Effect of duration of milk accumulation in the udder on milk composition, especially on milk fat globule

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ABSTRACT

Our objective was to study the effect of duration of milk accumulation on milk fat globules (MFG) secretion to better understand relationships between milk yield, milk fat, and MFG secretion. The modification of the milk accumulation duration in the udder is a tool to increase milk fat content. Four milking frequencies were studied on 6 dairy cows averaging 118 ± 22 d in milk: 2 milkings/d separated by 11- and 13-h or by 4- and 20-h intervals and 1 milking/d. The experimental trial was a double Latin square 3×3 with 2-wk periods. Postexperiment, a milking frequency of 36-h was repeated twice. Compared with 2 milkings with 11- and 13-h frequencies, 1 milking/d reduced milk and milk fat yields and increased fat content, without any effect on the size of MFG. Two milkings with 4- and 20-h intervals had no significant effect on milk fat yield and content but tended to increase the size of the MFG. Lipolysis, measured on morning milk, was weaker with 1 milking/d. When data were analyzed according to milk accumulation duration (4, 11, 13, 20, 24, and 36 h), the highest fat content and the largest diameters of MFG were obtained on milks from 4 and 36 h milkings (62.8 g/kg, 4.15 μm and 57.7 g/kg, 4.09 μm , respectively). Such observations could have 2 origins: the richness in residual milk of the 4-h milk and the coalescence of MFG related to the long milk accumulation duration in the 36-h milk. For each duration of milk accumulation, a relationship exists between MFG size and fat yield. The positive relation between MFG size and fat content was confirmed at each duration of milk accumulation. Rate of secretion of milk fat (milk accumulation of 4 h excluded) was also well correlated with MFG size. For the 36-h milk, this relationship was also observed but with a significantly different slope, assuming phenom-

ena of MFG coalescence in response to the supposed increased intramammary pressure or to slower secretion rate and, hence, fusion events of microlipids droplets in the cytoplasm. Duration of milk accumulation joined with large increases in milk fat content induces changes in MFG size.

Key words: milk composition, milk fat, milk accumulation duration, milk fat globule

INTRODUCTION

Milk from cows is composed of about 4% of fat. Milk fat structure and composition are particularly complex, with not less than 7 different kinds of lipids (neutral glycerides, phospholipids, sphingomyelin, cerebrosids, gangliosids, cholesterol, carotenoids, and vitamin A). Milk fat is organized as milk fat globules (MFG), which are droplets of triglycerides surrounded and stabilized in the aqueous phase by a membrane. The latter is derived from the plasma membrane and part of the mammary epithelial cells content. Whereas numerous studies have looked at the effect of farm management practices on fatty acid synthesis (Chilliard et al., 2000, 2001), very few papers focused on the effect of farm management factors on MFG size and structure (Logan et al., 2014; Lopez et al., 2014). Likewise, whereas the consequences of a change in milk fatty acid profile on technologic, sensory, and nutritional properties of milk fat are well known (CLA or *trans* fatty acids for instance; Chilliard et al., 2001), the understanding of the influence of MFG size on those properties are lacking. Most often the effect of MFG size on milk fat properties is studied using technological treatments (centrifugation, gravitational separation, cross-flow microfiltration) to select small and large fat globules from the same mixed or individual milks (Goudédranche et al., 2000; Spitsberg, 2005; Lopez, 2011). Individual characteristics of dairy cows and feeding management can be used to adjust MFG functionalities (Couvreur and Hurtaud, 2007). For instance, selection based on individual variability could be used to modify MFG size

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(Couvreur and Hurtaud, 2007; Logan et al., 2014). The same way, a change in milk fatty acid profile has been reported after a change in feeding management and, more specifically, a change of forage type or a change of the forage-to-concentrate ratio or the addition of fat in the diet (Couvreur et al., 2006, 2007; Hurtaud et al., 2010; Mesilati-Stahy et al., 2015). The effects of feeding management and individual variability on MFG size are additive, with no interaction (Couvreur and Hurtaud, 2007).

Couvreur and Hurtaud (2007) highlighted relationships between MFG size and milk composition variables such as milk proteins, calcium or the activity of fatty acids desaturation by the epithelial mammary cells. Briard et al. (2003), Couvreur and Hurtaud (2007), and Mesilati-Stahy et al. (2015) reported a positive relationship between MFG size and milk fat content. Wiking et al. (2004) showed a relationship between MFG size and only diurnal milk fat synthesis. Regarding these conflicting results, we can conclude that factors determining MFG size are not known. To study those relationships, it is necessary to modify MFG size. Milking frequency appears to be a good tool to do so, especially to modify milk fat content without changing milk fatty acid profile. Indeed, Rémond et al. (2009) demonstrated that atypical milking frequencies increased milk fat content from 30 to 65 g/kg. With milk fat content varying from 37.1 to 43.3 g/kg with milking frequencies, Delamaire and Guinard-Flament (2006a) found that changes in milk fatty acid composition were minor.

