6	26.9
Ash	6.0	5.8
Ether extract	4.3	4.2
NFC[2]	41.7	39.8
DM, % of as fed	50.8	50.9
Feed particles > 19 mm[3]	9.7 ± 3.3	8.3 ± 2.8
Feed particles 8 – 19 mm	43.5 ± 2.2	43.4 ± 4.5
Feed particles < 8 mm	46.8 ± 3.6	48.3 ± 5.5

[1]200.0 g/kg Ca; 156 g/kg P; 30.0 g/kg Mg; 35.0 g/kg S; 150 mg/kg Co; 2,000 mg/kg Cu; 5,000 mg/kg Mn; 11,900 mg/kg Zn; 82 mg/kg Se; 200 mg/kg I; 1,000 KUI/kg vit A; 220 KUI/kg vit D; 6.2 KUI/kg vit E.

[2]Nonfiber carbohydrates calculated as 100 – (CP + Ether extract + ash + NDF).

[3]Orifice diameter of sieves of the Penn State Particle Separator. Mean ± SD of 12 TMR samples.

Table 2. Intake, lactation performance, MUN, SCC, feed efficiency, BCS, and BW during 56 days of the comparison period on treatments Control and FlexyPro

	Treatments			*P*-value[1]		
	Control	FlexyPro	SEM	Treat.	Time	Interaction
DMI, kg/d	22.3	22.3	0.29	0.55	< 0.01	0.96
DMI (d 15 to 56), kg/d	22.2	22.1	0.32	0.82	< 0.01	0.89
Milk, kg/d	31.3	32.2	0.31	< 0.01	< 0.01	0.99
Milk (d 15 to 56), kg/d	30.8	31.6	0.35	< 0.01	< 0.01	0.96
4% FCM, kg/d	28.3	28.9	0.475	0.13	< 0.01	0.74
ECM, kg/d	29.9	30.6	0.48	0.10	< 0.01	0.79
Fat, kg/d	1.047	1.071	0.0157	0.21	< 0.01	0.75
Fat, %	3.37	3.38	0.035	0.91	0.06	0.63
Protein, kg/d	1.005	1.039	0.0182	0.15	< 0.01	0.93
Protein, %	3.22	3.25	0.017	0.23	< 0.01	0.77
Casein, kg/d	0.779	0.806	0.0134	0.13	< 0.01	0.92
Casein, %	2.50	2.52	0.015	0.44	< 0.01	0.77
Lactose, kg/d	1.442	1.477	0.0390	0.44	< 0.01	0.91
Lactose, %	4.58	4.55	0.023	0.33	< 0.01	0.66
Solids, kg/d	3.791	3.896	0.0890	0.33	< 0.01	0.88
Solids, %	12.1	12.1	0.036	0.78	0.19	0.52
MUN, mg/dL	17.1	14.1	0.35	< 0.01	< 0.01	0.43
Linear SCC,[2] 1 to 9	3.19	3.60	0.315	0.28	< 0.01	0.42
Milk/DMI	1.42	1.48	0.024	< 0.01	< 0.01	0.74
ECM/DMI	1.33	1.40	0.025	0.02	< 0.01	0.72
BCS, 1 to 5	3.06	3.10	0.051	0.51	< 0.01	0.16
BW, kg	660	663	2.3	0.49	< 0.01	0.76

[1]*P*-value for the effects of treatment, time (day or week), and the interaction between treatment and time.

[2]Equivalency of the linear scores: 3.18 = 114,000 cells/mL and 3.52 = 152,000 cells/mL.

Table 3. Total tract apparent digestibility (D) of DM, OM, NDF, and non-NDF OM, urinary allantoin excretion, pH and total protozoa concentration in rumen fluid on treatments Control and FlexyPro

	Control	FlexyPro	SEM	*P*-value
% of intake				
D DM	61.4	66.1	2.81	0.16
D OM	63.8	68.0	2.62	0.17
D NDF	41.2	48.3	0.03	0.12
D Starch	90.5	91.3	0.01	0.52
D non-NDF OM	80.9	80.7	0.01	0.87
Allantoin, mmol/d	338	316	32.1	0.62
DOMI, kg/d	13.4	13.7	0.71	0.72
Allantoin/DOMI, mmol/kg	26.6	23.4	2.60	0.38
ECM/DOMI, kg/kg	2.16	2.17	0.163	0.93
Protozoa, x 10^4/mL	15.56	15.13	3.249	0.92

