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PAPER

Effects of a delay in feed delivery on behaviour, milk yield and heamatological parameters of dairy cows

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Abstract

This study aimed to evaluate the effects of a delay (at 09:30 instead of 07:30 h) in Total Mixed Ration (TMR) delivery on the behaviour, milk yield, and haematological parameters in dairy cows. Twelve healthy Italian Holstein cows, divided into two homogeneous groups of six each, were involved. The TMR delay was applied either for one (on Tuesday; single delay, SD) or for three consecutive days (Tuesday to Thursday; repeated delay, RD) to one group at a time while the other acted as control. A week of pause was interposed between treatments and the order of the treatment was randomised. Behaviour was observed by instantaneous scan sampling every 2 min during seven half-hour periods on Tuesday and Thursday and blood samples were taken at 14:30 h on these days. Milk yield was recorded daily. Data were analysed by a mixed models for repeated measurements. The delay caused a marked increase in activities towards the feeding rack prior to feeding on day 1 when delay was applied both as SD ($P<0.01$) and RD ($P<0.05$). On the third day of delay, pre- and post-meal agonistic behaviour increased in delayed cows ($P<0.01$). Milk yield and blood parameters were not affected by the delay. Results suggest that a two hours delay in feeding can affect behaviour in cows, increasing behavioural patterns, which may be a sign of mild frustration.

Introduction

In farm conditions, unexpected changes in the management routine due to mechanical or electrical problems or attributable to human factors, such as a delay in food delivery, can occur frequently.

Unpredictable feeding schedules in primates have been shown to increase abnormal and self-directed behaviour, and are likely to represent a stressor (Waitt and Buchanan-Smith, 2001; Ulyan *et al*., 2006), although such a result was not always confirmed (Bloomsmith and Lambeth, 1995). However, few studies have investigated the effects of unpredictable changes of the daily management routine in farm animals, even if management procedures are widely recognized as representing an important potential stress source (Broom and Fraser, 2007), and to modify feeding and lying behaviour in daily cows (DeVries and von Keyserlingk, 2005). Johannesson and Ladewig (2000) reported that 8-week-old dairy calves that were used to a regular predictable milk feeding schedule showed various behavioural deviations when exposed to an unpredictable 3-hour delay in feeding. No great behavioural changes were reported by these Authors when the same treatment was applied to calves that were already used to an irregular feeding routine. It has long been known that one of the variables which greatly influences the consequences of applying a stressor is the lack of predictability and control (Weiss, 1971).

Other aspects of a delay in feeding, apart from its unpredictability, could be important. It has been known that the timing of food delivery could influence behaviour, milk composition and blood parameters in dairy cows (Bertoni *et al*., 2004). Cows fed during the night showed a slower food intake on the second meal in comparison to those fed during the day, and this in turn influenced insulin and urea levels. Insulin increased after first meal both in cows fed during the night and in those fed during the day, but reacted differently after the second meal: *i.e.* big increase in the cows fed during the day and very slight increase in cows fed during the night (Bertoni *et al*., 2004). Moreover, after a short raise, urea was reduced as a consequence of the first meal in both groups, while the changes after the second one were opposite in the two groups: *i.e.* increased in the cows fed during the night and slightly reduced in the cows fed during the daytime (Bertoni *et al*., 2004). Although accidents which could cause an unpredictable delay in the feeding delivery could happen in dairy farms, there seems to be little research on their possible effects on the behaviour and welfare of dairy cows. Moreover, the possible implications of unpredictable feeding disruption on milk yield have not been previously studied.

The aim of this study was to investigate the effects of mild unpredictable management change, *i.e.*, two-hour delay in Total Mixed Ration (TMR) delivery on behaviour, milk yield, and blood parameters of dairy cows kept under normal farm conditions.

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Materials and methods

Animals and management

The experimental protocol of the study and all procedures were carried out according to the Italian legislation on animal care (Legislative Decree No. 116 of January 27, 1992).

