The Effect of Exposing High Yielding Dairy Cows To Short Wavelength LED Illumination During The Night On Fatty Acid Profile

Aviv Asher

Northern R&D, MIGAL – Galilee Research Institute

Matan Fialko

Northern R&D, MIGAL – Galilee Research Institute

Florin Fares

Northern R&D, MIGAL – Galilee Research Institute

Uzi Moallem

Agricultural Research Organization

Shamai Yaacoby

Agricultural Research Organization

Roei Gutman (✉ roeeg@migal.org.il)

Tel-Hai College, Upper Galilee

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Abstract

Fatty acid levels vary between day and night milking. Many dairy cows are kept under whole light at night (LAN) illumination using a white light-emitting diode (W-LED) suspected of disrupting circadian rhythms. We investigated the effect of W-LED LAN and same lux level red LED (R-LED) LAN, which is known not to disrupt circadian rhythms in other mammals, on milk yield and circadian composition, compared to a natural light-dark (LD)-cycle of 10-11 h light. Cows under the natural LD-cycle showed circadian variation in milk-fat composition, characterized by higher health-promoting monounsaturated fatty acid (MUFA) and lower saturated fatty acid (SFA) levels in 13:30-h (day milk, DM) than 03:30-h (night milk, NM). W-LED did not affect milk and milk-fat yields, yet abolished the circadian pattern of milk-fat composition towards a less healthy fatty acid profile by lowering MUFA levels of DM. Switching W-LED to R-LED reversed these circadian disruptions. Therefore, R-LED and W-LED have no commercial advantage over the tested LD-cycle, while W-LED is disadvantageous, showing a circadian disruption. Accordingly, if the illumination is indispensable, the R-LED regime is preferable over W-LED for cow's wellbeing and for preserving the natural milk-fat profile; while differentiating DM from NM from a commercial-health-promoting perspective.

Introduction

Long-term dairy records of milk yield and composition (e.g., fat and protein level) show a cosine-like monthly variation. This is characterized by milk yield peaking around the vernal equinox (March to April), and fat and protein concentration peaking earlier, around the winter solstice (November to January)^{1,2}. Although changes in diet and environmental temperature contribute to these variations, the natural change in photoperiod (i.e., the daily change in the hours of illumination) is recognized as the dominant factor that drives these annual changes¹. In addition, artificial illumination has long been recommended as a means to increase daylight up to 16 or 18-h. This is known to increase milk yield, without affecting fat content, compared to a short day with 8 hours of illumination or natural daylight of ca. 9.5 to 14.5-h, regardless of the stage of lactation³⁻⁷. Notably, maximizing illumination hours to a 24-h illumination cycle does not further increase or decrease milk yield, or protein and fat content in Holstein cows compared with the 18-h illumination cycle⁸ or a natural photoperiod of 13-h of illumination⁹.

In addition to its not increasing cows' productivity, compared with the long-day illumination cycle^{8,10}, 24-hour illumination may have deleterious effects. The latter have been suggested from human and animal model studies that revealed the physiological harms of such a constant illumination (known as light at night, LAN)¹¹. In human and animal models, these LAN-induced impairments are due to disruption of the normal circadian physiological cycle, such as the melatonin hormone cycle; and have been associated with increased prevalences of metabolic diseases and probably cancer¹¹⁻¹³. Therefore, circadian disruption due to LAN following shift working has been declared a pollutant and suggested as a carcinogen by the World Health Organisation¹⁴.

In cows as in other organisms, behavioral and physiological processes show a daily rhythm, with a ca. 24-h period length (i.e., circadian rhythm) affected by the photoperiod^{15,16}. Notably, and as found in humans and in animal models, our previous research in cows showed LAN-induced circadian disruption in heart rate and in melatonin rhythms¹⁷. This was manifested by a lower milk-melatonin level in both day milk (DM) and night milk (NM), and a lower within-day difference in milk-melatonin level between milking times¹⁷. Despite these data, many dairy workers illuminate the barn all night for operational comfort, believing that this light regime also leads to higher milk yield, due to higher feed consumption. Therefore, the present study's first aim was to re-examine in a controlled manner the effect of LAN on the amount and composition of milk, compared to a light regime of about 10 hours of natural light.

A novel element of this study is the usage of light-emitting diode (LED) bulbs, which have gained popularity in the agricultural market due to their longer lifespan, higher illumination efficiency (illumination per watt of electric energy), and the option to control the illumination spectrum, compared to traditional bulbs^{18,19}. However, the use of white LED illumination raises several concerns^{9,20}. These are due to the domination of white-LED (W-LED) by short wavelengths (i.e., 'blue' illumination), which better activate the photoreceptors that synchronize the internal master clock, hence resulting in more significant disturbance of daily rhythms than longer wavelengths (i.e., 'yellow' illumination)^{9,11,20}. Indeed, LAN using 'blue' illumination was found to be more stressogenic to lactating cows than 'yellow' illumination⁹, and the circadian disrupting effect of 'blue' illumination (e.g., suppression of plasma melatonin level vs. 'yellow' illumination or red illumination) was even shown when these wavelengths were provided during the last hours of illumination^{21,22}.

In contrast to the above, long wavelengths (i.e., 'red' illumination) do not activate the photoreceptors that synchronize the internal master clock and minimally influence the circadian system of both humans and rodents¹¹. Therefore, red-LED (i.e., R-LED) was suggested as an option for barn illumination for dairy workers during night-dark hours. Notably, the lack of an R-LED LAN-induced circadian disruption was not fully demonstrated under long-term controlled conditions. Hence, the second aim of the current study was to examine the effect of R-LED vs. W-LED on milk yield and circadian parameters related to milk composition, especially milk-fat composition.