Table 2. Chewing activity and ingestion behavior on treatments Control and FlexyPro

	Control	FlexyPro	SEM	*P*-value
Ingestion, min/d	299	272	12.9	0.09
Rumination, min/d	458	463	18.6	0.87
Chewing,[1] min/d	758	735	25.4	0.41
Ingestion, min/kg DMI	13.6	12.8	0.68	0.37
Rumination, min/kg DMI	20.8	21.6	0.95	0.57
Chewing, min/kg DMI	34.4	34.3	1.40	0.97
Laying time, min/d	729	800	34.3	0.13
Meal size, kg of DM/meal	2.8	2.8	0.15	0.99
First meal duration, min	60	53	5.4	0.33
Meal duration, min	38.0	36.0	2.2	0.43
Meals/d	8.0	8.0	0.36	0.80

[1]Ingestion + rumination.

Table 2. Proportion of intake in periods of the day and feed sorting behavior on treatments Control and FlexyPro

	Control	FlexyPro	SEM	*P*-value
0700 to 1300 h, % of daily intake	38.1	37.4	0.03	0.79
1300 to 1900 h, % of daily intake	37.6	44.0	0.02	0.04
1900 to 0700 h, % of daily intake	23.9	20.5	0.02	0.18
Observed/Predicted,[1] %				
0700 to 1300 h				
> 19 mm	93.7	69.3	10.44	0.03
> 8 mm and < 19 mm	104.9	88.3	3.60	< 0.01
< 8 mm	100.9	120.0	3.59	< 0.01
1300 to 1900 h				
> 19 mm	68.9	63.9	7.24	0.61
> 8 mm and < 19 mm	96.4	99.5	1.32	0.05
< 8 mm	113.2	112.6	2.17	0.85
1900 to 0700 h				
> 19 mm	80.7	80.0	9.44	0.96
> 8 mm and < 19 mm	99.2	109.1	4.36	0.12
< 8 mm	110.7	105.9	4.22	0.43
TMR and orts, as fed basis				
TMR 0700 h, kg	30	30		
Orts 1300 h, kg	14.4	14.5	1.36	0.96
Orts 0700 to 1300 h, % of offered	48.0	48.3	0.04	0.96
TMR 1300 h,[2] kg	36.0	33.3	2.87	0.51
Orts 1900 h, kg	16.2	14.1	1.63	0.23
Orts 1300 to 1900 h, % of offered	46.7	42.3	0.03	0.25
TMR 1900 h, kg	16.2	14.1	1.63	0.23
Orts 0700 h, kg	5.6	6.0	0.66	0.65
Orts 1900 to 0700 h, % of offered	34.7	43.1	0.04	0.23
Daily orts,[3] % of offered	11.0	12.2	0.01	0.46

[1]< 100 % = rejection, > 100 % = preferential intake, 100 % = no selection. Sieves of the Penn State Particle Separator.

[2]TMR 1300 h = Orts 1300 h + Offered TMR 1300 h.

[3]Daily orts = (Orts 0700 h/Offered TMR per day) x 100.

Table 7. Skin surface and rectal temperatures, respiration rate, and sweating rate on treatments Control and FlexyPro

	Treatments			*P*-value		
	Control	FlexyPro	SEM	Treat.	Time	Interaction
	Skin surface, °C					
Rump						
0900 h	34.2	34.0	0.11	0.26	< 0.01	0.52
1500 h	34.7	34.9	0.08	0.13	< 0.01	0.33
2200 h	34.9	35.1	0.09	0.05	< 0.01	0.55
Shoulder blade						
0900 h	34.4	34.3	0.07	0.46	< 0.01	0.25
1500 h	34.9	35.1	0.67	0.05	< 0.01	0.34
2200 h	35.4	35.5	0.09	0.38	< 0.01	0.38
	Rectal, °C					
0900 h	38.4	38.4	0.06	0.74	< 0.01	0.49
1500 h	38.9	38.9	0.07	0.85	< 0.01	0.68
2200 h	38.6	38.6	0.07	0.69	< 0.01	0.25
	Respiration rate, breaths/min					
0900 h	51	52	2.0	0.61	< 0.01	0.06
1500 h	63	63	2.0	0.88	< 0.01	0.71
2200 h	56	55	1.9	0.71	< 0.01	0.82
	Sweating rate, $g/m^2/h$					
1600	219.9	200.6	11.43	0.25	< 0.01	0.07

