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# Effect of increased milking frequency and residual milk removal on milk production and milk fatty acid composition in lactating cows

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It has been well established that milk yield is affected both by milking frequency and due to the removal of residual milk, but the influence of a combination of these factors is unclear. In this study, four mid-lactation cows were used in a 4 × 4 Latin square design to test the hypothesis that the effects of more frequent milking and residual milk removal on milk yield and composition are additive and alter milk fatty acid composition. Treatments comprised two or four times daily milking in combination with (or without) residual milk removal over a 96 h interval preceded by a 2 d pretreatment period and followed by a 8 d washout in each 14 d experimental period. Milk was sampled at each milking for the analysis of gross composition and SCC. Samples of available and residual milk collected on the last milking during each treatment period were collected and submitted for fatty acid composition analysis. Increases in milking frequency and residual milk removal alone or in combination had no effect on milk yield or on the secretion of lactose and protein in milk. However, residual milk removal during more frequent milking increased milk fat yield. Milking treatments had no major influence on the fatty acid composition of available milk, but resulted in rather small changes in the relative abundance of specific fatty acids, with no evidence that the additive effects of treatments were due to higher utilisation of preformed fatty acids relative to fatty acid synthesis de novo. For all treatments, fat composition of available and residual milk was rather similar indicating a highly uniform fatty acid composition of milk fat within the mammary gland.

**Keywords:** milking, milking frequency, fatty acid composition, dairy cow, residual milk, udder emptying

During milking, the fat content of milk steadily increases from approximately 2% at the start to around 8% at the end, whereas protein and lactose concentrations remain relatively constant throughout milking (Ontsouka et al. 2003). Between 10 and 15% of the total milk volume is left in the mammary gland after milking, depending on several factors related to the milking routine (Brandsma, 1978), such as pre-stimulation, feeding (Johansson et al. 1999), and milking operator (Rushen et al. 1999). Residual milk retained in the gland, that only can be removed by administration of exogenous oxytocin (Bruckmaier et al. 1994), has a relatively high fat content of around 13% (Ontsouka et al. 2003). Several studies in cows have shown an effect on milk yield of repeated oxytocin administration with or without removal of residual milk (Adams & Allen, 1952; Sprain et al. 1954; Linzell & Peaker, 1971; Heap et al. 1986; Nostrand et al. 1991; Knight, 1994). However, prolonged oxytocin administration has been reported to have no effect on gross milk composition despite increases in milk yield of between 11 and 12% (Heap et al. 1986; Nostrand et al. 1991). Furthermore, the impact of repeated exposure to exogenous oxytocin on milk fatty acid (FA) composition is not known and possible specific effects of removing the residual milk fat on milk fat secretion have not been investigated.

Total milk yield can also be manipulated by milking frequency. More frequent milking may increase milk yield, whereas decreases in milking frequency can lower milk yield (Österman & Bertilsson, 2003; Wall & McFadden, 2012; Stelwagen et al. 2013). The influence of milking frequency on milk yield has been variously attributed to one of several mechanisms, including the action of an as yet unidentified protein Feedback Inhibitor of Lactation (FIL) (Peaker & Wilde, 1996) or serotonin (Hernandez et al. 2007, 2011) in milk. Both FIL and serotonin have been proposed to act *via* a negative feedback mechanism on

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secretory cells, such that their removal from the mammary gland allows further milk synthesis. Effects of milking frequency on milk yield have been reported to occur without major effects on gross milk composition (Amos et al. 1985) but possible effects on milk fat composition by milking frequency have not been evaluated. The relative proportions of saturated and unsaturated FA are known to differ between morning and evening milk (Ferlay et al. 2010), suggesting an association between milking frequency and milk removal on the regulation of milk fat synthesis in the lactating cow.

The present study tested the hypothesis that the effects of increased milking frequency and residual milk removal (RMR) on milk yield and composition are additive and alter milk FA composition mediated *via* changes in the utilisation of FA synthesised de novo and preformed FA for milk fat synthesis in the mammary gland. The aim was to investigate if removal of the residual milk has an effect on milk secretion in the dairy cow.

## Materials and methods

The study was conducted at the Kungsängen Research Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden. All experimental procedures were evaluated and approved by the Uppsala regional animal ethics committee and performed in accordance with EU Directive 2010/63 (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes).

### *Animals, management, and experimental design*

Four multiparous Swedish Red dairy cows of mean (SD) 149 (21) DIM, producing 36·8 (1·8) kg/d of ECM were randomly allocated to a 4 × 4 Latin Square with a 2 × 2 factorial arrangement of treatments. Treatments comprised twice or four times daily milking in combination with or without residual milk removal (2×, 2×RMR, 4× and 4×RMR, respectively). Each 14 d experimental period consisted of a 2 d baseline measurement followed by a 4 d interval when treatments were applied followed by an 8 d washout.

Cows were milked starting at 06·30 and 18·30 (2×) or at 06·30, 12·30, 18·30, and 00·30 (4×) during the 4 d treatment periods. During the baseline measurements and treatment washout periods, cows were milked at 06·30 and 16·00 according to the standard management procedures in the herd. During the wash-out periods, cows were milked using the DeLaval DelPro™ MU480 system (DeLaval International AB, Tumba, Sweden).

For baseline measurements and treatment periods, cows were milked with a custom milking machine (DeLaval International AB, Tumba, Sweden), which allowed milk from each quarter to be collected separately and the removal of teat cups for each udder quarter. Before milking, the teats were cleaned with a wet towel for 60 s, followed by stripping of fore-milk for 30 s. Milking of each individual quarter was stopped when the flow decreased to 0·2–0·3 kg/min. Residual milk was removed by the administration of 1 ml of 10 I.U. oxytocin (Partoxin vet. 17 µg/ml, Pharmaxim Sweden AB, Helsingborg, Sweden) in the jugular vein immediately after milking. Three minutes after the injection, the teat cups were reattached and the residual milk was removed without pre-stimulation of the udder. Milking of residual milk was stopped at milk flow 0·1–0·2 kg/min. Following the last milking for each treatment period, residual milk was collected from all cows.