Dairy products are primarily composed of saturated fatty acids (SFA)^{23,24}, of which high consumption is suggested as a risk factor for cardiovascular diseases (e.g., atherosclerosis)^{25,26}. Therefore, ongoing efforts have explored ways to reduce milk-fat SFA levels while increasing unsaturated fatty acids (UFA) levels²⁷. Notably, for some milk constituents (e.g., fat level), a circadian variation in milk-fat composition has been found [i.e., a difference between DM (milked at 12:30 or 15:00) and NM (milked at 04:30 or 05:00)]²⁸⁻³¹. Therefore, this study's third aim was to investigate the effects of W-LED and R-LED on the circadian variation in dairy cows' milk-fat composition (i.e., DM vs. NM), compared to the natural light regime. We hypothesized that whole night W-LED illumination would disrupt the natural circadian pattern of milk-fat composition, and that this would be rescued by whole night R-LED illumination. Our results supported this hypothesis and, more importantly, showed a less-healthy fatty acid profile of DM under W-LED but not under R-LED. This further promotes the differentiation of DM from NM and the use of R-LED as a source of LAN if needed.

Materials And Methods

Animals, housing conditions, and experimental protocol

The Israeli Committee for Animal Care and Experimentation (Bet-Dagan, Israel) approved all the animal involving procedures (Volcani file number 72917 IL). All experiments were performed in accordance with relevant guidelines and regulations. The experiments were conducted in the experimental dairy farm in the Volcani Center, Beit Dagan, located in the center district of Israel. We used two groups of Israeli high yielding Holstein cows, a control group (n=16) and a treatment group (n=41), housed in two separate yards on the same farm. The initial parameters for block subdivision, i.e., age (3.6 ± 1.3 years in both groups), days at milking (control, 175.3 ± 84.6 ; treatment, 163.3 ± 82.1), body weight, and daily milk yield (Table 1), did not differ between the groups (Table 1). Cow's feed (see Supplementary Table S1 for diet composition) was provided *ad-lib* and dispensed daily at 10:00 standard time (or one hour earlier during daylight savings time, which ended October 27th, 2019, the 30th day of the experiment). During the pre-experimental phase, the illumination conditions at the yard included fluorescent bulbs that were left on during the night (Fig. 1 and Table 1). The cows were regularly milked at 05:00, 13:00, and 20:00 hr. standard time throughout the pre-experimental phase and following all the experimental phases. During the milking that lasted ca. 1-hr., the cows were exposed to the following illumination conditions at the milking parlor: white fluorescent illumination, dominant wavelength: 545 nm at 140 lux (measured at cows' eye height using AvaSpec-2048-FTSDU, Avantes, Eerbeek, Netherlands).

Table 1
Illumination conditions and selected parameters collected at the experimental phases. Data are presented as mean $\pm$ SD.

Variable / Phase		White fluorescent (Pretreatment)		Natural light (Baseline)		White LED vs. natural light (Midphase)		Red LED vs. natural light (Endphase)	
		Control (n=16)	Treatment (n=41)	Control (n=16)	Treatment (n=41)	Control (n=16)	Treatment (n=41)	Control (n=16)	Treatment (n=41)
Body weight (kg)		692.7 ± 67.9	654.5 ± 42.1	714.2 ± 73.2*	670.3 ± 44.8	716.6 ± 80	676.0 ± 49.9	724.6 ± 80.2	686.4 ± 51.7
Milk yield (L/d)		48.4±6.7	43±6.3	45.6±8	44.9±7.2	38.8±11	38.8±7.6	39.5±9	40.3±10.7
Experimental period		- Sep' 26 , 2019		Sep' 27 – Oct' 28, 2019		Oct' 29 – Nov' 17, 2019		Nov' 18 – Dec' 06, 2019	
Sunrise ¹		05:32		05:52		06:12		06:30	
Sunset ¹		17:32		16:58		16:40		16:36	
Natural LD ratio		12:12		11:13		10.5:13.5		10:14	
Cowshed	Illumination type	White fluorescent		Natural light		Natural light	White LED	Natural light	Red LED
	Dominant wavelength (nm)	545		670		670	462	670	663
	Intensity (Lux)	140		1.7		1.7	125.4	1.7	124.6
	Illumination on-off (h.)	16:30 – 05:30		-		-	16:30 – 05:30	-	16:30 – 05:30
	LD ratio	24:0		11:13		10.5:13.5	24:0	10:14	24:0
*, p < 0.05 between treatment by T-test.									
¹ Sunrise and sunset hours on the sampling day are presented as standard time and indicate the end and the beginning of civil twilight, respectively. (https://mylush.net/)									
LED, light-emitting diode.									

To examine the effects of different types of LED illumination (i.e., different dominant wavelengths) on body weight, milk yield, milk constituents, and the composition of fatty acids in DM vs. NM, we first transferred all the cows to a natural light-dark cycle regime (LD-cycle) of ca. 11-h light and ca. 13-h dark (Fig. 1 and Table 1). This contrasts with the previous illumination conditions in which the cows were exposed to a whole day illumination regime due to fluorescent illumination during the dark hours (Fig. 1 and Table 1). The control group was held under this naturally changing LD-cycle throughout the experiment, during which the light-hours were shortened by ca. one hour to ca. 10:14 LD-cycle (Fig. 1, Table 1). For the treatment group, the illumination of the cowshed was changed twice during the experiment, as depicted in Figure 1 and Table 1. Following 32 days at the natural LD-cycle, the illumination conditions of the treatment group were changed to a white LED illumination regime (W-LED, dominant wavelength of 462 nm, day light 6500k, 34w, Soul LTD, 124.5 Lux). Accordingly, lights were turned on throughout the night, resulting in a 24-h illumination regime (1.5:22.5 LD-cycle, Fig. 1 and Table 1). Following 21 days at this illumination regime, the light bulbs were changed from W-LED to R-LED (dominant wavelength of 663 nm, Infraled LTD, Israel, 124.6 Lux), and the cows were followed for the final 18 days (Fig. 1 and Table 1).