We studied the effect of atypical milking frequencies on milk fat secretion and MFG characteristics. Cows were milked twice a day, with 11 and 13 h between each milking, and once a day to study the changes in milk fat synthesis and secretion. An atypical milking frequency with 2 milkings 4 and 20 h apart was done to study milk fat properties from residual milk. Finally, the effects of a long milk accumulation in the udder were studied with a 36-h interval between milking.

MATERIALS AND METHODS

Animals

This study was conducted at the Méjusseaux experimental farm (UMR 1348 PEGASE, Le Rheu, France), and used 6 mid-lactation dairy cows. At the beginning of the experiment, their lactation stage was 117 ± 22 d. They produced 34.3 ± 3.6 kg/d of milk yield with average milk fat content of 40.0 ± 4.8 g/kg and average milk protein content of 31.8 ± 2.1 g/kg. Their BW was 637 ± 56 kg. Milk fat globule diameter varied between 3.1 and 4.3 μm , with an average of 3.8 ± 0.4 μm .

Treatments

Milking frequencies were used to describe MFG under different milk accumulation durations. Four experimental treatments were applied: 2 milkings per day at 0645 and 1745 h (durations of milk accumulation in the udder: 11 and 13 h; **2M₁₁₋₁₃**); 1 milking per day at 0645 h (duration of milk accumulation in the udder: 24 h; **1M₂₄**); 2 milkings per day at 0645 and 1045 h (durations of milk accumulation in the udder: 20 and 4 h; **2M₄₋₂₀**); and, at the end of the experiment, a milking interval of 36 h (**1M₃₆**) was studied twice for all the dairy cows. Dairy cows were milked on Sunday at 1745 h, on Tuesday at 0645 and 1745 h, and on Thursday at 0645 h.

Experimental Design

The experimental design was a double Latin square design with four 2-wk periods (1 wk of adaptation to treatment and 1 treatment week with measurements) with 3 milking frequencies. Between each period (Saturday and Sunday), cows were milked twice (0645 and 1745 h). For **1M₃₆**, it was a continuous design with 2 repetitions.

Feeding

The composition of the experimental diets is given in Table 1. Diets were formulated to meet energy and protein requirements of cows according to the French Unité Fourragère Lait and protein digested in the small intestine (PDI; INRA, 2007). Energy and protein balances were calculated as the differences between energy and protein supplies and energy and protein needs (INRA, 2007). All diets were isoenergetic (0.32 MJ of NE_L/kg of DM) and isonitrogenous (104 g of protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen-fermented OM per kilogram of DM; INRA, 2007; PDIE). Diets contained 64.7% corn silage, 15% energy concentrate (20% wheat, 20% corn, 20% barley, 15% fine wheat bran, 20% beet pulp, 1% sunflower or rapeseed oil, 3% cane molasses, and 1% salt), 8% formaldehyde-treated soybean meal, 10% dehydrated alfalfa, 100 g of urea, and 300 g of 6% phosphorus, 24% calcium, and 4% magnesium mineral and vitamin mix as a TMR. The dairy cows were fed ad libitum.

Measurements and Sampling Scheme

Feeds were weighed, mechanically distributed twice daily at 0800 and 1700 h, and manually mixed. Volun-

Table 1. Diet composition

Item	Measurement
Corn silage, %	64.7
Energy concentrate, %	15
Formaldehyde treated soybean meal, %	8
Dehydrated alfalfa, %	10
Urea, g	100
Minerals (6% P, 24% Ca, and 4% Mg), g	300
NE _L , MJ/kg of DM	0.32
PDIE, ¹ g/kg of DM	105
PDIN, ² g/kg of DM	236

¹PDIE = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from rumen-fermented OM (INRA, 2007).

²PDIN = protein digested in the small intestine supplied by rumen-undegraded dietary protein and by microbial protein from RDP (INRA, 2007).

tary DMI was recorded daily for each cow throughout the experiment via individual electronic gating. Dry matter content of corn silage was determined (80°C, 48 h) every 3 d to adjust the proportion of corn silage in the diets. Orts were collected and weighed daily before the morning feeding. Orts were assumed to have a similar composition to the offered diet.

Milk yield was measured at each milking. Secretion rates were determined by dividing milk yield by the duration of milk accumulation. Milk protein and fat contents as well as SCC were measured at each milking 3 d/wk the first week and 5 d/wk (Monday, Tuesday, Wednesday, Thursday, and Friday) the second week. Milk fat and protein contents were determined on an infrared dairy analyzer (Milkoscan, Foss Electric, Hillerød, Denmark) using the ISO 9622 method (ISO, 1999). Somatic cell count was measured by Somatocount (Bentley Instruments, Chaska, MN). The free fatty acid content (lipolysis) of milk was measured by the copper soap method (Jellema, 1991) on morning milking 5 d/wk (Monday, Tuesday, Wednesday, Thursday, and Friday) the second week.