Experimental cows were housed in individual stalls fitted with rubber mats bedded with chopped straw. Cows were offered silage prepared from mixed grass-clover swards ad libitum and a commercial compound feed. During the course of the experiment, silage fed during experimental periods 1 and 2 (analysed composition; Metabolisable energy (ME) 11·8 MJ/kg DM, in vitro digestible organic matter (IVDOM) of 92·3% in rumen soluble organic matter, 125 g crude protein (CP)/kg DM) was replaced with an alternative grass silage of similar chemical composition (ME, 10·3 MJ/kg DM; 83·4% IVDOM, and 101 g CP/kg DM). Silage was supplemented with a standard concentrate (Solid 120, Lantmännen AB, Sweden) containing 195 g CP/kg DM, 57 g crude fat/kg DM, with a ME 13·4 MJ ME/kg DM and fed according to the Swedish feeding recommendations (Spörndly, 2003) to meet calculated nutrient requirements anticipating a 15% increase in milk yield on all treatments. The ME content of silage and concentrates was calculated based on 96 h in vitro incubations (Åkerlind et al. 2011). CP was determined using a fully automated Kjeldahl procedure (Kjeltec 1030, Tecator, Höganäs, Sweden). During treatment periods, fresh silage was offered four times daily at 06·30, 12·30, 18·30, and 00·30. Concentrates were fed at each milking time at the same time as pre-stimulation started.

### *Measurements and chemical analysis*

Milk yield at each milking was measured by weighing the amount of milk expressed from each udder quarter. Measurements of yield for each quarter at each milking over a 24 h interval were summed to generate daily milk yields. After milking, milk collected from each quarter was gently stirred, sub-sampled, preserved with 10% bronopol, 2-bromo-2-nitropropane-1·3-diol (VWR International AB, Stockholm, Sweden), and stored at 4 °C until submitted for analysis. Representative samples of composite available and residual milk were obtained by mixing milk from all quarters according to yield. Milk fat, true protein, and lactose concentration were analysed by mid-infrared spectroscopy (Fourier Transform Instrument, FT 120, Foss, Hillerød, Denmark) calibrated using samples for which reference measurements had previously been made. Milk somatic cell count (SCC) was determined using fluorescence-based cell counting (Fossomatic 5000, Foss).

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Samples of both available and residual milk obtained during the last milking of each treatment period ($n = 32$) were stored at −20 °C until submitted for the analysis of milk fatty acid composition. Lipid in a 1 ml milk sample was extracted in triplicate using a mixture of ammonia, ethanol, diethylether, and hexane (0·2:1·0:2·5:2·5 vol/vol). Organic extracts were combined, evaporated to dryness at 40 °C under oxygen-free nitrogen, dissolved in hexane, and stored at −80 °C prior to preparation of fatty acid methyl esters (FAME). After thawing at room temperature, lipid was transesterified to FAME by incubation with methyl acetate and methanolic sodium methoxide (Shingfield et al. 2003).

The FAME were analysed with a gas chromatograph (6890N, Agilent Technologies) equipped with a CP-Sil 88 column (100 m × 0·25 mm i.d., 0·2 μm film thickness, Agilent Technologies) and flame ionisation detector using a temperature gradient program (Shingfield et al. 2003). Hydrogen was used as a carrier gas operated at a nominal initial flow rate of 2·1 ml/min and initial pressure 206·8 kPa, held for 50 min, increased at a rate of 34·5 kPa/min to a final pressure 310·3 kPa that was maintained for 7 min. Isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170 °C at a constant pressure (158·6 kPa) and nominal initial flow rate of 0·9 ml/min. Injector and detector temperatures were maintained at 255 °C. Peaks were routinely identified based on retention time comparisons with authentic FAME standards (#463, Nu-Chek Prep Inc., Elysian, MN; L8404 and O5632; Sigma) and cross-referencing with samples for which FA had been identified based on GC-MS analysis of FAME and corresponding 4,4-dimethyloxazoline derivatives (Halmemies-Beauchet-Filleau et al. 2011; Hristov et al. 2011; Kairenius et al. 2015). Milk FA composition was expressed as a weight percentage of total FA using theoretical relative response factors (Halmemies-Beauchet-Filleau et al. 2011).

### *Calculations and statistical analysis*

Available milk was defined as the milk that could be removed following endogenous milk ejection, whereas residual milk was that recovered after the administration of oxytocin. Measurements of daily yields of milk and milk constituents were analysed by ANOVA for repeated measures for a 4 × 4 Latin Square with a 2 × 2 factorial arrangement of treatments using the Mixed procedure of SAS (Version 9·2, SAS institute, Cary, NC) with a model that included the fixed effects of period, sampling day, milking frequency, RMR, and their interaction, a covariate (measured over a 48 h period before treatments were applied), and interactions of the covariate with milking frequency and removal of residual milk, and the random effect of cow assuming an Auto Regressive Order One Covariance Structure. Denominator degrees of freedom were calculated using the Kenward-Rogers method.

Milk FA composition data were analysed by ANOVA for a 4 × 4 Latin Square with a 2 × 2 factorial arrangement of treatments with a model that included the fixed effects of period, milking frequency, removal of residual milk, and their interaction, and the random effect of cow using the Kenward-Rogers correction for denominator degrees of freedom. Differences between the FA composition of available and residual milk were evaluated using a student's t-test. Least squares means ± SEM are reported if not otherwise stated. Treatment effects were declared significant at $P \leq 0{\cdot}05$.