Table 8. Proportion of cows (% of total) with rectal temperature ≥ 39.2°C at 0900, 1500, and 2200 h on treatments Control and FlexyPro

	Control	FlexyPro	Est[1]	SE[2]	Odds ratio	95% CI[3]		*P*-value
0900 h	3.9	5.8	0.41	0.42	1.52	0.66	3.48	0.32
1500 h	29.4	30.1	0.03	0.20	1.03	0.70	1.52	0.86
2200 h	10.2	15.1	0.45	0.27	1.56	0.91	2.67	0.10

[1]Parameter estimate generated with the GENMOD procedure of SAS using logistic regression for binomial data. Control is zero.
[2]Standard error of the estimate.
[3]Profile likelihood 95 % confidence interval for odds ratio.

Table 3. Jugular blood acid-base balance at 6 h after morning feeding on treatments Control and FlexyPro

	Control	FlexyPro	SEM	*P*-value
pH	7.42	7.42	0.007	0.83
pCO_2,[1] mm Hg	46.57	43.90	0.691	0.01
pO_2,[2] mm Hg	52.34	47.64	5.473	0.47
HCO_3^-, mmol/L	29.74	27.79	0.566	< 0.01
Total CO_2, mmol/L	31.17	29.14	0.574	< 0.01
Base excess, mmol/L	4.64	2.89	0.591	0.02
$SatO_2$,[3] %	82.46	83.10	1.721	0.71

[1]Partial pressure of CO_2.
[2]Partial pressure of O_2.
[3]% oxygen saturation of hemoglobin.