During the second week of each experimental period, milk was sampled on Wednesday evening and Thursday morning for 2M₁₁₋₁₃ treatment, on Thursday morning for 2M₄₋₂₀ treatment (0645 and 1045 h), and on Thursday morning for 1M₂₄ treatment (0645 h). Crude protein (Kjeldahl), NPN (precipitation at pH 4.6 with TCA and filtration), noncasein N (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate), casein (CP minus noncasein N), and urea (colorimetric enzymatic reaction) were determined according to the methods described in Hurtaud et al. (1993) and Haque et al. (2015). Total and soluble calcium were analyzed by atomic spectrophotometry absorption on milk and

milk ultrafiltrate, respectively. Average MFG size was measured on 3 replicates from each cow during the second week of each period using a Coulter Multisizer II system (Coulter Electronics Limited, Luton, UK) fitted with a vacuum suction control unit pierced with a 70-μm diameter hole. This control unit measured the energy released by impacting particles as they pass through the hole. This gives a size class-stratified (0.177 μm class) count of MFG flowing through the hole in a given timeframe (20 s) and a size-stratified MFG distribution profile. Milk samples were diluted in an electrolytic solution (Isoton II, Coulter Electronics Limited, Luton, UK). Mean diameter $d_{10} = \Sigma(N_i \times d_i) / \Sigma N_i$, where N_i = number of MFG with diameter d_i ,

$$d_{3,2} = \sum (N_i \times d_i^3) / \sum (N_i \times d_i^2),$$

$$d_{4,3} = \sum (N_i \times d_i^4) / \sum (N_i \times d_i^3),$$

and specific membrane area $S = 6/(\rho \times d_{3,2})$, where ρ is fat density (0.92), were used to compare treatments. Fatty acid composition was determined during the second week of each period on 1 replicate by lipid extraction from a 0.5-mL sample of milk fat according to the method described in Hurtaud et al. (2014).

Statistical Analysis

For the animal performance parameters (DMI, BW, milk, FCM, milk fat content and protein content, and milk fat and protein yields) and milk composition, an ANOVA was carried out using the Proc GLM in SAS software (SAS 9.4, SAS Institute Inc., Cary, NC) according to a Latin square design. Adjusted means were compared with a Student's *t*-test. The 3 variables of the model were the cow factor (5 df), the period factor (3 df), and the milking factor (2M₁₁₋₁₃, 2M₄₋₂₀, and 1M₂₄; 2 df).

As period effect was not significant, data on the duration of milk accumulation in the udder of 36 h were compared with the results obtained in the first experiment with 2M₁₁₋₁₃ treatment for each cow. The explanatory variables were the cow factor and the milking factor treatment (2M₁₁₋₁₃ in average day and 1M₃₆).

As period effect was not significant, we compared the different durations of milk accumulation in the udder (4, 11, 13, 20, 24, and 36 h). An ANOVA was carried out using the Proc GLM in SAS software (SAS 9.4, SAS Institute Inc.). The 2 variables of the model were the cow factor (5 df), and the duration of milk accumulation in the udder (4, 11, 13, 20, 24, and 36 h; 5 df).

RESULTS AND DISCUSSION

Milking Frequency and Milk Production and Composition

Compared with 2M₁₁₋₁₃ treatment, the results of 1M₂₄ and 2M₄₋₂₀ treatments are close to the ones observed in the literature regarding the effect of once-a-day milking on milk production (O'Brien et al., 2002; Rémond and Pomiès, 2005, 2007; Clark et al., 2006; Delamaire and Guinard-Flament, 2006a; Rémond et al., 2009). For milk fat and protein yields, the effect of a 36-h interval between milking was stronger than the one observed with a 24-h interval (Guinard-Flament et al., 2011).

Treatment 1M₂₄. Milk production decreased by 22.3% (6.9 kg) with the 1M₂₄ treatment compared with the 2M₁₁₋₁₃ treatment (Table 2). These results are well known and comparable to what has been previously reported in the literature (Knight and Dewhurst, 1994; Tucker et al., 2009). Compared with the 2M₁₁₋₁₃, milk fat yield decreased with 1M₂₄, but to a lesser extent than milk yield so that milk fat content increased by 2.8 g/kg. These results have been previously reported in the literature, with an increase of 3 g/kg in milk fat content, on average, when cows were milked once a day for a short period of time (Davis et al., 1999; O'Brien et al., 2002; Rémond and Pomiès, 2005, 2007; Clark et al., 2006; Delamaire and Guinard-Flament, 2006a). The 1M₂₄ treatment led to a decrease in protein yield (−220 g/d). This decrease occurred concurrently to the decrease in milk production, resulting in no change in milk protein content. These results were similar to the ones reported by Delamaire and Guinard-Flament (2006a). Once a day milking reduced lipolysis (0.23 vs 0.35 mEq/100 g of milk fat). These results have been previously reported (Abeni et al., 2005; Rémond and Pomiès, 2005).