## Results

### *Milk yield and composition*

More frequent milking tended ($P = 0{\cdot}068$) to increase milk yield, whereas residual milk removal had no effect on milk yield (Table 1). There were few effects on the main milk components. Treatments had no effect on milk fat content, lactose content or SCC, while increased milking frequency tended to depress ($P = 0{\cdot}077$) milk protein content (Table 1). Removal of residual milk had no effect on milk yield or milk fat, true protein or lactose concentrations. However, residual milk removal increased milk fat yield in cows milked 4× compared with 2× daily ($P = 0{\cdot}018$ for milking frequency by residual milk removal interaction).

The proportion of residual milk was higher for 4× than 2× milking ($P = 0{\cdot}001$), and the proportion of residual milk increased ($P < 0{\cdot}001$) over the course of the 96 h treatment interval (Fig. 1). On the 2×RMR treatment the proportion of residual milk increased from 19·7 to 31·0%, and from 31·2 to 71·5% on the 4×RMR treatment.

Treatments had no effect on milk SCC that were low throughout the course of the experiment (Table 1).

### *Milk fatty acid composition*

Treatments had no substantive effects on the proportions of total SFA, MUFA, PUFA or *trans* FA in available and residual milk fat, but altered the relative abundance of specific FA for both milk sources. More frequent milking lowered ($P < 0{\cdot}05$) 24:0 and there was numerical but non-significant increase in 18:3n-6, unresolved *trans*-9, *cis*-12, *cis*-15 18:3 and *cis*-9, *cis*-12, *trans*-15 18:3 and *trans*-9 20:1 concentrations in available milk (Table 2). Residual milk removal was associated with significant decreases in 6:0, 8:0, and 20:2n-6 and an increase in 16:0 and total 16 carbon FA in available milk (all $P < 0{\cdot}05$, Table 2). Increases in milking frequency resulted in an increase in milk fat *cis* 16:1 and total 16:1 concentrations in available milk following the removal of residual milk ($P < 0{\cdot}05$ for milking frequency by residual milk removal interactions). Removal of residual milk elevated 18:0 concentrations in available milk during 2× milking, whereas the reverse was true for cows milked 4× daily ($P < 0{\cdot}05$ for milking frequency by residual milk removal interactions). Conversely, residual milk removal

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**Table 1.** Effect of two (2×) or four (4×) times daily milking combined without or with residual milk removal (RMR) on the yield of milk and milk constituents

| | Treatments† | | | | | *P*-values‡ | | |
|---|---|---|---|---|---|---|---|---|
| | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR |
| Milk yield (kg/d) | 36·6 | 38·3 | 38·3 | 40·9 | 0·64 | 0·068 | 0·629 | 0·318 |
| Milk component yields (g/d) | | | | | | | | |
| Fat | 1556 | 1600 | 1603 | 1711 | 33·2 | 0·218 | 0·478 | **0·018** |
| Protein | 1275 | 1338 | 1309 | 1408 | 23·6 | 0·136 | 0·427 | 0·708 |
| Lactose | 1725 | 1811 | 1797 | 1882 | 33·7 | 0·106 | 0·946 | 0·223 |
| Milk component concentrations (%) | | | | | | | | |
| Fat | 4·30 | 4·21 | 4·16 | 4·23 | 0·098 | 0·790 | 0·921 | 0·441 |
| Protein | 3·49 | 3·52 | 3·39 | 3·42 | 0·028 | 0·077 | 0·135 | 0·847 |
| Lactose | 4·68 | 4·72 | 4·65 | 4·58 | 0·017 | 0·100 | 0·278 | 0·670 |
| SCC (log10) | 4·33 | 4·47 | 4·28 | 4·66 | 0·060 | 0·254 | 0·490 | 0·983 |
| SCC antilog (cells/ml) | 21000 | 29 000 | 19 000 | 46 000 | | | | |

†Values represent least square means of measurements made for samples collected from four cows during a 96 h treatment period.
‡Probability of effects due to milking frequency (MF), residual milk removal (RMR) and their interaction (MF × RMR). Bold typeface indicates significant effects ($P \leq 0{\cdot}05$).