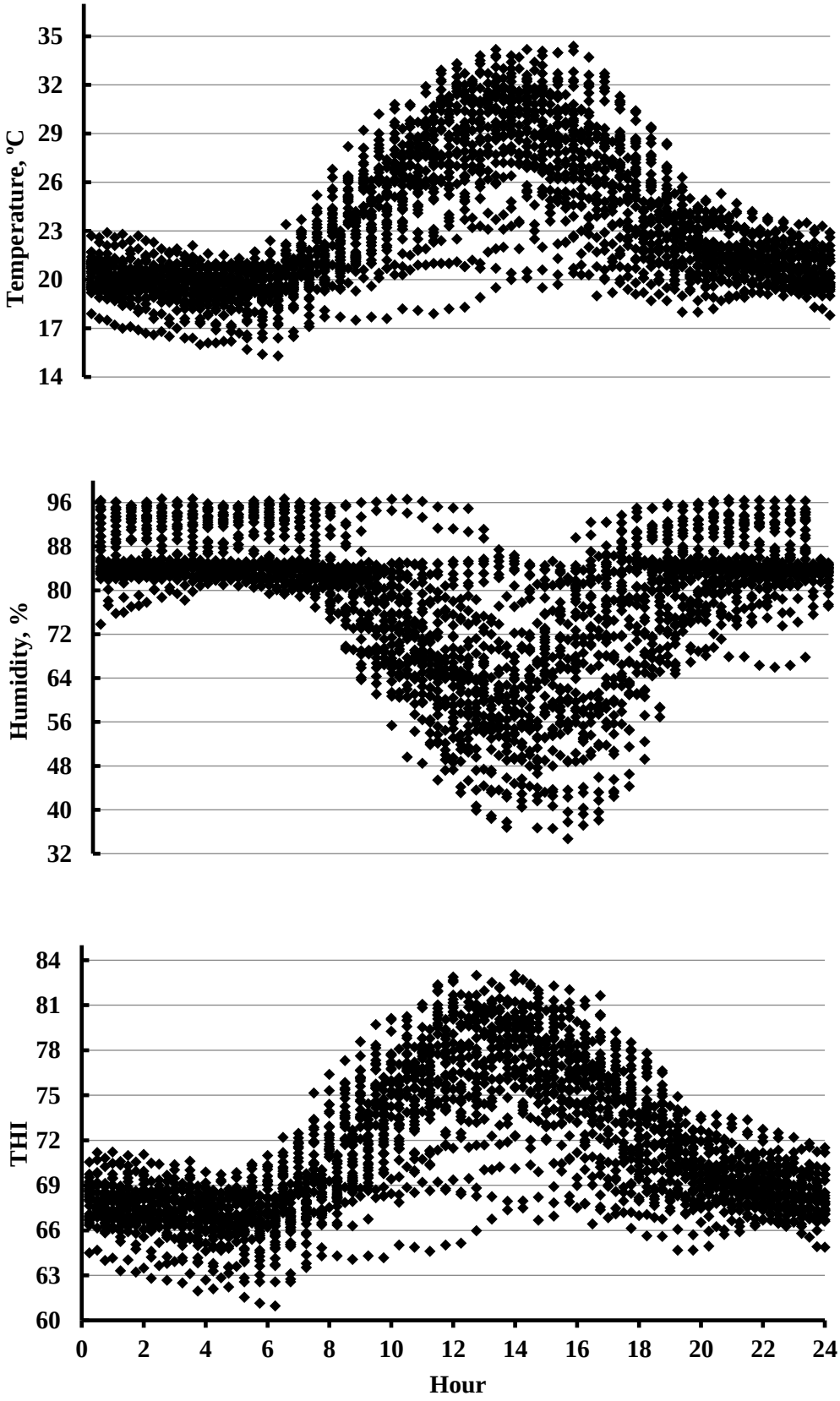

Figure 1. Temperature, humidity, and Temperature-Humidity Index (THI) inside the tie stall. Temperature: 23.2 ± 4.0 °C (Mean ± SD). Humidity: 78.4 ± 12.3 %. THI: 71.2 ± 4.5. THI ≥ 68: 71.5 % of time. THI ≥ 72: 36.9 % of time. Minimum daily THI: 65.9 ± 1.6. Maximum daily THI: 79.3 ± 3.1. 2733 recordings at 30-min intervals.