Sampling and analysis of the milk constituents

Two milk samples were collected 10 hours apart, 1-3 days before the end of each of the three experimental phases (Fig. 1). One milk sample was taken during the regular milking time of 13:30 (i.e., DM) and another during a novel milking time of 03:30 (i.e., NM). The latter time was set to enable sampling the milk formed in the mammary gland during the night hours, before sunrise. At each milking, one 50 ml milk tube from each cow was sent to an external ISO certified lab [Israeli Cattle Breeders' Association, Caesarea, Israel]. There, total protein, fat, lactose, and urea were analyzed using FTIR (Foss MilkoScan™), and milk somatic cell count (SCC) was evaluated using flow cytometry (Foss Fossomatic™). Additionally, two 15 ml tubes were sub-sampled and frozen at -20°C for further analysis of the fatty acid profile.

Analysis of the fatty acid profile of the milk

Samples were prepared as previously described³²: 0.5 ml of each milk sample was pipetted into a 15 ml tube, followed by 10 ml of hexane (analytical grade, J.T.Baker) spiked with 50 µg/ml of benzophenone (Cat #: 427551, Sigma) as internal standard, and 50 µg/ml Tridecanoin (T3882, Sigma) as transesterification standard. Then, 1 ml of a saturated solution of sodium methoxide (TCI Co., Ltd) in methanol (HPLC grade, J.T.Baker) was added. The sealed tubes were placed in a rack and vigorously shaken for 20 minutes while lying horizontally on an orbital shaker. An aliquot of the upper organic layer was filtered through a syringe filter (PTFE filter matrix, 13 mm Ø, 0.45 µm pore size) directly to a glass amber vial for gas chromatography (GC) analysis.

External calibration standards were prepared. First, a standard mix containing 37 fatty acid methyl esters (cat #: 18919, Sigma, Supplementary Table S2) was dissolved in hexane to a 20 mg/ml concentration and kept in a capped vial as a stock solution at -20°C. An aliquot from the stock was used to prepare standard dilutions of 4, 3.2, 2.4, 1.6, 0.8, 0.4, and 0.1 mg/ml in hexane. Additionally, a standard of conjugated linoleic acid (CLA, C18:2) methyl esters (cat #: 05632, Sigma) was dissolved in pentane to a 50 mg/ml concentration and kept in a capped vial as a stock solution at -20°C. An aliquot from this stock was used to prepare standard dilutions of 1, 5, 10, 15, 20, and 25 µg/ml in hexane. Each calibration solution, both the standard mix and CLA standard, contained 50 µg/ml of benzophenone as internal standard.

The fatty acid profile was analyzed on a GC instrument (7890A, Agilent Tech.) coupled with a mass spectrometer (5975C, Agilent Tech.). Separation was performed on a polar column (30 m length, 0.25 mm internal diameter, Zebron ZB-FAME, Phenomenex). The conditions were as follows: 1 µl of the sample or calibration standard was injected into the GC inlet, heated to 250°C, and set to a split ratio of 10:1. The oven program was set to 100°C for 2 minutes, then a 10°C/min increase to 140°C, then a 3°C/min increase to 180°C, ending with a 30°C/min increase to 260°C, with another 2 minutes holding time (the total runtime was 24 min). Fatty acids were quantified as methyl esters using individual calibration curves, as previously described³³. A calibration curve was plotted with values calculated for each fatty acid: $x_i = C_i / C_{is}$, $y_{es} = A_i / A_{is}$. Here, x_i is the calibrant's value for a given FAME (plotted on the x-axis); C_i is the calibrant concentration for a given FAME as mg/ml; and C_{is} is the standard internal concentration in the calibrant as mg/ml.

Statistical analysis

To examine the effects of treatment (natural illumination vs. W-LED or R-LED) and milking time (NM, 03:30, vs. DM, 13:30), and their interactions, on concentrations of various milk constituents (e.g., fatty acids, total protein, fat, lactose, urea, and SCC milk levels), we implemented a repeated measures ANOVA procedure on the results of each experiment period (natural light, W-LED, and R-LED). Accordingly, milking time was the within-subject factor, and treatment was the between-subject factor (Using IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Differences in concentrations of the various milk constituents between milking hours within the same treatment group were tested with the paired T-test. Differences in concentrations of different milk constituents between treatment groups at the same hour were tested using the two-sample unpaired T-test. The significance level was set at $p < 0.05$.

Results

Milk-fat composition under the natural illumination regime is time-dependent, showing higher SFA levels at night milking

Under natural LD-cycle conditions, milking hour affected the concentrations of most of the milk components analyzed, without interaction with the insignificant group-effect (Tables 1 and 2, Figs. 2 and 3, and Supplementary Table S3). Therefore, we pooled the data of the two groups. At the 03:30 milking (NM) compared to the 13:30 milking (DM), both groups showed higher fat levels, lower urea levels, and no differences in protein, lactose, and SCC levels (Figs. 2 and 4). Under natural light conditions, the cow groups did not differ in the levels of 14 of 15 quantified fatty acids (Table 2, Figs. 2 and 3, and Supplementary Tables 2 and 3). Therefore, we pooled the groups' fatty acid levels (Fig. 4).

Table 2

The effect of experimental phase (i.e., illumination condition) and milking time on whole milk constituents (g/100 ml, except for somatic cell count, cells*10³/ml composition (in percentage) in whole milk. Data are presented as mean ± SE.