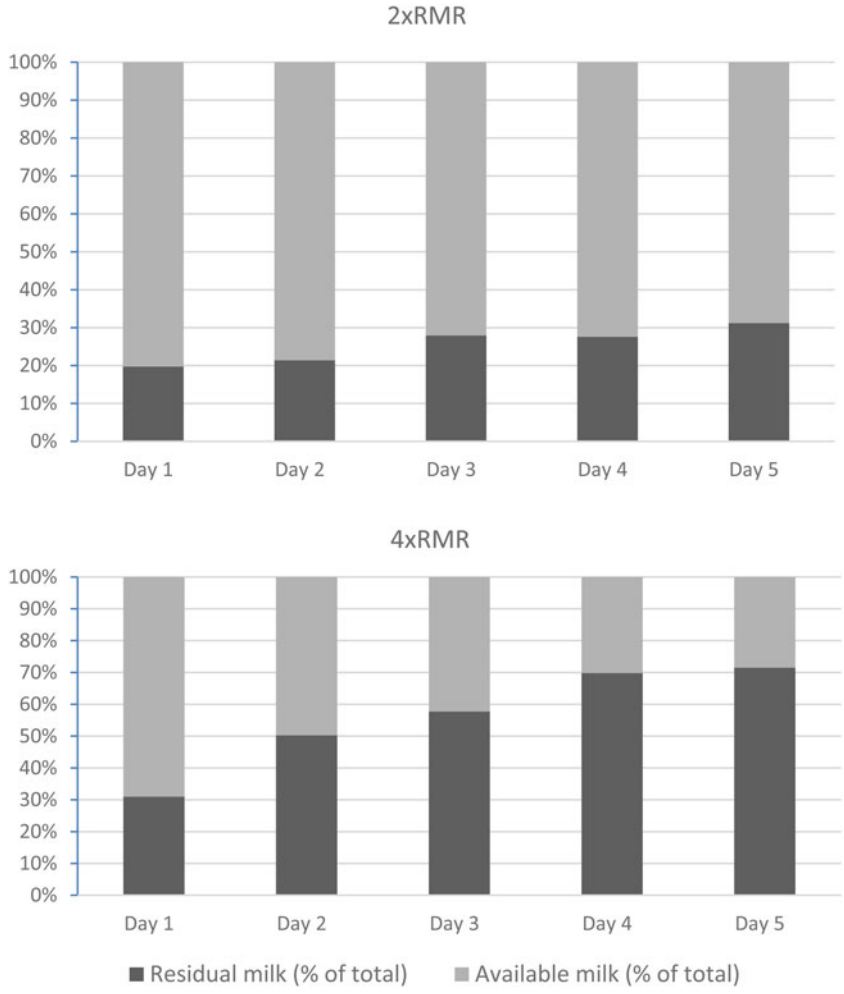


**Fig. 1.** Change in the proportion of available and residual milk (% of total milk) over five consecutive days with residual milk removal (RMR) after each milking in dairy cows milked two (2×) or four (4×) times daily with 12 h and 6 h milking intervals, respectively.

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**Table 2.** Effect of two (2×) or four (4×) times daily milking combined without or with residual milk removal (RMR) on milk fatty acid composition