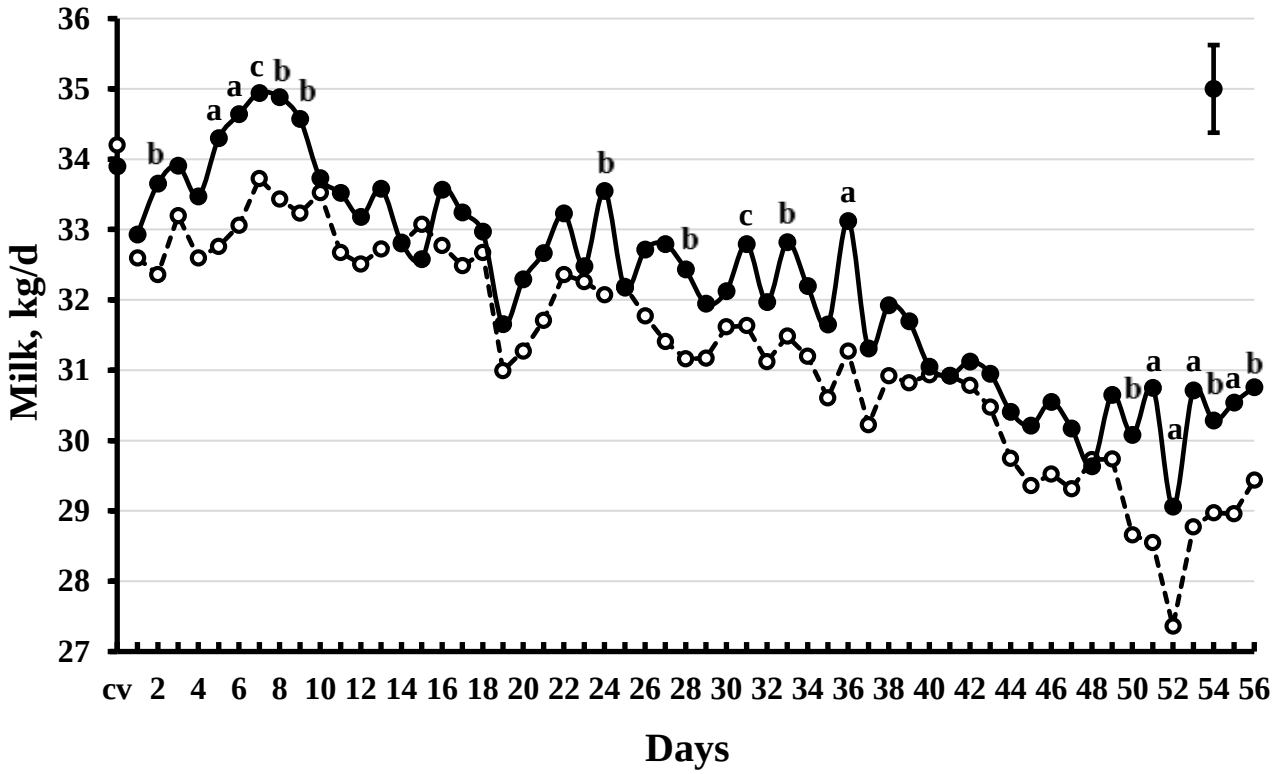

Figure 2. Milk yield on treatments Control (--o--) and FlexyPro (—●—). P = 0.01 for the effect of treatment, P < 0.01 for the effect of day and P = 0.99 for the interaction of treatment and day. cv = covariate (P = 0.71). Slice option of SAS: [a] $P \leq 0.05$, [b] $P \leq 0.10$, and [c] $P \leq 0.15$.

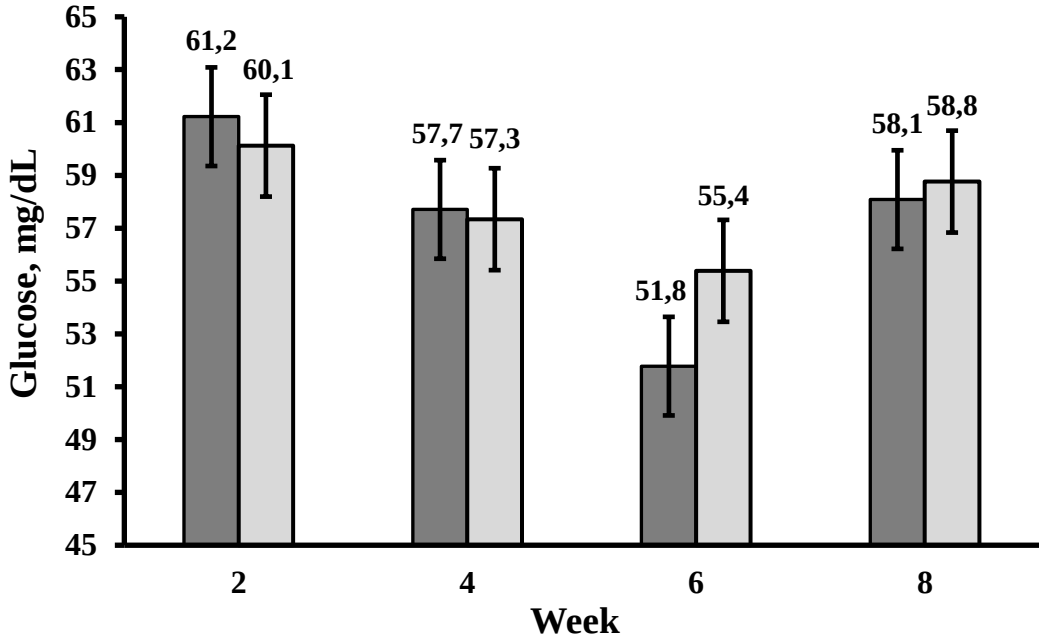

Figure 3. Plasma glucose at 12 h post-feeding on treatments Control (■) and FlexyPro (□). *P*-values: 0.61 for the effect of treatment, < 0.01 for the effect of week, and 0.58 for the interaction of treatment and week. Mean ± SEM: Control = 57.2 ± 1.05 and FlexyPro = 57.9 ± 1.09.