Treatment 2M₄₋₂₀. Milk production tended to be lower for cows in the 2M₄₋₂₀ treatment compared with cows in the 2M₁₁₋₁₃ treatment (−2.6 kg/d, $P = 0.065$; Table 2). Those results are similar to the ones reported by Rémond et al. (2009) with atypical milking frequencies. Milk composition from cows in the 2M₄₋₂₀ treatment was close to milk composition from cows in the 2M₁₁₋₁₃ treatment; milk fat content, milk fat yield, and milk protein content were not statistically different ($P > 0.05$), in accordance with the results reported by Rémond et al. (2009). However, milk protein yield was smaller for cows in the 2M₄₋₂₀ treatment compared with cows in the 2M₁₁₋₁₃ (−89 and −155 g/d, respectively). These decreases could be due to the negative effect of the 20-h interval between milking on protein synthesis. The reason could also be the opening of mammary tight junctions that occurred after 18 h of milk accumulation (Stelwagen et al., 1997). Lipolysis was lower for milk resulting from morning milking (after an interval of 20 h) for cows in the 2M₄₋₂₀ treatment (−0.06 mEq/100 g of milk fat).

Treatment 1M₃₆. In comparison to treatment 2M₁₁₋₁₃, treatment 1M₃₆ induced a decrease in milk production (−6.2 kg, −20.1%; Table 3). This reduction was similar to the one observed with the 1M₂₄ treatment. Somatic cell count was 5 times higher compared with the 2M₁₁₋₁₃ treatment. These results are well known, as SCC increases when interval between milking exceeds 24 h (Stelwagen et al., 2008). Milk fat yield was higher (+146 g) despite the fact milk production was lower; accordingly, milk fat content was largely increased (+17.9 g/kg). Milk protein yield decreased by 171 g; however, the decrease in protein yield was proportionally smaller than the decrease in milk production, resulting in increased concentration in milk proteins. Consequently, milk protein content increased by 1.4 g/kg.

Table 2. Milk production and composition

Item	Treatment ¹			RMSE ²	Effect
	2M ₁₁₋₁₃	1M ₂₄	2M ₄₋₂₀		
Milk yield, kg/d	31.0 ^a	24.1 ^b	28.4 ^a	2.49	<0.001
Milk yield, kg/h	1.29 ^a	1.01 ^b	1.19 ^{ab}	0.103	<0.001
FCM, kg/d	31.3 ^a	25.3 ^c	28.4 ^b	2.43	0.002
Fat content, g/kg	40.3 ^b	43.1 ^a	41.0 ^b	1.59	0.012
Fat yield, g/d	1,258 ^b	1,043 ^a	1,150 ^{ab}	99.5	0.004
Apparent fat secretion rate, g/h	52.4 ^a	43.5 ^b	48.0 ^{ab}	4.15	0.004
Lipolysis, mEq/100 g of fat	0.35 ^a	0.23 ^c	0.29 ^b	0.038	<0.001
Protein content, g/kg	32.6	33.0	32.8	0.68	0.489
Protein yield, g/d	1,011 ^a	791 ^c	922 ^b	74.97	<0.001

^{a-c}Means within rows with no common superscripts differ ($P < 0.05$).

¹2M₁₁₋₁₃ = 2 milkings at 0645 and 1745 h; 1M₂₄ = one milking at 0645 h; 2M₄₋₂₀ = 2 milkings at 0645 and 1045 h.

²RMSE = root mean square error.

Table 3. Milk production and composition

Item	Treatment ¹		RMSE ²	Effect
	2M ₁₁₋₁₃	1M ₃₆		
Milk yield, kg/d	30.9	24.7	2.92	<0.001
Milk yield, kg/h	1.29	0.69	0.085	<0.001
FCM, kg/d	30.8	30.5	1.48	<0.001
Fat content, g/kg	39.8	57.7	6.86	<0.001
Fat yield, g/d	1,230	1,376	60.1	<0.001
Apparent fat secretion rate, g/h	51.2	38.2	3.49	<0.001
Lipolysis, mEq/100 g of fat	0.34	0.40	0.258	0.859
Protein content, g/kg	32.6	34	0.96	0.007
Protein yield, g/d	1,005	834	90.8	<0.001

¹2M₁₁₋₁₃ = 2 milkings at 0645 and 1745 h; 1M₃₆ = one milking at 0645 h after 36 h of milk storage in the udder.

²RMSE = root mean square error.

Milk Accumulation in the Udder and Milk Production and Composition

Milk yield increased as accumulation duration in the udder increased up to 20 h after milking. Milk yield increased from 5.4 kg after 4 h of milk accumulation, to 23.1 kg after 20 h of milk accumulation, and up to 24.7 kg after 36 h of milk accumulation in the udder (Table 4). Past 20 h of milk accumulation, milk production did not significantly increase. Milk rate secretion was largely reduced with a 36-h interval, from 1.37 to 0.92 kg/h. These results were previously reported by Ayadi et al. (2004), Stelwagen et al. (2008), and Guinard-Flament et al. (2011). The fact that milk production did not increase after 20 h of accumulation results from a reduction in milk rate secretion after this point (Rémond et al., 2009; Table 4). About 16 to 18 h are necessary to fill the alveolar compartment in the udder (Davis et al., 1999).