| | Available milk | | | | | | | | Residual milk | | | | | | | | Available vs. residual | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatments[†] | | | | | *P*-values[‡] | | | Treatments[†] | | | | | *P*-values[‡] | | | *P*-values[§] | | | |
| g/100 g fatty acids | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR | 2× | 2×RMR | 4× | 4×RMR |
| 4:0 | 3·20 | 3·19 | 3·28 | 3·15 | 0·162 | 0·829 | 0·509 | 0·561 | 3·75 | 3·49 | 3·55 | 3·28 | 0·111 | **0·023** | **0·008** | 0·910 | 0·105 | **0·011** | 0·432 | 0·359 |
| 6:0 | 2·21 | 2·12 | 2·22 | 2·16 | 0·076 | 0·303 | **0·018** | 0·597 | 2·40 | 2·25 | 2·36 | 2·24 | 0·072 | 0·490 | **0·003** | 0·741 | 0·228 | **0·003** | 0·349 | 0·345 |
| 8:0 | 1·46 | 1·33 | 1·46 | 1·40 | 0·064 | 0·354 | **0·025** | 0·379 | 1·52 | 1·41 | 1·53 | 1·43 | 0·069 | 0·690 | **0·025** | 0·838 | 0·610 | **0·002** | 0·524 | 0·622 |
| 10:0 | 3·53 | 3·17 | 3·59 | 3·45 | 0·183 | 0·217 | 0·085 | 0·396 | 3·53 | 3·30 | 3·66 | 3·46 | 0·192 | 0·272 | 0·121 | 0·913 | 0·982 | **0·009** | 0·790 | 0·984 |
| 10:1 | 0·37 | 0·32 | 0·34 | 0·36 | 0·028 | 0·847 | 0·237 | 0·061 | 0·39 | 0·34 | 0·36 | 0·35 | 0·031 | 0·597 | 0·057 | 0·335 | 0·754 | **0·000** | 0·714 | 0·494 |
| 12:0 | 4·36 | 3·95 | 4·40 | 4·35 | 0·165 | 0·226 | 0·218 | 0·318 | 4·26 | 3·99 | 4·41 | 4·27 | 0·160 | 0·218 | 0·239 | 0·697 | 0·612 | 0·108 | 0·786 | 0·763 |
| *trans*-9 12:1 | 0·11 | 0·09 | 0·10 | 0·11 | 0·012 | 0·518 | 0·469 | 0·085 | 0·11 | 0·09 | 0·10 | 0·10 | 0·012 | 0·890 | 0·090 | 0·374 | 0·970 | 0·718 | 0·904 | 0·269 |
| *cis*-12:1 | 0·13 | 0·11 | 0·12 | 0·12 | 0·012 | 0·564 | 0·402 | 0·149 | 0·13 | 0·11 | 0·12 | 0·11 | 0·013 | 0·802 | 0·116 | 0·384 | 0·990 | 0·836 | 0·878 | 0·064 |
| 14:0 | 12·66 | 12·60 | 12·79 | 12·90 | 0·225 | 0·229 | 0·873 | 0·601 | 12·26 | 12·48 | 12·62 | 12·84 | 0·247 | **0·029** | 0·133 | 0·977 | 0·465 | 0·075 | 0·452 | 0·821 |
| *cis*-9 14:1 | 1·09 | 1·03 | 1·01 | 1·13 | 0·133 | 0·677 | 0·221 | **0·009** | 1·07 | 1·01 | 1·01 | 1·03 | 0·139 | 0·435 | 0·384 | 0·125 | 0·904 | **0·027** | 0·993 | 0·572 |
| 16:0 | 31·39 | 32·42 | 30·81 | 33·31 | 0·901 | 0·827 | **0·040** | 0·318 | 31·44 | 32·37 | 30·89 | 32·17 | 0·901 | 0·580 | 0·133 | 0·792 | 0·966 | 0·758 | 0·510 | 0·254 |
| Σ *trans* 16:1 | 0·14 | 0·16 | 0·14 | 0·15 | 0·010 | 0·438 | 0·150 | 0·551 | 0·17 | 0·15 | 0·16 | 0·15 | 0·007 | 0·614 | 0·221 | 0·479 | 0·060 | 0·471 | 0·221 | 0·348 |
| Σ *cis* 16:1 | 1·44 | 1·46 | 1·33 | 1·58 | 0·117 | 0·939 | **0·015** | **0·023** | 1·42 | 1·36 | 1·31 | 1·42 | 0·119 | 0·596 | 0·530 | 0·073 | 0·444 | **0·004** | 0·513 | **0·011** |
| Σ 16:1 | 1·58 | 1·62 | 1·46 | 1·72 | 0·119 | 0·902 | **0·011** | **0·034** | 1·58 | 1·51 | 1·47 | 1·57 | 0·119 | 0·551 | 0·687 | 0·067 | 0·969 | **0·019** | 0·961 | **0·021** |
| C18:0 | 8·52 | 9·19 | 9·10 | 8·46 | 0·524 | 0·736 | 0·964 | **0·028** | 8·43 | 9·19 | 8·87 | 9·03 | 0·550 | 0·513 | 0·066 | 0·192 | 0·853 | 0·932 | 0·819 | **0·040** |
| 10-oxo-18:0 | 0·13 | 0·11 | 0·10 | 0·09 | 0·028 | 0·223 | 0·406 | 0·962 | 0·15 | 0·13 | 0·11 | 0·09 | 0·033 | 0·138 | 0·441 | 0·903 | 0·806 | 0·092 | 0·926 | 0·675 |
| 13-oxo-18:0 | 0·018 | 0·018 | 0·011 | 0·015 | 0·0050 | 0·771 | 0·299 | 0·846 | 0·021 | 0·014 | 0·024 | 0·010 | 0·0049 | 0·892 | 0·057 | 0·443 | 0·086 | 0·746 | 0·303 | 0·450 |
| 15-oxo-18:0 | 0·006 | 0·006 | 0·012 | 0·019 | 0·0076 | 0·226 | 0·667 | 0·622 | 0·025 | 0·020 | 0·022 | 0·010 | 0·0050 | 0·192 | 0·093 | 0·474 | 0·194 | **0·027** | 0·227 | 0·415 |
| Σ *trans* 18:1 | 3·68 | 3·64 | 3·83 | 3·45 | 0·254 | 0·864 | 0·191 | 0·266 | 3·48 | 3·46 | 3·68 | 3·71 | 0·210 | 0·063 | 0·939 | 0·805 | 0·565 | 0·080 | 0·672 | 0·599 |
| Σ cis 18:1 | 16·69 | 16·54 | 16·34 | 15·40 | 0·644 | 0·105 | 0·212 | 0·350 | 16·56 | 16·33 | 16·12 | 15·80 | 0·592 | 0·247 | 0·500 | 0·906 | 0·767 | 0·101 | 0·815 | 0·721 |
| Σ 18:1 | 20·37 | 20·19 | 20·17 | 18·85 | 0·875 | 0·181 | 0·188 | 0·306 | 20·04 | 19·80 | 19·80 | 19·51 | 0·748 | 0·547 | 0·546 | 0·960 | 0·599 | **0·033** | 0·772 | 0·679 |
| Σ 18:2[¶] | 2·33 | 2·14 | 2·36 | 2·20 | 0·195 | 0·812 | 0·321 | 0·942 | 2·31 | 2·10 | 2·38 | 2·18 | 0·189 | 0·645 | 0·226 | 0·960 | 0·952 | **0·032** | 0·938 | 0·941 |
| Σ CLA | 0·68 | 0·65 | 0·69 | 0·61 | 0·035 | 0·646 | 0·067 | 0·471 | 0·69 | 0·66 | 0·70 | 0·63 | 0·039 | 0·884 | 0·110 | 0·435 | 0·971 | 0·370 | 0·801 | 0·547 |
| 20:0 | 0·15 | 0·16 | 0·16 | 0·15 | 0·007 | 0·595 | 0·961 | 0·250 | 0·15 | 0·17 | 0·15 | 0·16 | 0·008 | 0·776 | **0·017** | 0·468 | 0·553 | 0·050 | 0·598 | 0·236 |
| *trans*-9 20:1 | 0·0013 | 0·0028 | 0·0035 | 0·0050 | 0·00119 | 0·092 | 0·240 | 1·000 | 0·0035 | 0·0325 | 0·0048 | 0·0005 | 0·00143 | 0·506 | 0·078 | 0·108 | 0·098 | 0·863 | 0·722 | 0·073 |
| *trans*-10 20:1 | 0·0060 | 0·0028 | 0·0073 | 0·0055 | 0·00163 | 0·248 | 0·160 | 0·648 | 0·0063 | 0·0070 | 0·0065 | 0·0048 | 0·00163 | 0·554 | 0·766 | 0·462 | 0·886 | 0·246 | 0·650 | 0·391 |
| *trans*-13 20:1 | 0·012 | 0·007 | 0·011 | 0·011 | 0·0031 | 0·640 | 0·535 | 0·441 | 0·038 | 0·009 | 0·012 | 0·010 | 0·0035 | **0·005** | **0·002** | **0·003** | **0·001** | 0·213 | 0·830 | 0·669 |
| 20:2 n-6 | 0·027 | 0·017 | 0·029 | 0·018 | 0·0025 | 0·474 | **0·003** | 0·917 | 0·030 | 0·021 | 0·027 | 0·019 | 0·0033 | 0·383 | **0·030** | 0·784 | 0·395 | **0·047** | 0·288 | 0·783 |
| 20:3 n-6 | 0·12 | 0·12 | 0·12 | 0·11 | 0·007 | 0·371 | 0·658 | 0·355 | 0·12 | 0·11 | 0·12 | 0·11 | 0·007 | 1·000 | 0·128 | 0·588 | 0·966 | 0·378 | 0·377 | 0·971 |
| 20:4 n-3 | 0·049 | 0·041 | 0·049 | 0·042 | 0·0044 | 0·952 | 0·121 | 0·858 | 0·049 | 0·049 | 0·054 | 0·061 | 0·0094 | 0·378 | 0·738 | 0·682 | 1·000 | 0·534 | 0·542 | 0·313 |
| 20:4 n-6 | 0·071 | 0·075 | 0·075 | 0·075 | 0·0060 | 0·710 | 0·772 | 0·679 | 0·075 | 0·075 | 0·077 | 0·077 | 0·0049 | 0·661 | 0·929 | 0·976 | 0·606 | 1·000 | 0·846 | 0·450 |
| 20:5 n-3 | 0·063 | 0·059 | 0·066 | 0·064 | 0·0038 | 0·355 | 0·455 | 0·753 | 0·072 | 0·063 | 0·069 | 0·068 | 0·0043 | 0·889 | 0·269 | 0·396 | 0·100 | 0·065 | 0·109 | 0·533 |
| *cis*-9 22:1 | 0·0073 | 0·0025 | 0·0083 | 0·0020 | 0·00303 | 0·929 | 0·085 | 0·789 | 0·0108 | 0·0090 | 0·0053 | 0·0025 | 0·00210 | **0·009** | 0·206 | 0·764 | 0·506 | 0·137 | 0·594 | 0·391 |
| 22:2 n-6 | 0·0050 | 0·0033 | 0·0048 | <0·0001 | 0·00150 | 0·264 | 0·062 | 0·332 | 0·0030 | 0·0023 | 0·0023 | 0·0015 | 0·00143 | 0·582 | 0·582 | 1·000 | **0·041** | 0·252 | 0·206 | 0·391 |
| 22:4 n-6 | 0·0093 | 0·0090 | 0·0078 | 0·0093 | 0·00239 | 0·800 | 0·800 | 0·723 | 0·0113 | 0·0075 | 0·0095 | 0·0073 | 0·00210 | 0·604 | 0·152 | 0·696 | 0·572 | 0·182 | 0·518 | 0·500 |
| 22:5 n-3 | 0·099 | 0·109 | 0·102 | 0·104 | 0·0092 | 0·763 | 0·086 | 0·254 | 0·10 | 0·10 | 0·10 | 0·10 | 0·010 | 0·962 | 0·379 | 0·602 | 0·925 | 0·206 | 0·971 | 0·872 |
| 22:6 n-3 | 0·0030 | 0·0023 | 0·0030 | 0·0020 | 0·00092 | 0·839 | 0·189 | 0·839 | 0·0048 | 0·0038 | 0·0050 | 0·0025 | 0·00081 | 0·544 | 0·065 | 0·372 | 0·133 | 0·215 | 0·116 | 0·813 |
| 24:0 | 0·038 | 0·036 | 0·035 | 0·030 | 0·0041 | **0·025** | 0·072 | 0·387 | 0·030 | 0·045 | 0·034 | 0·030 | 0·0068 | 0·443 | 0·443 | 0·188 | 0·537 | 0·459 | 0·943 | 0·964 |
| Σ Unidentified | 0·25 | 0·23 | 0·27 | 0·26 | 0·019 | 0·277 | 0·419 | 0·753 | 0·37 | 0·26 | 0·36 | 0·27 | 0·036 | 1·000 | **0·019** | 0·764 | **0·017** | 0·150 | 0·066 | 0·856 |
| Σ SFA | 71·10 | 71·75 | 71·48 | 72·82 | 1·263 | 0·314 | 0·179 | 0·621 | 71·25 | 72·22 | 71·67 | 72·35 | 1·146 | 0·654 | 0·201 | 0·804 | 0·898 | **0·021** | 0·931 | 0·811 |
| Σ MUFA | 24·09 | 23·75 | 23·63 | 22·69 | 1·086 | 0·185 | 0·257 | 0·571 | 23·79 | 23·27 | 23·30 | 23·07 | 0·972 | 0·442 | 0·417 | 0·750 | 0·760 | **0·025** | 0·856 | 0·839 |
| Σ PUFA | 4·38 | 4·08 | 4·43 | 4·06 | 0·267 | 0·940 | 0·229 | 0·893 | 4·39 | 4·05 | 4·47 | 4·12 | 0·262 | 0·760 | 0·195 | 0·973 | 0·767 | 0·126 | 0·225 | 0·057 |
| Σ Trans FA | 4·89 | 4·80 | 5·03 | 4·59 | 0·301 | 0·861 | 0·190 | 0·365 | 4·74 | 4·61 | 4·93 | 4·86 | 0·255 | 0·166 | 0·489 | 0·862 | 0·679 | 0·053 | 0·818 | 0·647 |
| Σ 4–14 | 31·25 | 30·02 | 31·47 | 30·81 | 0·664 | 0·354 | 0·109 | 0·591 | 31·47 | 30·52 | 31·48 | 31·18 | 0·768 | 0·558 | 0·287 | 0·561 | 0·812 | **0·003** | 0·521 | 0·955 |