The study was conducted at the dairy productive units of the experimental farm belonging to the University of Padova. The experiment was carried out between the middle of September to the middle of November with a mean daily temperature of 14.8±4.14°C (range 5.0°C to 20.4°C), and a relative humidity of 82.5±10.6% (range 48% to 97%) using 12 healthy Holstein dairy cows in late lactation, divided in two groups of six each (group A and B; Table 1), homogeneous for age, parity, days in milk (DIM) and body condition score (BCS). The two groups were loose housed with cubicles in two contiguous pens in the same building. Each pen consisted in both an indoor and an outdoor area and was provided with an automatic feeder and a drinking bowl. There were 1.5 cubicles and 70 cm feed bunk space per head. Water was always available *ad libitum*. TMR was distributed once a day (at 07:30 h) and was available *ad libitum*. Residual feed, when present, was carried away just prior to the next TMR delivery. In addition to receiving TMR, the cows were also individually fed concentrate supplementation by transponder-operated automatic feeders. This supplementation was delivered whenever the cow went to the apparatus (if the cow had not already eaten all the supplement allotted to it). The cows were milked twice daily at 05:30 and 17:00 h. This type of management represents a common practice of most of the Italian dairy farms. The TMR contained [% on dry matter (DM) basis] grass hay (44.2%), commercial mixed feed for lactating cows (Petrini Group, Italy; 12.3%), dehydrated alfalfa hay (10.2%), dried beet pulp (10.1%), barley meal (9.8%), corn meal (9.8%), commercial calcium esterified fatty acids from palm oil (Maxifat, Consorzio Agrario Padova, Italy; 2.7%), and sodium bicarbonate (0.8%). The composition of the TMR was 51% of DM and contained 13.8% of CP, 43.3% of NDF and 0.88 Milk Feed Units on a DM basis (*i.e.,* 1496 kcal of net energy for lactation, as 1.0 Milk Feed Unit/Kg DM equals to 1700 kcal of Net Energy for lactation, on DM).

Experimental procedure

The 2-hour delay in feed distribution, which was applied to the cows as a form of environmental challenge, concerned the morning TMR meal, distributed at 09:30 instead of 07:30 h either for one (Tuesday; single delay, SD) or for three consecutive days (Tuesday to Thursday, repeated delay, RD) during subsequent weeks, as detailed in Table 2. All other management procedures (*e.g.*, milking, cleaning, concentrate feeding) were kept constant. All the cows not involved in the experiment which were housed in the same barn always received their morning TMR meal at 07:30 h. The same applied to the experimental cows when acting as controls (*i.e.*, CTR_SD when acting as control for SD; and CTR_RD when acting as control for RD) and during the weeks of pause, which were interposed between experimental periods in order to avoid carry over effects (Table 2). The baseline periods were labelled baseline_control (BAS_C) if they preceded control periods (*i.e.*, CTR_SD or CTR_RD) and baseline_treatment (BAS_T) if a delay condition (*i.e.*, SD or RD) was applied in the experimental week immediately following them.

Behavioural observations

Behavioural data were collected twice during the six experimental weeks, *i.e.*, Tuesday and Thursday, which were day 1 (D1) and day 3 (D3) when SD or RD were applied. The cows were directly observed by instantaneous scan sampling (Martin and Bateson, 1986) every two minutes. In order to assess how behaviour changed during the day, seven half-hour periods (from 07:00 to 07:30, 07:30 to 08:00, 09:00 to 09:30, 09:30 to 10:00, 11:00 to 11:30, 11:30 to 12:00 and from 13:00 to 13:30 h) were selected for observation. These time periods were specifically selected in order to observe animals before TMR delivery time, immediately after TMR delivery, and far from TMR meals both in days in which the TMR delivery was delayed and in days in which the normal routine was applied. For statistical analysis the half-hour periods were categorized as pre-meal, when they preceded the morning TMR delivery, and post-meal when they followed it. Therefore, in control groups in which no delay was applied the 07:00 to 07:30 h period was considered pre-meal, whereas the other six periods were considered post-meal; in the days in which the feeding delay was applied the first three periods (*i.e.*, from 07:00 to 07:30, from 07:30 to 08:00, from 09:00 to 09:30 h) were considered pre-meal, whereas the others were considered as post-meal. Because the time the cows were observed for (and thus the number of scans taken) was not even before and after meal (half an hour pre-meal versus 3 hours post-meal during baseline and control conditions), the data to be analysed were calculated as percentages of the total number of scans in a given phase (*i.e.*, if a cow was recorded to be inactive in 5 scans out of the total 15 in the pre-meal phase during a control day, it was counted as being inactive for 33.33% of the scans, same as if it was inactive for 30 scans out of 90 in the post-meal phase of the same day).

Sixteen behavioural categories were selected on the basis of preliminary observations and are presented in Table 3. Each behavioural category included similar behavioural patterns. For example the single behaviour rubbing against structures or other body parts has been grouped into the more general behaviour category of self-grooming together with licking a part of its own body and scratching a part of its own body with a limb.

Milk yield and blood parameters

Data on milk yield were automatically recorded daily at every milking session (*i.e.*, morning and evening) during all nine weeks of the experiment.