	Baseline – natural light				Midphase - white LED illumination vs. natural light				Endphase – red LED illumination vs. natural light		
	Control (n=15)		Treatment (n=18)		Control (n=15)		Treatment (n=18)		Control (n=15)		Treatment (n=18)
	03:30	13:30	03:30	13:30	03:30	13:30	03:30	13:30	03:30	13:30	03:30
Protein	3.4±0.1	3.5±0.1 [#]	3.5±0.1	3.5±0.1	3.6±0.0 ^a	3.7±0.1	3.3±0.1	3.5±0.9 [*]	3.6±0.1	3.7±0.1 ^{\$}	3.3±0.2
Lactose	4.9±0.05	5.0±0.0 [*]	5.0±0.0 [*]	4.9±0.1	4.9±0.0 ^a	4.8±0.1	4.6±0.1	4.9±0.1 [*]	4.8±0.1 ^a	4.8±0.1 [`]	4.3±0.18
Urea	33.0±1.9	36.8±2.4	33.7±1.2	39.8±1.9 ^{\$}	24.5±1.0	43.5±2.8 ^{\$}	37.3±1.5 ^c	46.8±1.9 ^{\$}	20.3±1.7	31.5±1.3 ^{\$}	24.2±1.2
SCC	466±298	261±139	313±91	217±50	389±251	588±253	322±95	288±108	216±30	292±49 [*]	700±211
Fat	3.8±0.4	3.1±0.2	3.9±0.2	3.6±0.2	3.4±0.2	3.9±0.3 [*]	3.6±0.2	4.1±0.1 [*]	3.6±0.2	4.4±0.2 ^{\$}	3.7±0.2
SFA	71.8±0.7 [#]	70.3±0.8	73.0±0.5 ^{\$}	71.9±0.5	71.0±0.8 [*]	69.0±0.8	72.9±0.5	72.1±0.5 ^b	72±0.7 [*]	70.0±0.7	73.2±0.5
Butyric	6.2±0.2	6.3±0.2	6.4±0.3	6.1±0.2	6.9±0.2 ^{a,\$}	5.8±0.2	6.2±0.2	6.6±0.1 ^b	6.9±0.2	6.5±0.2	6.5±0.2
Caproic	3.0±0.1	2.9±0.1	3.2±0.1	3.0±0.1	3.5±0.1 ^{a,\$}	2.7±0.1	3.1±0.1	3.3±0.1 ^b	3.4±0.1 [*]	3.1±0.1	3.3±0.1 [#]
Caprylic	1.5±0.1 [*]	1.4±0.1	1.7±0.1	1.6±0.1	1.9±0.1 ^{a,\$}	1.4±0.1	1.6±0.1	1.8±0.0 ^c	1.7±0.1	1.6±0.1	1.7±0.1
Capric	3.2±0.1 [#]	2.9±0.1	3.5±0.1 ^{\$}	3.2±0.1	3.2±0.1 [#]	2.8±0.1	3.2±0.2	3.3±0.1 ^a	3.5±0.2	3.3±0.1	3.6±0.2
Lauric	3.5±0.1 [#]	3.3±0.1	3.8±0.1 [#]	3.6±0.2	3.4±0.1	3.2±0.1	3.5±0.2	3.4±0.2	3.8±0.1	3.7±0.1	3.8±0.2
Myristic	10.0±0.1 [*]	10.0±0.2	10.0±0.2 [*]	10.0±0.2	9.8±0.2	9.6±0.3	10.0±0.2	9.8±0.2	10.3±0.9	10.0±0.2	10.1±0.1
Pentadecylic	1.1±0.1	1.1±0.1	1.0±0.1	1±0.1	1.1±0.0	1.0±0.1	1.0±0.0	1.0±0.0	1.0±0.1	1.0±0.1	0.9±0.1
Palmitic	32.2±0.5	31.0±0.4	32±0.5	32.2±0.5	31.8±0.5	32.2±0.5	33.2±0.5 ^a	32.5±0.4	31.5±0.5	30.5±0.4	31.7±0.4
Margaric	0.6±0.0	0.6±0.0	0.7±0.1	0.6±0.0	0.7±0.0 [#]	0.6±0.0	0.7±0.1	0.6±0.0	0.6±0.0	0.5±0.0	0.6±0.0
Stearic	10.2±0.3	10.0±0.2	10.6±0.3	10.7±0.3	9.1±0.2	9.8±0.3	10.2±0.3 ^b	9.8±0.3	9.3±0.3	9.9±0.2	10.9±0.3
MUFA	23.7±0.6	25±0.7 [*]	22.8±0.4	23.8±0.5 [#]	24.3±0.7	26.7±0.9 ^b	23.1±0.4	23.8±0.4	23.5±0.7	25.3±0.6	22.6±0.5
Myristoleic	1.0±0.1	1.0±0.1 [*]	1.0±0.1	1.0±0.1	1.1±0.1	1.2±0.1	1.1±0.1	1.1±0.1	1.1±0.1	1.2±0.1	0.9±0.1
Palmitoleic	1.7±0.1	1.8±0.1 [#]	1.6±0.1	1.6±0.1	2.0±0.2	2.0±0.2	1.7±0.1	1.8±0.1	1.8±0.1	2.0±0.1 ^a	1.5±0.1
Oleic	21.1±0.6	21.9±0.6 ^{\$}	20.3±0.4	21.1±0.4 [#]	21.1±0.6	24±1 ^{a*}	20.3±0.4	20.9±0.4	20.6±0.6	22.2±0.6	20.2±0.4
PUFA	4.5±0.2	5±0.2 ^{b,\$}	4.2±0.1	4.4±0.1 [*]	4.3±0.1	4.3±0.3	4.1±0.1	4.2±0.1	4.4±0.2	4.7±0.2	4.2±0.1
Linoleic	3.7±0.1	4.1±0.2 ^{a,\$}	3.5±0.1	3.6±0.1	3.6±0.1	3.6±0.3	3.4±0.1	3.5±0.1	3.6±0.1	3.8±0.1	3.5±0.1
CLA	0.7±0.0	0.8±0.1 ^{b,\$}	0.6±0.0	0.7±0.0 [#]	0.7±0.0	0.8±0.1	0.7±0.0	0.7±0.0	0.8±0.1 ^a	0.8±0.0	0.7±0.0
SCC, somatic cell count; SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids. CLA, conjugated linoleic acid. *, p < 0.01; \$, p < 0.001, between milking hours, within treatments, by paired T-test. a, p < 0.05; b, p < 0.01; c, p < 0.001, between treatments, within milking hours, by test.											