**Table 2.** (*Cont.*)

| | Available milk | | | | | | Residual milk | | | | | | Available vs. residual | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatments† | | | | *P*-values‡ | | | Treatments† | | | | *P*-values‡ | | | | *P*-values§ | | |
| | 33·15 | 34·24 | 32·48 | 35·21 | 0·934 | **0·029** | 0·263 | 33·20 | 34·07 | 32·55 | 33·93 | 0·954 | 0·567 | 0·132 | 0·707 | 0·839 | 0·361 | 0·309 | **0·021** |
| Σ 16 | 35·35 | 35·52 | 35·78 | 33·30 | 0·938 | 0·271 | 0·165 | 0·120 | 34·98 | 35·15 | 35·23 | 34·63 | 0·813 | 0·821 | 0·731 | 0·531 | 0·114 | 0·108 | 0·088 | **0·021** |
| >16 | 94·50 | 94·53 | 94·50 | 94·49 | 0·027 | 0·152 | 0·432 | 0·353 | 94·45 | 94·49 | 94·46 | 94·49 | 0·023 | 0·648 | **0·031** | 0·783 | **0·006** | **0·004** | 0·081 | 0·642 |
| Total FA, g/100 g milk fat | | | | | | | | | | | | | | | | | | | | |

CLA, Conjugated Linoleic Acid.
†Values represent least square means of measurements made for samples collected from four cows at the end of a 96 h treatment period.
‡Probability of effects due to milking frequency (MF), residual milk removal (RMR) and their interaction (MF × RMR). Bold typeface indicates significant differences ($P \leq 0{\cdot}05$).
§Probability of differences between available and residual milk collected on the last milking during each treatment period. Bold typeface indicates significant differences ($P \leq 0{\cdot}05$).
¶Total 18:2 excluding isomers of CLA.

decreased the proportion of *cis*-9 14:1 in available milk during 2×, but not 4× milking daily ($P < 0{\cdot}05$ for milking frequency by residual milk removal interactions; Table 2).