Blood samples were collected from the jugular vein into heparinized evacuated tubes at 14:30 h on Tuesday and Thursday of the six experimental weeks (Table 2) in order to

Table 1. Characteristics of cows belonging to the two experimental groups

	Group A	Group B
Cows, n	6	6
Age, yrs	3.5±1.4	3.8±1.4
Parity, n	1.8±1.3	2.0±1.1
DIM, n	234.8±43.8	250.0±57.3
Milk yield, kg/d	29.6±5.3	28.0±5.3
BCS, points°	3.2±0.2	3.2±0.2

DIM, days in milk; BCS, body condition score. °Using the method suggested by Edmonson *et al.* (1989)

Table 2. Schedule of the experimental design adopted in the study in both groups of cows. In brackets there is the abbreviation for the 6 different treatment considered

Days	Experimental week	Group A	Group B
1-7	1	Baseline_control - no delay (BAS_C)	Baseline_treated - no delay (BAS_T)
8-14	2	Control - no delay (CTR_SD)	Single delay (SD)
15-21	-	Pause - no delay	Pause - no delay
22-28	3	Control - no delay (CTR_RD)	Repeated delay (RD)
29-35	-	Pause - no delay	Pause - no delay
36-42	4	Baseline_treated - no delay (BAS_T)	Baseline_control - no delay (BAS_C)
43-49	5	Repeated delay (RD)	Control - no delay (CTR_RD)
50-56	-	Pause - no delay	Pause - no delay
57-63	6	Single delay (SD)	Control - no delay (CTR_SD)

assess possible alterations of plasma cortisol and metabolic profiles (urea, glucose, cholesterol, triglycerides, beta-hydroxybutirrate, NEFA, lactate dehydrogenase (LDH; L-lactate) potentially related to the feeding delay.

Cortisol levels were analysed using a validated solid-phase microplate RIA procedure after diethyl ether extraction and subsequent sample dilution (Gabai *et al*., 2006). Briefly, a 96-well micro-titre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with anti-rabbit γ–globulin serum raised in a goat, by incubating overnight the antiserum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4°C. The plate was then washed twice with PBS 0.1% BSA, pH 7.4 (RIA buffer) and incubated overnight at 4°C with 200 L of the anti-cortisol serum diluted 1:8000. The antiserum (Centro Medico Diagnostico Emilia, Bologna, Italy) was raised in the rabbit against cortisol-3 carboxymethyloxime–BSA and showed the following cross reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.9%, corticosterone 3.5%, progesterone <0.01%. The plate was carefully washed with RIA buffer, and standards (1.56–400 pg/well), quality control, unknown extracts and tracer (1,2,6,7–^{3}H-cortisol, Perkin-Elmer Life Sciences, 30 pg/well, specific activity: 3700 GBq/mmol) were added (final volume: 200 L). The plate was incubated overnight at 4°C, the incubation mixture was decanted and wells washed with RIA buffer, added with 200 L scintillation cocktail (Microscint 20, Perkin-Elmer Life Sciences) and counted on the beta-counter (Top-Count, Perkin-Elmer Life Sciences). All samples were assayed in duplicate. The intra- and inter-assay coefficients of variation (CV) were 3.1% and 2.8%, respectively. The sensitivity of the assay was defined as the dose of hormone at 90% binding (B/B0) and was 3.125 pg/well (Gabai *et al*., 2006). The values for the other haematological parameters were obtained by enzymatic colorimetric methods using an automatic biochemical analyser (Hitachi 911, Roche Boheringer; Mannheim, Germany) (van Suijlen *et al*., 2000).

Statistical analysis

All behavioural data recorded (*i.e.*, incidence of behavioural categories observed on each cow) were analysed using the same hierarchical linear model for repeated measures using the MIXED procedure of SAS. After checking through the Akaike information criterion (AIC) for possible (co)variance structures between repeated measures, the compound symmetry was retained for all variables. The model accounted for the fixed effects of treatment (T; BAS_C, BAS_T, CTR_SD, CTR_RD, SD, and RD), the day of week in which behavioural data were observed (D; Tuesday=D1, and Thursday=D3), the interaction TxD, the meal phase (M; pre-meal, and post-meal), and the interactions TxM, DxM, and TxDxM. In this model, the variance of the cow within TxD was directly used by proc MIXED as error term for all other fixed effects in the main plot (*i.e*., T, D and T x D). To evaluate the effects of meal delay (SD or RD) on the occurrence of animal behaviour, contrasts were carried by decomposing the degrees of freedom of TxD interaction aiming to compare SD *vs* CTR_SD and RD *vs* CTR_RD. Because of in the model the effect which best represented the environmental change (*i.e.*, the delay in TMR distribution) was linked to the following levels: SD-pre-meal phase-D1, RD-pre-meal phase-D1, RD-pre-meal phase-D3, the incidence of behavioural changes in these 3 specific moments were compared to the correspondent control observation by contrasting the delay *vs* control in the pre-meal phase and in the specific day of delay (*i.e*., D1 for SD and D1 and D3 for RD), by decomposing the variance of the interaction TxDxM.