Lactose yield followed the same trend as milk production and increased up to 20 h after milking (Table

4). Those results have previously been observed by several authors (Ayadi et al., 2004; Delamaire and Guinard-Flament, 2006a; Stelwagen et al., 2008; Guinard-Flament et al., 2011). After 36 h of milk accumulation, milk lactose content decreased by 4.2 g/kg compared with 13 h of milk accumulation. Protein yield followed the same trend and increased up to 20 h of milk accumulation in the udder (Table 4). For 24 and 36 h of accumulation, unlike lactose yield, protein yield continued to increase, in line with previous reports (Ayadi et al., 2004; Delamaire and Guinard-Flament 2006a; Stelwagen et al. 2008). Except for durations of 4 and 36 h of milk accumulation, protein content followed the same trend as lactose content and did not vary with milk accumulation duration in the udder. Milk protein content and milk lactose content after 4 h of milk accumulation in the udder were smaller than the ones observed for other milk accumulation durations. Similar results were previously reported, and changes are perhaps related to residual milk from the preceding 20-h milking interval; the high fat content probably

Table 4. Effect of milk storage duration in the udder (in h) on milk production and composition

Item	Duration of milk storage in the udder, h						RMSE ¹	Effect
	4	11	13	20	24	36		
Milk yield, kg/d	5.4 ^d	13.4 ^c	17.1 ^b	23.1 ^a	23.5 ^a	24.7 ^a	2.63	<0.001
Milk yield, kg/h	1.37 ^a	1.22 ^{ac}	1.34 ^a	1.14 ^{bc}	1.00 ^{bd}	0.92 ^d	0.178	<0.002
FCM, kg/d	7.3 ^e	14.1 ^d	16.3 ^c	21.9 ^b	24.9 ^a	30.6	2.21	<0.001
SCC, ×10 ³ /mL	107.3 ^b	97.1 ^b	24.3 ^b	37.6 ^b	54.8 ^b	241.2 ^a	100.86	<0.001
Fat content, g/kg	62.8 ^a	43.7 ^b	37.1 ^b	36.3 ^b	43.7 ^b	57.7 ^a	7.75	<0.001
Fat yield, g/d	342 ^c	584 ^b	633 ^b	844 ^a	1034 ^a	1376 ^a	111.0	<0.001
Apparent fat secretion rate, g/h	87.8 ^a	54.4 ^b	49.7 ^b	41.7 ^{bc}	43.1 ^c	38.2 ^c	11.43	<0.001
Lipolysis, mEq/100 g of fat	—	—	0.33	0.29	0.25	0.39	0.171	0.530
Protein content, g/kg	30.9 ^c	32.7 ^b	32.7 ^b	33.3 ^{ab}	33.0 ^b	34.0 ^a	0.96	<0.001
Protein yield, g/d	169 ^d	440 ^c	559 ^b	767 ^a	775 ^a	834 ^a	77.5	<0.001
Lactose content, g/kg	48.8 ^b	50.9 ^a	50.7 ^{ab}	50.7 ^{ab}	49.5 ^b	46.5 ^c	1.27	<0.001
Lactose yield, g/d	263 ^d	690 ^c	867 ^b	1171 ^a	1161 ^a	1134 ^a	108.7	<0.001

^{a-e}Means within rows with no common superscripts differ ($P < 0.05$).

¹RMSE = root mean square error.

Table 5. Effect of milk storage duration in the udder on milk protein and calcium contents

Item	Duration of milk storage in the udder, h						RMSE ¹	Effect
	4	11	13	20	24	36		
CP content, g/kg	32.7 ^b	33.6 ^{ab}	33.1 ^b	33.5 ^b	33.3 ^b	34.7 ^a	1.23	0.019
NPN content, g/kg	1.72	1.58	1.60	1.61	1.59	1.63	0.094	0.064
Noncasein N content, g/kg	7.26 ^c	7.26 ^c	7.43 ^{bc}	7.92 ^b	7.95 ^b	8.50 ^a	0.520	<0.001
Casein content, g/kg	25.5	26.4	25.7	25.6	25.3	26.2	1.37	0.568
Soluble protein content, g/kg	5.55 ^c	5.68 ^c	5.84 ^{cd}	6.31 ^{bd}	6.36 ^b	6.88 ^a	0.486	<0.001
Total Ca content, mg/kg	1,266 ^{bc}	1,326 ^a	1,244 ^c	1,293 ^{ab}	1,239 ^{cd}	1,254 ^{cd}	41.2	<0.001
Soluble Ca content, mg/kg	290 ^c	346 ^a	312 ^{bc}	318 ^{bd}	300 ^{cd}	317 ^{bd}	22.5	<0.001
Colloidal Ca content, mg/kg	976 ^a	980 ^a	932 ^b	975 ^{ac}	939 ^{bc}	937 ^b	36.9	0.016
Colloidal Ca-to-casein ratio, mg/g	38.3	37.1	36.3	37.9	36.9	35.8	2.35	0.208
Casein-to-true protein ratio, g/g	0.822 ^a	0.823 ^a	0.814 ^{ac}	0.803 ^{bc}	0.799 ^{bc}	0.790 ^b	0.0183	0.002

^{a-d}Means within rows with no common superscripts differ ($P < 0.05$).