Increases in milking frequency lowered the proportions of 4:0 and *cis*-9 22:1, and increased 14:0 concentration in residual milk ($P < 0{\cdot}05$, Table 2). Removal of residual milk had more pronounced effects on the fat composition of residual milk resulting in an increase ($P < 0{\cdot}05$) in the relative abundance of 20:0 and total FA content and a decrease in the relative proportions of 4:0, 6:0, 8:0, unresolved *trans*-9, *cis*-12, *cis*-15 18:3 and *cis*-9, *cis*-12, *trans*-15 18:3, 20:2n-6, and unidentified FA (Table 2). Furthermore, decreases in *trans*-13 20:1 due to residual milk removal were greater ($P < 0{\cdot}05$) during 2× than 4× milking. Relative proportions of certain FA and the FA content of milk fat were found to differ between available and residual milk depending on milking treatment (Table 2). In cows milked 2× daily the relative proportions of *trans*-13 20:1 and *cis*-9 22:1 were higher ($P < 0{\cdot}05$) and that of 14:0 was lower ($P < 0{\cdot}05$) in residual than available milk. Residual milk removal in combination with 2× milking resulted in elevated 4:0, 6:0, 8:0, 10:0, 10:1, 15-oxo-18:0, 20:2n-6, and total SFA concentrations, but decreased relative proportions of *cis*-9 14:1, *cis* 16:1, total 4 to 14 carbon FA, 16:1, 18:1, and MUFA (all $P < 0{\cdot}05$). It also lowered ($P < 0{\cdot}01$) the FA content of milk fat in residual relative to available milk. However, milk fat composition of available and residual milk did not differ during 4× milking. Similarly, residual milk removal in combination with 4× daily milking resulted in relatively few differences in the FA composition of available compared with residual milk, other than decreasing the proportions of *cis* 16:1, total 16:1, and total 16 carbon FA and increasing the relative abundance of 18:0 and total >16 carbon FA ($P < 0{\cdot}05$, Table 2).

Milking treatments resulted in minor, albeit significant changes in the relative abundance of 16 carbon unsaturated FA in available and residual milk (Supplemental Table S1). More frequent milking had no effect on the concentrations of 16:1 or 16:2 isomers in available or residual milk. Removal of residual milk altered the proportions of a few 16:1 and 16:2 isomers, as did the milking frequency by residual milk removal interaction. Irrespective of treatment, few differences in the 16:1 and 16:2 isomer profile of available and residual milk were detected (Supplemental Table S1).

Increases in milking frequency had no effect on 18:1 isomer concentrations in available or residual milk, other than increasing ($P < 0{\cdot}05$) the proportion of *trans*-13-14 18:1 in residual milk (Table 4). Removal of residual milk lowered ($P < 0{\cdot}05$) *trans*-16 18:1 and *cis*-16 18:1 in available milk but had no effect on the abundance of 18:1 isomers in residual milk. Removal of residual milk increased the proportion of *trans*-11 18:1 during 2× milking, but decreased the concentration of *trans*-11 18:1 during 4× milking ($P = 0{\cdot}033$ for milking frequency by residual milk removal interactions). Relative abundance of 18:1 isomers of available and residual milk were similar, other than on the 2× milking treatment (Table 3).

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**Table 3.** Effect of two (2×) or four (4×) times daily milking combined without or with residual milk removal (RMR) on milk C18:1 concentrations