Daily milk yield recorded during the nine weeks of experiment and the bi-weekly blood sample collected in the six experimental weeks were analysed with a hierarchical linear model for repeated measures using the MIXED procedure of SAS, but assuming different (co)variance structures as suggested by the AIC parameter measure in preliminary analysis. Specifically, the (co)variance structures used were the unstructured type for milk yield and the compound symmetry for all blood parameters. The linear model used for these variables accounted for the fixed effects of treatment (T;

Table 3. Behavioural categories used for data recording and analysis, their abbreviation and explanation of meaning for each recorded behaviour

Behavioural category	Abbreviation	Explanation
Activities towards structures	ACT_STR	Licking, nibbling, biting the structures and sniffing the structures or air
Activities towards the feeding rack	ACT_RACK	Licking, nibbling, biting and sniffing the feeding rack/trough
Inactivity (standing)	I_S	Standing without performing any apparent specific behavioural pattern
Inactivity (lying)	I_L	Being recumbent without performing any apparent specific behavioural pattern
Movement	MOV	Movements which were not part of other studied behavioural patterns, such as locomotion, lying down, standing up, *etc.*
Feeding (TMR)	FTMR	Eating TMR standing at feeding rack
Feeding (integration)	FINT	Eating concentrate at automatic feeding station
Ruminating (standing)	RM_S	Self-explanatory
Ruminating (lying)	RM_L	Self-explanatory
Other maintenance behavior	OMAINT	Drinking, micturition, defecation
Self grooming	SGROOM	Licking the surface of one's body, rubbing against structures or other body parts (body care)
Tongue playing (standing)	TP_S	Performing stereotyped tongue movements
Agonistic activities towards cows	AGON	Butting, displacing another cow
Non-agonistic activities towards other cows	ACT_COW	Licking, nibbling, biting, sniffing, mounting, observing or playing with another cow
Vocalizations	VOC	Self-explanatory. The emission of vocalizations by the animal had to happen while the animal was not performing any other studied behavioral pattern.
Other	OTHER	Behavioural patterns not belonging to the any other categories (*e.g.* coughing)

as described above), the week of experiment in which data were recorded (W) and the interaction TxW. Also in this case the random effect of cow within TxW was use as error term for T, W and TxW effects. In order evaluate differences in milk yield and in blood parameters before the treatment and during the weeks of treatment, the following contrasts were carried out by decomposing the variance of the TxW effect: 1) BAS_C *vs* BAS_T; 2) SD *vs* CTR_SD and 3)RD *vs* CTR_RD.

Results and discussion

Behavioural data

On the whole, the cows spent most of their time during observation either feeding (37.1% of the total of the scans) or ruminating (23.0%) or simply remaining inactive (27.9%), and this pattern was not changed by the delay in TMR delivery (data not shown).

Results of mixed model ANOVA (Table 4) indicated that the meal phase (M) had the greatest magnitude (*i.e.*, grater F values), as compared to the other effects accounted for in the model. With only some minor exceptions (*e.g.*, activities classified as social behaviour; Table 3), the F statistics for the M effect were the greatest and often associated to a very slight probability for the null hypotheses (P<0.001). In the following paragraphs the prevalence of the different behavioural categories in the different conditions will be given

Table 4. Result of ANOVA for fixed effect (degrees of freedom in brackets) in the mixed model analysis. Data reported are F values and their significance.

Behavioural category	Effect							Residual
	Treatment T (5)	Day D (1)	TxD (5)	Meal M (1)	TxM (5)	DxM (1)	TxDxM (5)	variance (132)
ACT_STR	1.43	2.96	2.74*	29.77***	1.34	2.62	3.37***	0.0011
ACT_RACK	1.11	0.72	2.07	43.00***	0.81	0.58	1.97	0.0022
I_S	1.72	3.67	0.34	99.69***	1.77	6.21*	0.40	0.0261
I_L	1.11	0.15	0.48	60.87***	0.51	3.56	0.50	0.0235
MOV	0.71	0.20	0.78	42.26***	0.90	0.09	0.41	0.0032
FTMR	2.26*	1.72	4.64***	303.81***	4.83***	24.80***	4.04***	0.0221
FINT	0.48	0.05	0.54	3.76	1.24	0.09	0.68	0.0025
RM_S	0.64	0.02	2.21	85.39***	1.30	0.01	2.65*	0.0422
RM_L	1.11	0.47	0.95	29.75***	0.45	0.37	0.55	0.0246
OMAINT	1.41	0.13	1.19	0.00	0.18	1.44	1.40	0.0006
SGROOM	1.14	0.50	2.02	23.50***	1.25	0.22	2.23	0.0020
TP_S	0.80	1.31	0.58	1.71	0.86	0.36	0.81	0.0001
AGON	1.76	8.44***	0.82	5.32*	1.81	4.92*	2.26	0.0002
ACT_COW	1.03	1.11	1.81	1.00	1.77	0.57	4.26***	0.0004
VOC	0.70	0.18	0.90	0.18	1.04	1.22	1.53	0.0001
OTHER	1.39	2.43	2.56*	2.61	2.34*	2.15	2.10	0.0042

ACT_STR, activities towards structures; ACT_RACK, activities towards the feeding rack; I_S, inactivity (standing); I_L, inactivity (lying); MOV, movement; F_{TMR}, feeding (TMR); F_{INT}, feeding (integration); RM_S, ruminating (standing); RM_L, ruminating (lying); OMAINT, other maintenance behaviour; SGROOM, self grooming; TP_S, tongue playing (standing); AGON, agonistic activities towards other cows; ACT_COW, non-agonistic activities towards other cows; VOC, vocalizations. *P<0.05; **P<0.01; ***P<0.001; when not reported P>0.05.