Milking-hour affected the pooled and the un-pooled fatty acid composition of the milk (Table 2, Figs. 2 – 4, and Supplementary Table S3). At NM compared to DM, SFA levels were higher, while MUFA and polyunsaturated fatty acids (PUFA) levels were lower (Table 2, Figs. 2 – 4). The higher level of SFA at the NM milking was mainly due to the higher level of four short-to-mid chain fatty acids [caprylic (C8:0), capric (C10:0), lauric (C12:0), and myristic acid (C14:0)] (Table 2, Figs. 3 and 4, and Supplementary Table S3). The higher levels of MUFA and PUFA at the DM were due to the higher levels of all the detected MUFA and PUFA [i.e., myristoleic (C14:1), palmitoleic (C16:1), oleic (C18:1) acids, for MUFA; and linoleic (C18:2) and CLA (C18:2) acids, for PUFA] (Table 2, Figs. 3 and 4, and Supplementary Table S3).

The experimental phase affected the body weight and the milk yield, fat, urea, and protein levels of the control cows, but not lactose and SCC levels of the milk. Milk yield and milk urea decreased continually, while body weight and milk protein increased continually (Tables 1 and 2, Fig. 2, and Supplementary Tables 4 and 5). Nevertheless, though milk-fat showed an incremental increase throughout the experiment, this effect interacted with milking hour, thus indicating that fat incrementation was due to an increase in DM level (Fig. 2 and Supplementary Table S4). During the experiment, the levels of 7 of 15 quantified fatty acids changed, yet total SFA and MUFA levels remained constant (Fig. 3 and Supplementary Table S4). Milking hour affected the levels of most of the milk constituents, such as protein and urea levels, and 11 of the 15 quantified fatty acid levels (Table 2, Figs. 2 and 3, and Supplementary Table

S4). As found for natural light conditions, SFA levels were higher at NM, while MUFA and PUFA levels were higher at DM throughout the experiment, without interaction with the experimental phase (Figs. 2 and 3, and Supplementary Tables 3 and 4). In all, the milking-hour-related differences in milk-fat composition, under natural fall-illumination conditions of ca 10-11 illumination hours, were found to be robust. The differences were not affected by the combination of a 1-h reduction in the hours of natural light, an increase in days-at-milking, a reduction in milk yield, and the fat increment level at DM.

Whole-night white-LED illumination results in a higher level of SFA at day milking and abolishes the circadian variation in milk-fat composition found under the natural light regime

Bodyweight was similar for cows exposed to W-LED illumination and R-LED illumination (Table 1 and Fig. 1) and for control cows exposed to the natural LD cycle (Table 1 and Supplementary Table S5). In addition, between-treatment differences in milk yield were not found in analyses of data collected during and at the end of the W-LED phase or the subsequent R-LED phase (Table 1, Fig. 2E, and Supplementary Table S5). Similarly, total SCC, milk-fat, and protein levels were not affected by W-LED (Table 2, Supplementary Table S6, and Fig. 2A, B, and F). W-LED, however, affected total lactose and urea levels (Table 2, Supplementary Table S6, and Fig. 2C, and D). Notably, these effects were limited to NM, and affected the levels of between-milking-hour differences (Table 2 and Fig. 2C and D). For example, W-LED resulted in lower lactose levels in NM than in the control cows under the natural LD-cycle, thus increasing the between-milking-hour difference. However, the opposite was found in the NM urea level (Table 2, Fig. 2A and C). W-LED also resulted in lower protein levels at NM but did not result in total lower protein levels (Table 2, Supplementary Table S6, and Fig. 2A).

Notably, W-LED illumination affected milk-fat composition, especially of DM, without affecting the fat percentage at each milking hour (Table 2, Figs. 2 and 3, and Supplementary Table S6). These W-LED-induced changes in milk-fat composition at DM abolished the time-related difference in milk-fat composition that was still evident in control cows (Table 2, Figs. 2 and 3, and Supplementary Table S6). Exposure to W-LED specifically resulted in a higher SFA level due to a higher SFA level during DM than in control cows, which showed a declining trend in SFA levels compared to baseline (Table 2, Fig. 2G, and Supplementary Table S6). Hence, cows exposed to W-LED illumination did not show a time-related difference in SFA levels, in contrast to control cows exposed to a natural LD-cycle (Table 2 and Fig. 3). This lack of a time-related difference in SFA levels was due to the abolishment of time-related differences in butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), lauric (C12:0), and margaric (C17:0) acid levels, which were still present in the control cows (Table 2 and Fig. 3). The complementary effect was found in MUFA levels, which were now lower in the DM of the treated than the control cows (Table 2 and Fig. 3A). This W-LED induced lower MUFA level at DM was mainly due to a lower oleic acid (C18:1) level than that found in the control cows exposed to the natural LD-cycle. This resulted in a lower time-related difference in oleic acid level (Table 2 and Fig. 3A and G). In all, these results indicate that whole-night W-LED illumination resulted in higher SFA levels of DM and, hence, the abolishment of the circadian variation in milk-fat composition found under the natural LD cycle.