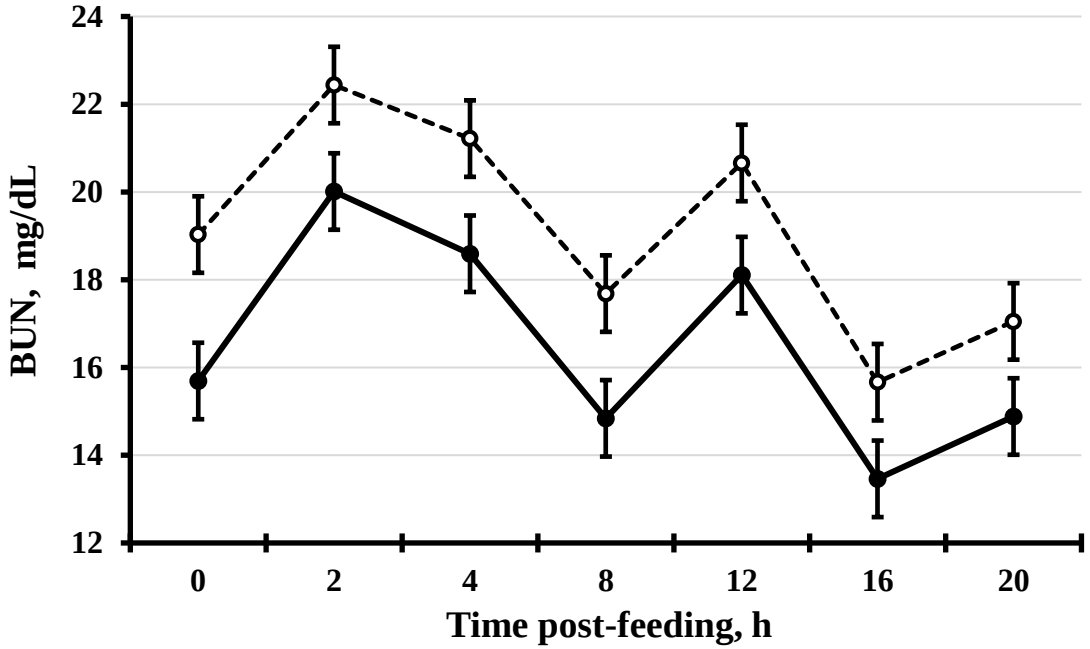

Figure 4. Blood urea nitrogen (BUN) over time on treatments Control (-○-) and FlexyPro (—●—). *P* < 0.01 for the effect of treatment, *P* < 0.01 for the effect of hour and *P* = 0.84 for the interaction of treatment and hour. Mean ± SEM: Control = 19.1 ± 0.78 and FlexyPro = 16.5 ± 0.78.