Table 5. LS Means for all the behavioural categories in the different conditions

Behaviour	BAS_C	BAS_T	CTR_RD	CTR_SD	RD	SD
ACT_STR	0.01343	0.01134	0.01829	0.00694	0.02002	0.006017
ACT_RACK	0.01250	0.02083	0.01968	0.009725	0.02812	0.02569
I_S	0.1379	0.1104	0.1743	0.2051	0.1531	0.1725
I_L	0.1837	0.1523	0.1493	0.1104	0.1956	0.1506
MOV	0.05134	0.05578	0.05949	0.03772	0.04791	0.05107
FTMR	0.2571	0.2664	0.1676	0.2002	0.1812	0.1958
FINT	0.02244	0.01874	0.01921	0.03263	0.02175	0.02476
RM_S	0.1374	0.1812	0.1405	0.1567	0.1058	0.1445
RM_L	0.09832	0.1058	0.1722	0.1491	0.1545	0.1465
OMAINT	0.01456	0.02338	0.01296	0.01550	0.01447	0.01111
SGROOM	0.04696	0.03426	0.03819	0.02662	0.03425	0.03194
TPS	0.003700	0.000000	0.003008	0.000694	0.001273	0.000925
AGON	0.002315	0.003006	0.005785	0.005094	0.01088	0.005325
ACT_COW	0.004829	0.01296	0.003935	0.006944	0.01088	0.008706
VOC	0.000000	0.000462	0.000925	0.000231	0.001273	0.000462
OTHER	0.01341	0.003008	0.01458	0.03634	0.01887	0.02399

ACT_STR, activities towards structures; ACT_RACK, activities towards the feeding rack; I_S, inactivity (standing); I_L, inactivity (lying); MOV, movement; FTMR, feeding (TMR); FINT, feeding (integration); RM_S, ruminating (standing); RM_L, ruminating (lying); OMAINT, other maintenance behaviour; SGROOM, self grooming; TP_S, tongue playing (standing); AGON, agonistic activities towards other cows; ACT_COW, non-agonistic activities towards other cows; VOC, vocalizations.

as LSMeans between brackets, as in Table 5. Particularly during the pre-meal phase compared to the post-meal phase, cows had a tenfold greater prevalence of activities toward structures (0.023 *vs* 0.002, respectively; P<0.001), showed a greater prevalence of activities toward racks (0.038 *vs* 0.0001, respectively; P<0.001), greater prevalence of standing inactivity (0.254 *vs* 0.064, respectively; P<0.001), greater movement (0.072 *vs* 0.029, respectively), ruminating activity (0.334 *vs* 0.221, respectively as the sum of both standing and lying rumination; P<0.001 for both behavioural categories), and self-grooming (0.048 *vs* 0.023, respectively; P<0.001). On the other hand, the post-meal phase (*i.e.*, from 07:30 to 08:00, 09:00 to 09:30, 09:30 to 10:00, 11:00 to 11:30, 11:30 to 12:00 and from 13:00 to 13:30 in the baseline and control phases and from 09:30 to 10:00, 11:00 to 11:30, 11:30 to 12:00 and from 13:00 to 13:30 h during delay days) was characterized by a 6-times greater prevalence of eating TMR activity at feeding racks than the pre-meal phase (0.364 *vs* 0.059, respectively; P<0.001) and by a greater prevalence of laying inactivity that in the pre-meal (0.227 *vs* 0.087, respectively; P<0.001). The prevalence of feeding (TMR) was significantly different also considering the treatment (*i.e.*, greater in both baseline phases, 0.26 for BAS_C and 0.27 for BAS_T, as compared to the treatment weeks; *i.e.*, 0.20 for SD, 0.18 for RD) and all the interactions accounted in the model, due, as expected, to the very small value for this activity registered before feeding, *i.e.*, when TMR was almost absent at the feeding rack.