Whole-night R-LED illumination rescues the W-LED-disrupted circadian variation in milk-fat composition

At the last experimental phase, we replaced the W-LED bulbs in the cowsheds of the treatment group with same-lux level R-LED bulbs, which are characterized by relatively long wavelengths. The aim was to examine whether the circadian disruptions observed at the second experimental phase were related to the dominant wavelength (i.e., short vs. long-wavelength given at the same intensity) rather than the inducement of LED illumination. Notably, shifting from W-LED to R-LED reversed most illumination-related circadian disruptions due to W-LED (Fig. 3). For example, it restored NM protein level and lowered NM urea level, but did not restore NM lactose levels (Table 2 and Fig. 2).

R-LED illumination restored DM SFA levels by lowering them to a level below that of NM, as found under natural light (baseline) conditions and in the same-age control cows that were continually exposed to the natural LD-cycle (Table 2, Fig. 2G, and Supplementary Tables 6 and 7). This R-LED-induced rescue-effect in SFA levels was due to a change in milk from DM, such that several SFA levels [butyric (C4:0), caprylic (C8:0), and capric (C10:0) acids] resembled DM levels found in the same-age control cows (Fig. 3 and Supplementary Table S2). In parallel, R-LED illumination resulted in control-like DM levels of MUFA, which yielded significantly higher MUFA levels than in NM found under natural light (baseline) conditions (Table 2, Fig. 3A, and Supplementary Table S7). The R-LED illumination-induced changes, especially the elevation in DM MUFA level, may be consequent to higher oleic acid levels in DM than in NM, as found under natural light (baseline) conditions, and in same-age controls (Table 2, Fig. 3H, and Supplementary Table S7). Lastly, switching from W-LED to same-lux level R-LED rescued, to some extent, the pattern of higher CLA level at DM vs. NM (Fig. 3H).

Discussion

In cows and other organisms, many behavioral and physiological outputs show a circadian rhythm that is disrupted by exposure to artificial illumination at otherwise dark hours (i.e., LAN) ^{11,17}. Our present study shows that holding high-yield Holstein cows under a natural LD-cycle of ca. 10-11 h of natural light is accompanied by a circadian variation in milk-fat composition. This is reflected in higher levels of the health-promoting UFA in DM (milked at 13:30) than in NM (milked at 03:30). This natural circadian rhythm was abolished under whole night short-wavelength illumination by W-LED. However, the circadian disruption was rescued by same-intensity (lux level) of R-LED illumination. These results highlight the distinction between DM and NM from a commercial-health-promoting perspective; and suggest that if illuminating the barn during the night is indispensable, the R-LED regime is preferable over W-LED, for both the quality of the milk and the cow's wellbeing.

In dairy cows as in other organisms, most physiological parameters show a circadian output affected by the photoperiod ^{15,16}. This has prompted investigations of within-day variations in milk composition, to differentiate between DM and NM from health-promoting, nutritional, and commercial perspectives ^{17,28–31,34}. As expected, most of these studies found circadian oscillation in milk yield and in some of the milk's constituents, yet the presence and extent of the circadian variation varied between studies and depended on milking and feeding frequency ^{17,28–31,34}.

In this study, in which all the cows were fed *ad-libitum* and exposed to 10 to 11 h of natural light conditions, we found within-day variations in milk urea and fat, and to a lesser degree, in protein levels. During most of the experiment, higher levels of these milk components were found in DM than NM, as reported by others^{29–31}. On the other hand, lactose and SCC levels did not differ significantly between NM and DM, corroborating other studies^{17,28,34}. Regardless, we mainly focused on the composition of fatty acids, due to their highly nutritional-commercial value³⁵. We found a robust circadian pattern in fatty acid composition, characterized by higher UFA levels and lower complimentary SFA levels, in DM than NM of cows exposed to natural light conditions. This higher level of SFA in NM was due to a difference in several short-to-mid chain SFA (C8 to C14), while the higher level of UFA at DM was due to different levels of all the detected UFA, as was found by others³¹. These circadian rhythms in milk-fat level and composition are suggested to be under the control of the mammary circadian clock, which synchronizes to the central circadian clock, which synchronizes to the photoperiod¹⁶. These results show higher nutritional value of DM milk-fat over NM milk-fat. This supports separating DM from NM at the farm and dairy level, or linking the dairy to the milk or cheese production plant. Such separation enables the utilization of DM, which is richer in UFA, to create end-products with a health-related commercial benefit.

Our demonstration of circadian variation in several milk constituents set the ground for exploring our main aim. This entailed examining, in a controlled manner, the effect of whole night illumination using W-LED, on milk yield and composition, and especially milk-fat composition, compared to a natural illumination regime of about 10 hours of natural light. The result of this comparison is of scientific importance for several reasons. For example, although illumination throughout the night is inefficient for milk production, some dairy farms still practice it and, more recently, even use advanced LED lighting of various spectrums and intensities. The use of LED lighting highlights another important aspect of this study, namely, the examination of the effect of W-LED LAN, which is dominated by short wavelengths, on productivity and the existence of daily endogenous rhythms.