¹RMSE = root mean square error.

diluted the protein and lactose contents (Ayadi et al., 2004; Stelwagen et al., 2008).

Milk accumulation duration in the udder had no effect on CP (except for 36 h accumulation), NPN, and casein content (Table 5). Delamaire and Guinard-Flament (2006b) reported no differences in milk casein content with different accumulation durations in the udder. However, soluble protein content increased from 5.54 g/kg with an interval of 4 h to 8.51 g/kg with an interval of 36 h. This increase may have resulted from the opening of tight junctions (Stelwagen et al., 1997; Delamaire and Guinard-Flament, 2006a), which resulted in the transfer of protein from blood to milk.

Total calcium content, soluble calcium content, and colloidal calcium content changed with milk accumulation duration in the udder. The maximum differences observed between the different milk accumulation durations were 85, 57, and 49 mg/kg for total calcium content, soluble calcium content, and colloidal calcium content, respectively. Soluble calcium content decreased between 11 and 24 h of milk accumulation in the udder; soluble calcium could have passed in the blood when epithelial permeability increased because normally ionized calcium concentration in blood is lower than in milk (around 50 mg/L; Charbonneau et al., 2006).

Milk SCC was high after 4 h of milk accumulation in the udder (107,300 cells/mL), decreased until 13 h of milk accumulation in the udder (24,300 cells/mL), and increased up to 24 h of milk accumulation in the udder (54,800 cells/mL). The highest SCC was observed after 36 h of milk accumulation in the udder (241,200 cells/mL; Table 4). Stelwagen et al. (2008) reported no differences in SCC for different milk accumulation durations in the udder (6, 12, 18, and 24 h). Nielsen et al. (2005) believed that the release of somatic cells could follow the release of milk fat which could explain the higher SCC after a short milk accumulation duration in the udder (4 h for our study). The increase in

milk cells after 36 h is consistent with the fact that 36 h of milk accumulation corresponds to the first hours of involution of udder tissue during depletion, and this phase is characterized by elevated levels of SCC (Oliver and Sordillo, 1989).

Fat and MFG

Milk fat yield also increased with the duration of milk accumulation in the udder (from 342 g after 4 h of milk accumulation to 1,376 g after 36 h of milk accumulation). Milk fat content was significantly higher after 4 and 36 h of milk accumulation duration in the udder. Changes in milk fat content with different milk accumulation durations in the udder could result from 2 factors: (1) the difference between the rate of secretion in milk and milk fat and (2) the residual effect of previous milking for short milk accumulation duration (4 h). Indeed, apparent hourly milk fat secretion rate was initially lower in relation to hourly milk secretion rate, which resulted in the dilution of milk fat and a decrease in milk fat content between 4 and 13 h of milk accumulation in the udder (Table 4). Apparent hourly milk fat secretion rate was stabilized between 13 and 24 h of milk accumulation in the udder. For milk accumulation duration in the udder of 13, 20, and 24 h, apparent hourly milk fat secretion rate was 49.7, 41.7, and 43.1 g/h, respectively, and it decreased at 36 h (38.2 g/h). Concurrently, milk secretion rate decreased, resulting in an increase of 6.6 g/kg for milk fat content between 13 and 24 h and 20.6 g/kg between 13 and 36 h. The high milk fat content after 4 h of milk accumulation in the udder could be linked to milk fat content of residual milk (Ayadi et al., 2004). Indeed, milk fat content from milk collected at the end of milking was high (Dutreuil et al., 2009). At 4 h, the mammary gland can only synthesize a small quantity of milk; consequently, residual milk fat cannot be diluted. These results have

previously been observed by Schmidt (1960), Ayadi et al. (2004), and Stelwagen et al. (2008). Given the quantities of milk produced, no milk synthesis seemed to have occurred for 4 h. But if we remove the amount of milk fat produced after 4 h of milk accumulation, which could correspond to the residual fat, the fat secretion rate was not statistically different between treatments (11 h = 39.0 g/h, 13 h = 35.7 g/h, 20 h = 31.4 g/h, 24 h = 32.8 g/h, and 36 h = 32.2 g/h).

Saturated fatty acid content was lower after a milk accumulation duration of 11 h in the udder, increased significantly ($P < 0.05$) with a 13-h milk accumulation duration in the udder, and decreased after this point. These changes were linked to the simultaneous variation in short-chain fatty acid content ($P = 0.001$; Table 6). Delamaire and Guinard-Flament (2006a) reported a linear decrease in milk C4:0 to C10:0 fatty acids and a linear increase in palmitic acid content between 8 and 24 h of milk accumulation in the udder with no effect on milk C11:0 to C14:0 and C18:0 fatty acids. Observed changes in milk fatty acid composition with the increase of milk accumulation duration in the udder between 13 and 36 h could be due to the decrease in synthesis activity of fatty acids with carbon chain length of less than 16 atoms. This hypothesis is supported by Delamaire and Guinard-Flament (2006b),