The interaction Treatment x Day x Meal phase, which could reflect the application of the delay, influenced the prevalence of activities toward structures, of eating TMR, activity at feeding racks, of rumination while standing, and of non-agonistic activities towards other cows (Table 4). In particular, greater prevalence of activities toward racks were found in SD cows in comparison to control cows (CTR_SD) exactly at the moment when the delay was applied, *i.e.*, in the pre-meal phase (0.089 *vs* 0.011 for SD and CTR_SD in pre-meal, respectively; P<0.01; Table 6). When RD was applied, RD cows showed greater activity than CTR_RD in day 1 (Table 6), as regards the prevalence of behaviour toward structures (0.048 *vs* 0.001; P<0.01), racks (0.063 *vs* 0.022; P<0.05), other cows (*i.e.*, non-agonistic activities; 0.026 *vs* 0.006; P<0.05) and self-grooming (0.057 *vs* 0.011; P<0.05). When the feeding delay was repeated, the comparison between RD and CTR_RD cows in the pre-meal phase at the last day of delay (*i.e.*, on Thursday, day 3 of delay), still indicated differences in behaviour between groups, but some behavioural categories changed in the opposite direction as compared to D1. Indeed, cows in RD were recorded less often engaged than CTR_RD cows (Table 5), in activity toward structures (0.028 *vs* 0.067; P<0.01) and self-grooming (0.052 *vs* 0.089; P<0.01) during the delay, but they were recorded more often engaged in ago-

Table 6. Comparison of different behavioural categories between control (CTR_SD and CTR_RD) and delayed treatment (single, SD and repeated delay, RD) in the pre-meal phase during the appliance of feeding delay and on different days (day 1, D1; or day 3, D3) of scan sampling (data presented are least squares means for the incidence of each behaviour and are reported only for comparisons showing significance differences).

Behavioural category	Day 1 of delay				Day 3 of delay		Pooled SE
	CTR_SD	SD	CTR_RD	RD	CTR_RD	RD	
ACT_STR			0.001^{A}	0.048^{B}	0.067^{B}	0.028^{A}	0.0095
ACT_RACK	0.011^{A}	0.089^{B}	0.022^{a}	0.063^{b}			0.0136
SGROOM			0.011^{a}	0.057^{b}	0.089^{b}	0.052^{a}	0.0129
AGON					0.001^{A}	0.017^{B}	0.0044
ACT_COW			0.006^{a}	0.026^{b}			0.0064
VOC					0.001^{a}	0.004^{b}	0.0011

ACT_STR, activities towards structures; ACT_RACK, activities towards the feeding rack; SGROOM, self grooming; AGON, agonistic activities towards other cows; ACT_COW, non-agonistic activities towards other cows; VOC, vocalizations. [a, b]Means in the same row with different superscripts differ (P<0.05); [A,B]means in the same row with different superscripts differ (P<0.01).

Table 7. Milk yield and haematological parameters measured in different treatment during the weeks with baseline (*i.e.*, no delay) or with short or repeated delay treatment of the Total Mixed Ration feeding.

	Baseline		Short delay		Repeated delay		Pooled SE
	BAS_C	BAS_T	CTR_SD	SD	CTR_RD	RD	
Cows, n	6	6	6	6	6	6	-
Milk yield, kg/d	23.6	23.5	22.1	22.5	21.3	22.3	2.0
Haematological parameters							
Urea, mmol/L	2.89	2.71	2.77a	3.10b	2.48a	2.82b	0.13
Glucose, mmol/L	2.75	2.73	2.63	2.77	2.71	2.73	0.10
Cholesterol, mmol/L	5.89	5.77	5.35	5.56	5.37	5.33	0.38
Triglycerides, mmol/L	0.14	0.13	0.18	0.13	0.13	0.12	0.01
Beta-hydroxybutirrate, mmol/L	0.60	0.57	0.55	0.60	0.59	0.56	0.03
NEFA, mEq/L	0.14	0.16	0.15	0.14	0.15	0.14	0.02
LDH4, U/L	1509	1472	1455	1468	1464	1452	92
L-Lactate, mmol/M	0.75	0.70	0.75	0.65	0.64	0.74	0.07
Cortisol, ng/mL	5.90	8.20	7.34	8.39	8.57	9.20	0.98

[a,b]Means in the same row with different superscripts differ (P<0.05).

nistic activities (0.017 *vs* 0.001; P<0.01), and vocalizing (0.004 *vs* 0.001; P<0.05). It is worth noting that although all these behavioural patterns had low prevalence, a detailed examination of the data (data not shown) showed that the difference was not due to the behaviour of few very sensitive individuals in the group.