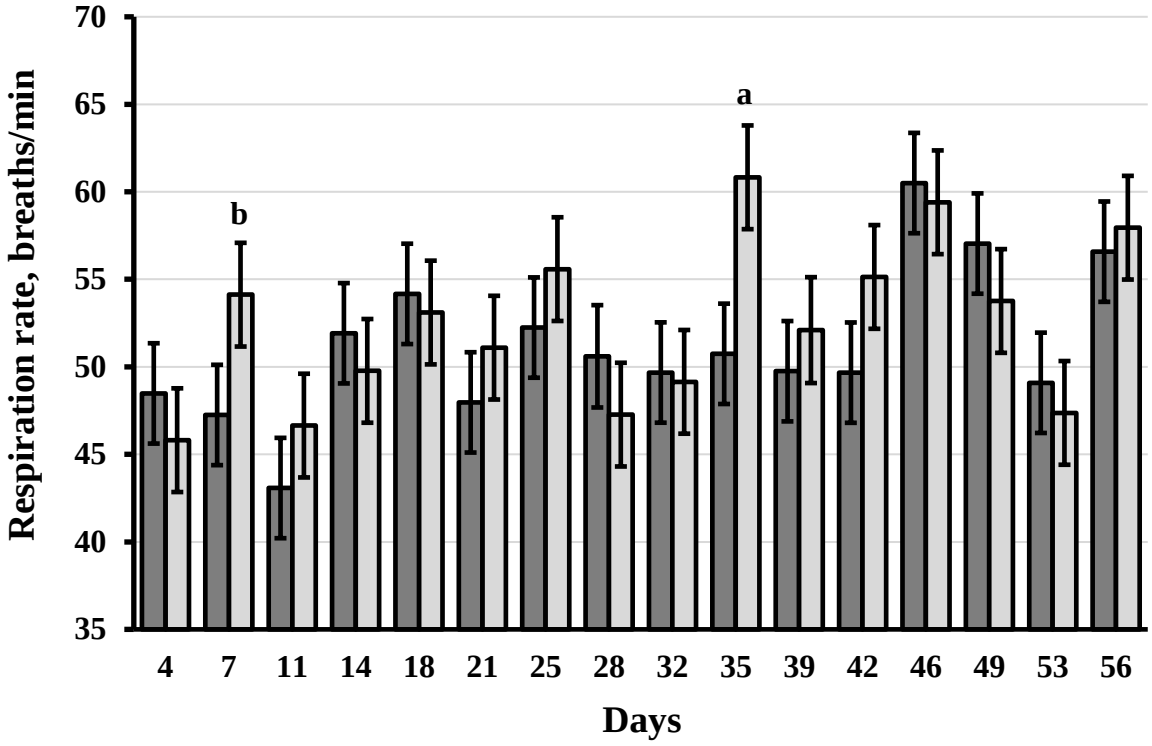

Figure 5. Respiration rate at 0900 h on treatments Control (■) and FlexyPro (□). *P*-values: 0.61 for the effect of treatment, < 0.01 for the effect of day, and 0.06 for the interaction of treatment and day. Slice option of SAS: $^{a}P = 0.01$ and $^{b}P = 0.07$.

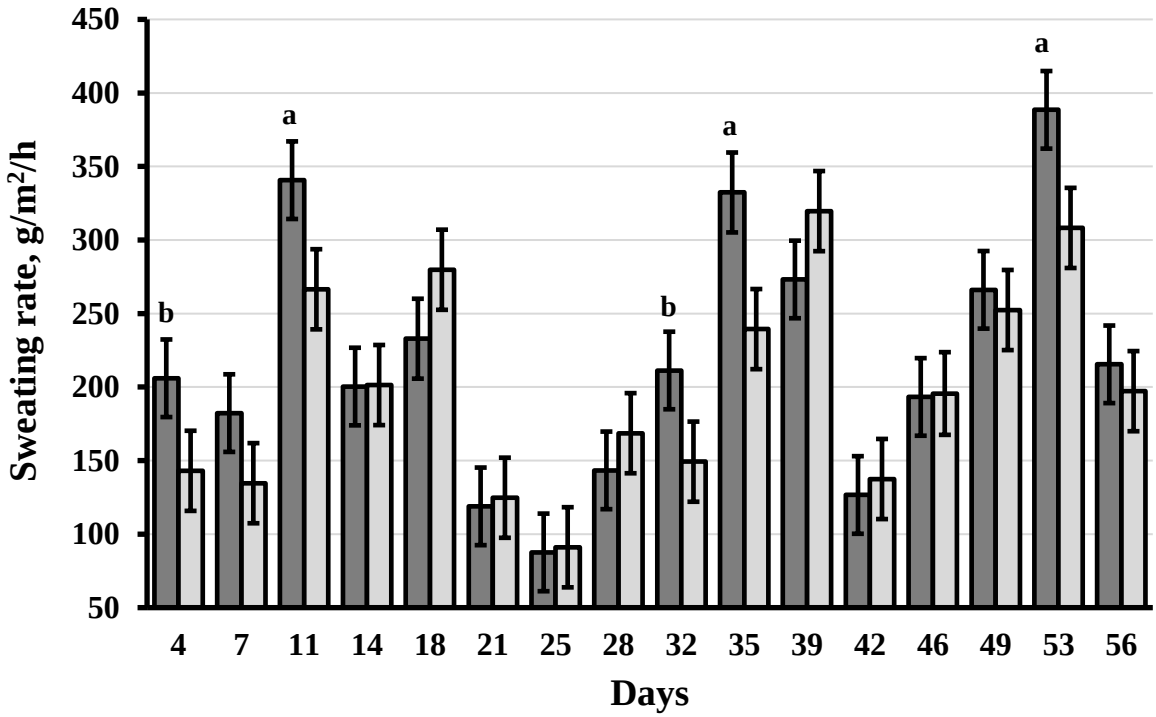

Figure 6. Sweating rate at 1600 h on treatments Control (■) and FlexyPro (□). *P*-values: 0.25 for the effect of treatment, < 0.01 for the effect of day, and 0.07 for the interaction of treatment and day. Slice option of SAS: $^{a}P \leq 0.05$ and $^{b}P = 0.10$.