who reported a decrease in acetate and BHB mammary uptakes, precursors of milk fatty acid de novo synthesis, when milk accumulation duration in the udder increased. A decrease in expression of genes implicated in milk fat synthesis has also been reported after 24 h of milk accumulation (Boutinaud et al., 2013). The decrease in milk SFA after 11 h of milk accumulation was consistent with results reported by Ferlay et al. (2010), who compared milk from morning milking (13 h) to milk from evening milking (11 h). These changes could be linked to a daylight effect or to a larger consumption of milk fatty acid precursors for other functions during daytime. The interpretation of milk fatty acid composition after 4 h of milk accumulation in the udder is difficult, assuming there was a greater proportion of residual milk in the milk collected after a 4-h interval and that this residual milk was issued from a preceding 20-h milking interval characterized by a different fatty acid composition (Table 6).

Various milk accumulation durations in the udder have significantly affected MFG size. Milk fat globule size decreased linearly between 4 and 20 h of milk accumulation in the udder, did not change between 20 and 24 h of milk accumulation in the udder, and increased after 36 h of milk accumulation in the udder. Differences in MFG size reached 0.74 μm for the MFG

Table 6. Effect of milk storage duration in the udder on milk fatty acid composition and yield and on milk fat globule (MFG) properties

Item	Duration of milk storage in the udder, h						RMSE ¹	Effect
	4	11	13	20	24	36		
SFA								
%	71.1 ^{bc}	70.4 ^c	73.5 ^a	72.6 ^a	71.8 ^{ab}	71.7 ^{ab}	1.10	<0.001
g/d	209 ^e	355 ^d	402 ^d	530 ^c	640 ^b	851 ^a	70.3	<0.001
UFA								
%	27.2 ^a	27.7 ^a	24.9 ^c	25.6 ^{bc}	26.3 ^{ab}	26.7 ^a	1.00	<0.001
g/d	80 ^e	138 ^d	134 ^d	183 ^c	233 ^b	314 ^a	25.6	<0.001
MUFA								
%	23.2 ^{ac}	23.7 ^a	21.1 ^b	21.9 ^d	22.6 ^{cd}	23.0 ^{ac}	0.82	<0.001
g/d	69 ^e	118 ^d	113 ^d	157 ^c	200 ^b	270 ^a	21.5	<0.001
Odd-chain fatty acids								
%	2.33	2.16	2.26	2.16	2.32	2.15	0.261	0.521
g/d	6.8 ^e	11.0 ^d	12.5 ^d	15.8 ^c	20.1 ^b	25.4 ^a	2.43	<0.001
Short-chain fatty acids								
%	16.7 ^{bc}	16.4 ^{bc}	18.1 ^a	16.9 ^b	16.7 ^{bc}	16.1 ^c	0.80	<0.001
g/d	49 ^e	84 ^d	100 ^d	124 ^c	149 ^b	191 ^a	17.8	<0.001
MFG								
d _{1,0} , μm	4.15 ^a	3.88 ^b	3.84 ^{bc}	3.72 ^c	3.81 ^{bc}	4.09 ^a	0.139	<0.001
d _{3,2} , μm	5.56 ^a	5.17 ^b	5.13 ^{bc}	4.94 ^c	5.06 ^{bc}	5.48 ^a	0.195	<0.001
d _{4,3} , μm	6.21 ^a	5.74 ^b	5.67 ^{bc}	5.47 ^c	5.62 ^{bc}	6.08 ^a	0.219	<0.001
Membrane, m ² /g of fat	1.18 ^c	1.27 ^b	1.29 ^{ab}	1.33 ^a	1.30 ^{ab}	1.20 ^c	0.052	<0.001
Membrane, g/milking	4.46 ^e	8.16 ^d	8.81 ^d	12.15 ^c	14.64 ^b	18.00 ^a	1.380	<0.001
Membrane, g/kg of fat	13.0 ^c	14.0 ^b	14.2 ^{ab}	14.6 ^a	14.3 ^{ab}	13.2 ^c	0.57	<0.001
Membrane, g/kg of milk	0.804 ^a	0.608 ^{bd}	0.519 ^c	0.528 ^{cd}	0.620 ^b	0.752 ^a	0.086	<0.001

^{a-e}Means within rows with no common superscripts differ ($P < 0.05$).

¹RMSE = root mean square error.

²Average diameter $d_{3,2} = \sum (N_i \times d_i^3) / \sum (N_i \times d_i^2)$, where N_i = number of MFG in class of diameter d_i .

³Average diameter $d_{4,3} = \sum (N_i \times d_i^4) / \sum (N_i \times d_i^3)$, where N_i = number of MFG in class of diameter d_i .