Not surprisingly, the meal (*i.e*., being in the pre- *vs* the post-meal phase), both as single effect and in its interactions, had an influence on the majority of the recorded behavioural patterns. In particular, activities towards the feeding rack were greater in the pre-meal phase on the first day when TMR was delayed both in the SD and in the RD condition. This increase in activities towards the feeding rack during delay days could be similar to the increase in the head through barriers behavioural pattern found by Johannesson and Ladewig (2000) in eight-week-old calves, when the regular feeding routine was occasionally disrupted and milk replacer delivered later than usual. Eight-week-old calves also showed increased eating and comfort behaviour when the regular feeding routine was occasionally disrupted, but a similar increase was not found in the present study. However the delay was longer for calves (3 h) than for cows in the present study (2 h), and the length of feed deprivation, together with different body weight, has been demonstrated as affecting eating motivation in lactating dairy cows (Schütz *et al*., 2006). Moreover in the experiment by Johannesson and Ladewig, calves were not fed *ad libitum*, unlike TMR feeding in the present experiment. Indeed, the behavioural changes shown by the calves were explained as anticipatory behaviour, due to the fact that the animals had learned to predict feed delivery, and that their expectations were not fulfilled (Johannesson and Ladewig, 2000). The increase in activities towards the feeding rack during SD and RD, found in the present study, could, therefore, have had a similar meaning. An unfulfilled anticipation would, in most cases, lead to some levels of frustration in the animals (Johannesson and Ladewig, 2000), although it is difficult to ascertain whether such a level of frustration could cause significant stress to the cows in the present study. Many factors may have contributed to the perception of the delay as a source of frustration. Cows may have been disturbed because they were hungrier, as they had had to wait longer to obtain feed, or they could have been disturbed by the change of the routine *per se* or/and by lack of expectation fulfilment. However one has to bear in mind that in the present experiment the delay was compounded by the fact that the delayed cows could see that other cows were eating while they were not (see below for a detailed discussion).

As far as the delay *per se*, is concerned, it is already known that both light/dark cycles and periodic food access are factors which can act on the biological clock and influence activity cycles in rats (Stephan, 1986; 2002), and that plasma corticosteroid circadian periodicity is also affected by periodic feed access (Moreira and Kreiger, 1982). Although behavioural circadian rhythms are less studied in farm than in laboratory animals, preferential periods of the day dedicated to grazing and other behaviours have been found also in free ranging cattle (Broom and Fraser, 2007).

Apart from chance factors, whose likelihood was low due to the presence of the control group in the same environment, other hypotheses, not related to circadian rhythms, could also be formulated. For example, different activities could also have been due to the not simultaneous feeding for the control and delayed cows in the adjacent pens, so the control cows ate while the delayed did not. Although this situation created alternative hypotheses, it was necessary in order to have an effective control group in which routine was kept as usual. For example, cows that were not eating were likely to be observing those that were eating or trying to reach the food in the feeding rack of the other pen. Social facilitation may also have played a role, as cows could have been motivated to go to the rack and eat while seeing other cows doing so. It is known that feeding behaviour within a group of intensively managed cows is usually highly synchronized, with delivery of fresh feed appearing to be the primary factor stimulating feeding (von Keyserlingk and Weary, 2010). Therefore, seeing other cows eating, while not getting anything to eat, may have been frustrating and possibly stressful for the cows undergoing the delay procedure, and this may be the major cause of the results found in the present study. Also, seeing other cows eating could have acted similarly to a form of local enhancement, increasing the interest of the delayed cows for the feeding rack. Although local enhancement and social facilitation would have played a role also when the delayed cows were fed and the controls were not, it is possible that effects were less when the cows which were not receiving their meal had already eaten.

Moreover, delivering feed at 07:30 h to the non-delayed cows and at 09:30 h to delayed ones may be considered to be similar to having an increased frequency of delivery, even if the cows in each pen received feed once daily (*i.e*., every cow had its meal once like before, but feed was delivered in total two times a day, instead of one). The frequency of food delivery has been shown to have significant effects on cows' behaviour (DeVries *et al*., 2005; Phillips and Rind, 2001; Robles *et al*., 2007), although these findings have not been replicated by Bava *et al.* (2012). Also, the moment of food delivery was shown to be associated with increased aggressive behaviour among cows (Jezierski and Podluzny, 1984). Increased aggression is reputed to be a sign of stress (Broom and Johnson, 1993), and a potential stressor in itself for confined animals.