Illumination with W-LED showed mixed effects. On the one hand, whole night illumination using W-LED bulbs did not affect cows' body weight or milk yield; both resembled those of control cows, which continued to be exposed to natural LD cycle. This corroborates other studies that compared outcomes of W-LED LAN to those of a natural photoperiod of 13-h of illumination⁹. The lack of effect was also evident concerning milk-fat and SCC, and to some extent, protein level, as others found^{8,9,17}. On the other hand, W-LED LAN resulted in higher urea levels, consistent with other studies⁹, and lower lactose levels. These differences were due to differences in NM urea and lactose levels, which corresponded to reductions and increases in the between-milking-hour differences in their levels. This W-LED-induced decrease in milk lactose level, together with the trending decrease in protein levels and the corresponding increase in urea level, may indicate a temporal negative energy (and protein) balance^{36,37}. In conclusion, W-LED LAN, which requires a high energy investment in illumination overnight, did not yield a commercial advantage, for example, higher milk yield than a natural lighting regime of 10-11 hours of light. Therefore, we can not cite any advantage in its usage.

Our lack of finding a robust W-LED-induced circadian disruption, or any disruption, in most of the measured parameters is somewhat surprising. This contrasts with LAN disruption that was observed over a range of physiological parameters and circadian rhythms in humans and in animal models¹¹, and in the circadian rhythm of milk melatonin and heart-rate in cows¹⁷. Hence, macro parameters, such as body weight and milk yield; and milk fat, protein, and SCC levels appear more resistant to a disturbance resulting from W-LED LAN exposure, at least one that lasts for three weeks. This resistance may result from the short, only three-week long manipulation, or from the endless selection of high-yielding cows. The latter favors cows that produce high amounts of milk, milk fat, and milk protein, despite their exposure to LAN and its detrimental effects. Nevertheless, the 'resistance' to W-LED exposure observed in this study does not infer sweeping resistance to W-LED. This is because W-LED bulbs differ in the composition of their wavelengths. The dominant wavelength in our W-LED illumination was 462 nm ('blue'), yet it included other wavelengths. The use of W-LEDs with a higher representation of short wavelength has been described to significantly affect such macro parameters as body weight, milk yield, and milk components⁹.

Despite the above, the lack of a W-LED-induced circadian disruption did not apply to milk fat composition. Milk and its products are a substantial source of dietary fat in many human populations³⁸. However, milk-fat comprises a high proportion of SFA^{23,24}, which are suggested as a risk factor for cardiovascular diseases (CVD, e.g., atherosclerosis)^{25,26}. Nevertheless, reducing SFA consumption by reducing milk consumption may not be the best approach since milk is rich in essential minerals and amino acids, and milk fat per se is a carrier for fat-soluble vitamins and a source of several essential fatty acids³⁹. Both feed-related factors, i.e., dietary intake and seasonal and regional effects, and animal-related factors, i.e., genetics (breed and selection), stage of lactation, mastitis, and ruminal fermentation, are capable of modifying the fatty acid composition, as well as the overall quantity of lipids present in milk⁴⁰. Therefore, manipulating fatty acid composition in milk-fat by genetic selection or dietary modification is more beneficial for increasing UFA percentage on account of SFA percentage^{27,35}.

Another option for obtaining a higher level of UFA in milk fat could be manipulating the photoperiod. Notably, numerous studies searched for an effect of the natural photoperiod and long-day and even whole night illumination on body weight, milk yield, and milk constituents, and on their circadian variation (reviewed above). Moreover, many studies investigated the effect of season-induced change in photoperiod on milk fat composition [e.g.,^{41,42}]. Yet, only a few recent studies investigated a circadian rhythm in milk fat composition^{28,31}. Thus, the novelty in this study is our aim of bridging this gap in existing knowledge. Our results showed that W-LED LAN increased SFA levels by increasing SFA levels in DM. This abolished the circadian variation in SFA and MUFA levels, maintained under a natural LD regime in control cows. This elevation in SFA and the circadian disruption were due to elevations in DM levels of butyric (C4:0), caproic (C6:0), caprylic (C8:0), capric (C10:0), and lauric (C12:0) acids, compared to control cows. The complementary effect for MUFA levels in DM was mainly due to an effect on oleic acid (C18:1) level, which was lower in DM of W-LED exposed cows than in the control cows. In all, these results show that W-LED LAN does not affect milk and milk-fat yields, yet results in a less healthy fatty acid profile, by increasing SFA levels of DM, hence abolishing the circadian pattern of milk-fat composition.

The biochemical mechanisms by which photoperiod, specifically W-LED LAN, affects milk fat composition is yet to be understood. Notably, the main effect on SFA levels was on the less abundant short to mid-chain ($\leq$ C12) fatty acid levels, and not on the most abundant palmitic acid (C-16). This suggests several implications. In contrast to palmitic acid, short to mid-chain fatty acids are mainly de-novo synthesized within the mammary gland rather than partly

transferred from the blood to the mammary gland²⁴. Therefore, the observed LAN-induced change in fatty acid composition may be due to a LAN-induced effect on the within-mammary gland synthesis of short to mid-chain fatty acid. Mammary gland cells obtain an endogenous circadian clock and show a circadian rhythm in about 7% of their transcriptome, including core-clock-related and metabolic genes¹⁶. As these genes are known to be affected by changes in photoperiod¹⁶, we suggest that the LAN-induced changes in fatty acid SFA levels are mainly due to a LAN-induced change in mammary gland clock-controlled de-novo anabolism of short to mid-chain fatty acids. Notably, the oleic acid level in DM, which was also affected by W-LED LAN, originates mainly from feed²⁴ rather than the mammary gland. Accordingly, the source of the difference in the level of oleic acid in milk may be external to the mammary gland. For both oleic acid and short to mid-chain fatty-acids, changes in the mammary circadian clocks are probably mediated by the already shown photoperiod-induced changes in blood melatonin¹⁷ and feeding patterns⁵. These patterns were shown to affect milk levels of both oleic acid and short to mid-chain fatty acids⁴³.