Figure 1. Relationship between milk fat content and milk fat globule diameter ($d_{4,3}$).

diameter ($d_{4,3}$) between milk collected after 4 and 20 h and $0.61 \mu\text{m}$ for milk collected after 36 and 20 h (Table 6). The effects of milk accumulation duration in the udder on MFG size could be explained by its effect on milk production parameters. Indeed, MFG size was correlated with milk production ($r = -0.35$, $P = 0.026$), milk fat content ($r = 0.74$, $P < 0.001$; Figure 1), milk protein ($r = -0.32$, $P = 0.043$), and milk calcium content ($r = 0.53$, $P < 0.001$). Relationships between MFG size and milk production, milk fat content, and milk protein have previously been established by Wiking et al. (2004) and Couvreur and Hurtaud (2007). Couvreur and Hurtaud (2007) reported lower colloidal calcium content associated with small MFG. A study by Valivullah et al. (1988) in rat and cow mammary cells suggested that the intracellular level of calcium might influence the cytoplasmic lipid droplets fusion process and the MFG diameter. The positive relationship between MFG size and milk fat content could be explained by 2 distinct hypotheses involving different mechanisms of regulation. First, large MFG associated with high milk fat content could result from the merging of MFG with each other (Guinard-Flament et al., 2001). Second, the quantity of MFG membrane secreted each day could be linked to milk production. In this case, the increase in milk production would result in the increase in MFG membrane and, consequently,

in more MFG membrane available for the synthesis of smaller MFG secretion. For a fixed amount of fat secreted, the MFG diameter may be mainly linked to the membrane turnover (Wiking et al., 2004; Couvreur and Hurtaud, 2007; Lopez, 2011).

Milk fat quantity was positively correlated with MFG size, but only intratreatment (milking frequencies, Figure 2), as reported by Wiking et al. (2004). Coefficient of correlation was higher with nocturnal milk fat secretion (13 and 20 h of milk accumulation in the udder).

We studied a new criterion: the rate of secretion per hour of milk fat (except after 4 h of milk accumulation in the udder, which corresponded to residual milk fat and not to milk fat synthesized during 4 h). This criterion seemed to explain the changes in MFG size well (Figure 3). The rate of milk fat secretion per hour was positively correlated with MFG size. Likewise, it could vary among individuals, which would explain the large individual differences in MFG size reported by Couvreur and Hurtaud (2007). It was also modified with milking frequency: lower milk fat secretion rate was observed when cows were milked once a day ($1M_{24}$) compared with twice a day ($2M_{11-13}$; 43.5 vs 52.4 g/h, respectively; Table 2).

Milk fat globule size observed after 36 h of milk accumulation in the udder was surprising (Figure 3). For this long milk accumulation duration, a factor other

Figure 2. Relationship between milk fat yield and milk fat globule diameter ($d_{4,3}$; $\square$ = 11 h, $\blacksquare$ = 13 h, $\circ$ = 4 h, $\bullet$ = 20 h, $\blacktriangle$ = 24 h, $\blacklozenge$ = 36 h).

Figure 3. Relationship between fat yield produced per hour and milk fat globule diameter ($d_{4,3}$; $\times$ = 11, 13, 20, and 24 h, $\blacklozenge$ = 36 h).

than milk fat secretion rate seemed to have modified MFG size. The increase in MFG size could result from a physical factor, which led to the coalescence of MFG with each other. This phenomenon was probably due to the increase in intramammary pressure, indicated by the increased mammary epithelium permeability supported by the increase in soluble proteins and the decrease in lactose content in milk (Delamaire and Guinard-Flament, 2006a). Another hypothesis could be that increased MFG size could be attributed to slower secretion rate and, hence, fusion events of microlipid droplets in the cytoplasm of the secreting epithelial cells.

Milk fat globule size was positively correlated with milk SFA content, milk short-chain fatty acid content, and negatively correlated with unsaturated milk fatty acid content. These results are in agreement with the ones reported by Briard et al. (2003), Michalski et al. (2005), and Mesilati-Stahy et al. (2015), who showed that small MFG contained more C18:1 *cis*-9, C16:1, and CLA *cis*-9,*trans*-11 and less C18:0 and C16:0 than large MFG. Milk with small MFG had higher unsaturated milk fatty acids content and lower short milk fatty acids content, as previously reported by Couvreur and Hurtaud (2007). Differences in MFG could be linked to differences in milk fat synthesis, especially to differences in the activity of desaturation of the udder. Boutinaud et al. (2013) reported a decrease in *SCD* mRNA level with once daily milking.

CONCLUSIONS

Milk fat globule diameter varied in response to variations in milking intervals; it largely increased when milk accumulation duration increased. This increase was joined with large increases in milk fat content. Milk fat globule diameter decreased when milk accumulation duration in the udder increased to 20 h. After this point, the increase in MFG size could result from MFG coalescence or to slower secretion rate, especially after 36 h of milk accumulation in the udder. Milk fat globule size was positively correlated with milk fat within each milking frequency and an individual relationship was preserved and independent from milk production level. Milk fat globule size was also positively correlated with the rate of secretion of milk fat. This relationship seems to be true for all milk accumulation durations in the udder except 36 h, for which multiple factors could have an effect on MFG. Some research will be necessary to explain this variability in the size of MFG. Particularly, some research needs to be done regarding intracellular formation of MFG (especially its membrane) and its evolution in the udder during milk accumulation.

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