In our study there was a significant increase in agonistic activities towards other cows only when the pre-meal condition on the third day of the RD treatment was compared to its control. In this situation the cows also showed a decrease of activities towards structures, and an increase in vocalizations which could be interpreted as a sign of stress in itself (Bristow and Holmes, 2007; Watts and Stookey, 2000), These changes in behaviour were not present on the first day of RD, and could be due to the persistence of the altered routine. The different magnitude of behavioural changes between the pre-meal phase in D1 of the SD condition and the pre-meal phase in D1 of the RD condition (*i.e*., increased activities toward racks, but also greater activities toward structures, non-agonistic activities towards other cows, and self-grooming) has no easy explanation. Indeed, despite SD and RD being administered in different orders during the experiment, cows of both groups experienced the environmental challenges. Moreover, despite differences due to individual perceptions and to the hypothetical response to environmental challenges in cows (Koolhaas *et al.,* 1999), individual variability was appropriately accounted for in the statistical analysis, attempting at avoiding possible animal effects. However, the response of some individuals in each group to the first time they experienced the delay (this being D1 of RD for group A and D1 of SD for Group B) may have affected the behaviour of the others in the same group, especially when exploratory and social behaviours are concerned. Nevertheless, to our knowledge, no experiment has been carried out up to date to assess the extent of interdependence of exploratory and social affiliative behaviour, such as those performed by Cooper *et al*. (2008) on feeding, lying, or standing behaviour in cattle. As for the other variables influencing behaviour, there was a clear effect of the pre-meal vs. post-meal condition, which might reflect a circadian rhythm in the observed behavioural categories, although pre-meal and post-meal observations had a different timing during the delay conditions (*i.e*., the periods from 07:30 to 08:00 h and from 09:00 to 09:30 h were considered post-meal when no delay was applied and pre-meal when it was). However, the timing of food delivery is known to pay a role in this cyclic pattern, as food can be a *Zeitgeber* (*i.e.*, synchroniser; Stephan, 2002). Also, during this study, cows were more often engaged in feeding activity immediately after TMR delivery, to the expense of the performance of other behavioural categories during that period.

Milk yield and blood parameters

Milk yield was not significantly different between the two groups of cows during the whole experiment, with a mean individual production of almost 23 kg/d during the nine weeks of trial. Comparison between treatment groups before the feeding delay (*i.e.,* baseline weeks) showed no differing daily milk yields in animals addressed to became controls (BAS_C) or treated with SD or RD delay (BAS_T). Indeed, these theses produced 23.6 and 23.5 kg/d of milk during the pre-treatment weeks (Table 6). Also the comparison between controls and animals that underwent a delay treatment (both single or repeated) had no different milk production considering the week in which the delay was administered (Table 7). Milk yield was thus not significantly influenced by the delay procedure. The simpler explanation for this is that the procedure could simply not have been a severe enough stressor to alter the daily average group production. Blood parameters, on the whole, did not appear to have been significantly influenced by the procedure. Among blood parameters, the only significant differences were observed in the urea concentration that was always greater ($P<0.05$) in treated cows during the delay weeks, *i.e.* 3.10 *vs* 2.77 mmol/L for SD and CTR_SD, and 2.82 vs. 2.48 mmol/L for RD and CTR_RD, respectively (Table 7). The greater urea level in delayed groups at blood sampling is not surprising. Indeed, Bertoni *et al*. (2004) reported a rapid increase in the urea level of dairy cows within 4-5 h after feeding. The delayed cows were blood sampled around five hours after their meal, while still in the phase in which the urea level is physiologically increased, while the non delayed cows seven hours after it, when the level could have started to decline to baseline levels. The post meal increase in urea was also much greater when the feeding interval was longer (Bertoni *et al*., 2004), as in the case of the delayed cows in this study. The distance between meal and blood sampling could thus account for the difference found in urea levels in this study, without the delay having a direct effect. The cortisol level measured ranged from 5.9 to 9.2 ng/mL, indicating a slightly elevated basal concentrations of this hormone as respect to other findings in the same environmental situation and using the same analytical method (Marinelli *et al*., 2007), although greater concentration could be due to a stress situation generated by managing and immobilizing cows for bleeding (Abeni *et al*., 2005).

Nevertheless, the finding that cortisol levels were not influenced by the delay was rather unexpected, although blood sampling was scheduled at 14:30 h, *i.e*., five hours from the end of the delay. This bleeding schedule was planned in order to avoid interference with the behavioural observation, but it might have masked the effect of feeding delay, especially if the procedure was perceived by the animals only as an acute stressor and not as a chronic one. Indeed, vocalizations (also linked to anxiety and increased cortisol; Bristow and Holmes, 2007, and generally to impaired welfare; Watts and Stookey, 2000), self-grooming (possible sign of stress; Broom and Johnson, 1993) and agonistic behaviour (increased in stressful situations; Broom and Johnson, 1993) were significantly affected by the delay, and thus cortisol increase could be hypothesized at the time of delay, but not after five hours. This is even more true because the overall results of this study suggest that it was unlikely that the cows experienced a chronic stress due to the experimental procedure, which could cause an alteration of cortisol levels after several hours after the stressor itself.

Conclusions

At a behavioural level, the most specific change following the disruption of the usual daily routine, caused by a delay of two hours in TMR delivery, was an increase of activities towards the feeding rack. Also, an increase in agonistic behaviour was found when the altered routine was maintained for three days. These behavioural changes suggest that some part of the procedure applied in the present study may be a source of mild frustration for cows, and may be perceived as a stressor by them, even without a significant increase in plasma cortisol levels. However, neither milk yield nor blood parameters appeared to be influenced by the procedure.

The importance of having a control group in the same environment notwithstanding, it would be interesting to perform a further study in order to separately assess the effects of the delay *per se vs* those of seeing the control cows eat while waiting for the delayed meal.

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