Previous studies in humans and in animal models showed that LAN-induced circadian disruption depends on the illumination spectrum, with short-wavelength illumination ('blue') resulting in the most robust circadian disruption¹¹. In contrast, long-wavelength illumination ('red') has minimal, if any, effect on the endogenous circadian rhythm¹¹. Cow studies also showed that 'blue' illumination results in a more robust circadian disruption than W-LED, 'yellow'-LED, and natural light^{9,20–22}. However, to the best of our knowledge, the lack of long-term effects of R-LED LAN on cow circadian rhythm and performance were not previously studied in a controlled manner. Hence, the novelty of our study is in demonstrating that continuous same-lux level R-LED LAN neither attenuated nor increased milk yield and milk-fat level compared to a natural LD-cycle with ca. 10 to 11 hours light. Moreover, R-LED LAN restored the circadian disruption observed in fatty acid levels under W-LED LAN. This supports a causal relation between W-LED LAN and circadian disruption in cows. This result was expected, considering the data from human and animal models¹¹, yet it was less documented in dairy cows.

Despite the above, cows showed lower protein and lactose levels at NM under R-LED LAN than did control cows held under a natural LD-cycle, as found in the previous phase – under W-LED LAN. Therefore, it is not straightforward to conclude that the differences in these parameters under the mid-phase resulted from W-LED LAN. Certainly, this may have been the mechanism of action, and the difference persisted well into the R-LED LAN period. Alternatively, illumination from R-LED LAN may have decreased protein levels and lactose in NM. With either possibility, R-LED LAN did not show any advantage (nor a disadvantage) over the natural LD-cycle of ca. 10 h. light, regarding milk yield, fat level, and composition; and did not attenuate the cows' body weight.

Conclusion

This study adds another layer of knowledge on milk yield and composition, above the known effects of illumination regimes and wavelength. We showed no commercial advantage in red or white LED LAN compared to the natural LD-cycle during the autumn months, at 32 degrees north latitude, consisting of ca. 10 to 11 h light. Moreover, W-LED LAN showed a significant disadvantage – a circadian metabolic disruption that yielded a higher level of SFA of DM, fatty acids whose increased dietary consumption is suggested as a risk factor for metabolic diseases. Hence, assuming that barn illumination is indispensable due to non-milk production-related reasons (e.g., dairy operation and maintenance of cows' health), the R-LED regime is preferable over W-LED from the perspective of the cow's wellbeing and the milk-fat profile that is most consistent with the natural circadian cycle. This variance in circadian milk-fat composition, which is reflected in a higher nutritional value of DM, highlights DM differentiation from NM, in regard to commercial-health promoting aspects. Our results support the separation of DM from NM to obtain 'premium milk' for health-promoting purposes.

Declarations

Data availability

The dataset used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

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Authors' contributions

RG, AA, UM, and SY designed the experiment; MF and FF acquired the data; MF and RG analyzed and interpreted that data; RG drafted the manuscript; AA, UM, and SY revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The experimental timeline and sampling

Figure 2

The effect of illumination condition (i.e., experimental phase) and milking time on whole milk constituents and milk-fat composition in whole milk. Compared to natural light (control group), W-LED LAN resulted in lower levels of protein (a), lactose (c), and urea (d) during nighttime milking (done at 3:30), and a higher SFA level (g) during daytime milking (done at 13:30). The W-LED LAD-induced effects on protein (a), urea (d), and SFA (g) levels were not detected under R-LED LAN. Neither W-LED nor R-LED LAN affected milk yield (e), SCC (b), and milk-fat (f) levels. The data are presented as mean $\pm$ SE. SCC, somatic cell count; control, n=15; treatment, n=18. *, p < 0.05; #, p < 0.01; \$, p < 0.001, between milking hours, within treatments, by paired T-test. a, p < 0.05; b, p < 0.01; c, p < 0.001, between treatment, within milking hours, by the unpaired T-test.

Figure 3

The effect of illumination condition (i.e., experimental phase) and milking time on milk-fat composition in whole milk. Compared to natural light (control group), W-LED LAN, but not R-LED LAN, resulted in a lower level of MUFAs during daytime milking (done at 13:30). The effect of W-LED LAN on day-milk SFA level (see Fig. 2 g) was due to changes in butyric (c), caproic (d), caprylic (e), and capric (f) acid levels. The effect of W-LED LAN on day-milk MUFA level (a) was due to a change in oleic acid levels (g). Compared to natural light (control group), W-LED LAN, but not R-LED LAN, resulted in a lower level of CLA during daytime milking (done at 13:30) (h). The data are presented as mean ± SE. control, n=15; treatment, n=18. CLA, conjugated linoleic acid. *, $p < 0.05$; #, $p < 0.01$; \$, $p < 0.001$, between milking hours, within treatments, by paired T-test. a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$, between treatment, within milking hours, by the unpaired T-test.

Figure 4

The effect of milking time on whole milk constituents and the milk-fat composition of the treatment and control groups combined during the natural light (i.e., baseline) phase. Night milk, milked at 03:30, showed higher fat level (a) and lower urea level (b) than day milk, milked at 13:30, with no difference in protein (a), lactose (a), and SCC levels (c). Night milk-fat showed higher SFA (d) and lower MUFA (e) and PUFA (f) levels than day milk-fat. The difference in SFA level was due to caprylic, capric, lauric, and myristic acid levels (g and i). The difference in MUFA level was due to myristoleic, palmitoleic, and oleic acid levels (h and i). The difference in PUFA level was due to linoleic and CLA levels (j). The data are presented as mean $\pm$ SE. $n=33$. SCC, somatic cell count; CLA, conjugated linoleic acid. *, $p < 0.05$; #, $p < 0.01$; \$, $p < 0.001$, between milking hours, by the paired T-test.

Supplementary Files

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