| | Available milk | | | | | | | | Residual milk | | | | | | | | Available *vs.* residual | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatments† | | | | | *P*-values‡ | | | Treatments† | | | | | *P*-values‡ | | | *P*-values§ | | | |
| g/100 g fatty acids | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF*RMR | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR | 2× | 2×RMR | 4× | 4×RMR |
| *trans*-4 18:1 | 0·034 | 0·037 | 0·040 | 0·034 | 0·0058 | 0·733 | 0·733 | 0·377 | 0·033 | 0·032 | 0·036 | 0·035 | 0·0040 | 0·441 | 0·810 | 0·952 | 0·915 | 0·462 | 0·558 | 0·783 |
| *trans*-5 18:1 | 0·030 | 0·034 | 0·037 | 0·030 | 0·0049 | 0·750 | 0·703 | 0·193 | 0·027 | 0·029 | 0·031 | 0·032 | 0·0034 | 0·301 | 0·671 | 1·000 | 0·666 | 0·323 | 0·253 | 0·362 |
| *trans*-6-8 18:1 | 0·33 | 0·32 | 0·32 | 0·31 | 0·034 | 0·407 | 0·426 | 0·685 | 0·33 | 0·30 | 0·33 | 0·34 | 0·033 | 0·285 | 0·618 | 0·170 | 0·953 | **0·042** | 0·925 | 0·638 |
| *trans*-9 18:1 | 0·29 | 0·29 | 0·31 | 0·27 | 0·035 | 0·923 | 0·303 | 0·475 | 0·26 | 0·28 | 0·28 | 0·28 | 0·026 | 0·342 | 0·632 | 0·632 | 0·570 | 0·089 | 0·677 | 0·846 |
| *trans*-10 18:1 | 0·39 | 0·34 | 0·38 | 0·38 | 0·063 | 0·648 | 0·387 | 0·311 | 0·37 | 0·32 | 0·38 | 0·38 | 0·064 | 0·230 | 0·372 | 0·473 | 0·792 | 0·323 | 0·931 | 0·986 |
| *trans*-11 18:1 | 1·02 | 1·08 | 1·11 | 0·96 | 0·083 | 0·687 | 0·279 | **0·033** | 1·02 | 1·07 | 1·09 | 1·04 | 0·094 | 0·463 | 0·955 | 0·079 | 0·978 | 0·324 | 0·803 | 0·213 |
| *trans*-12 18:1 | 0·32 | 0·32 | 0·34 | 0·30 | 0·043 | 0·945 | 0·387 | 0·522 | 0·32 | 0·31 | 0·33 | 0·32 | 0·034 | 0·469 | 0·567 | 0·965 | 0·915 | 0·249 | 0·899 | 0·805 |
| *trans*-13-14 18:1 | 0·58 | 0·58 | 0·60 | 0·53 | 0·047 | 0·669 | 0·384 | 0·332 | 0·45 | 0·47 | 0·51 | 0·60 | 0·042 | **0·019** | 0·121 | 0·272 | 0·249 | **0·037** | 0·365 | 0·341 |
| *trans*-15 18:1 | 0·33 | 0·32 | 0·33 | 0·30 | 0·024 | 0·699 | 0·315 | 0·539 | 0·30 | 0·31 | 0·32 | 0·32 | 0·015 | 0·060 | 0·806 | 0·736 | 0·501 | 0·263 | 0·820 | 0·712 |
| *trans*-16 18:1¶ | 0·35 | 0·32 | 0·36 | 0·32 | 0·014 | 0·552 | **0·020** | 0·442 | 0·37 | 0·35 | 0·37 | 0·36 | 0·013 | 0·598 | 0·419 | 0·729 | 0·481 | **0·033** | 0·866 | 0·357 |
| *cis*-9 18:1 | 15·86 | 15·76 | 15·45 | 14·65 | 0·565 | 0·079 | 0·260 | 0·366 | 15·72 | 15·51 | 15·25 | 14·99 | 0·525 | 0·214 | 0·532 | 0·933 | 0·738 | 0·070 | 0·804 | 0·737 |
| *cis*-11 18:1 | 0·35 | 0·34 | 0·37 | 0·32 | 0·036 | 0·859 | 0·170 | 0·388 | 0·35 | 0·35 | 0·36 | 0·34 | 0·032 | 0·944 | 0·517 | 0·643 | 0·923 | 0·628 | 0·850 | 0·505 |
| *cis*-12 18:1 | 0·22 | 0·18 | 0·23 | 0·19 | 0·039 | 0·691 | 0·143 | 0·781 | 0·22 | 0·20 | 0·23 | 0·20 | 0·036 | 0·760 | 0·412 | 0·824 | 0·990 | **0·016** | 0·967 | 0·666 |
| *cis*-13 18:1 | 0·068 | 0·051 | 0·078 | 0·067 | 0·0107 | 0·081 | 0·069 | 0·701 | 0·075 | 0·087 | 0·074 | 0·081 | 0·0098 | 0·606 | 0·190 | 0·795 | 0·512 | **0·003** | 0·752 | 0·499 |
| *cis*-15 18:1 | 0·13 | 0·14 | 0·14 | 0·12 | 0·011 | 0·590 | 0·954 | 0·133 | 0·13 | 0·12 | 0·13 | 0·13 | 0·009 | 0·880 | 0·496 | 0·741 | 0·683 | 0·184 | 0·809 | 0·605 |
| *cis*-16 18:1 | 0·071 | 0·066 | 0·079 | 0·064 | 0·0047 | 0·540 | **0·041** | 0·263 | 0·070 | 0·065 | 0·073 | 0·065 | 0·0048 | 0·705 | 0·076 | 0·599 | 0·914 | 0·704 | 0·588 | 0·913 |

†Values represent least square means of measurements made for samples collected from four cows at the end of a 96 h treatment period.
‡Probability of effects due to milking frequency (MF), residual milk removal (RMR) and their interaction (MF × RMR). Bold typeface indicates significant differences ($P \leq 0{\cdot}05$)
§Probability of differences between available and residual milk collected on the last milking during each treatment period. Bold typeface indicates significant differences ($P \leq 0{\cdot}05$).
¶Contains cis-14 18:1 as a minor component.

More frequent milking had no effect on 18:2 isomer concentrations in available or residual milk (Table 4). Removal of residual milk decreased ($P < 0{\cdot}05$) the proportions of several 18:2, 18:3 and CLA fatty acids in available and residual milk. However, the 18:2 isomer profile of available and residual milk did not differ (Table 4).

More frequent milking had no effect on the concentrations of odd and branched chain FA (OBCFA) in available and residual milk (Supplemental Table S2). Removal of residual milk resulted in few changes in OBCFA concentrations, as did residual milk removal by milking frequency interactions. For all treatments, the OBCFA composition of available and residual milk was similar, although the concentrations of specific minor FA were found to differ, particularly on the 2×RMR treatment (Supplemental Table S2).

## Discussion

Novel features of the present investigation included the detailed assessment of fat composition of both available and residual milk in response to increases in milking frequency and the removal of residual milk. Milk fat globules consist of high proportions of triacylglycerols (96–98% of total milk lipids) with small amounts of 1,2-diacylyglycerols, monoacylglycerols, (0·02%), free fatty acids (0·22%), phospholipids, and retinol esters (Jensen, 2002). The base catalysed transesterification of milk lipid into fatty acid methyl esters used in the present investigation does not result in the production of allylic methoxy artefacts or isomerisation of CLA isomers but does not methylate free fatty acids or N-acyl lipids including sphingolipids and glycosphingolipids (Kramer et al. 1997). Determination of fatty acid composition did not discriminate between neutral and polar lipids but provided a weighted mean that reflected the composition of triacylglycerols. Residual milk is known to have a higher fat content than cisternal milk (Dill et al. 1974; Ontsouka et al. 2003) and a higher proportion of larger milk fat globules (MFG) (Kernohan & Lepherd, 1969). However, it remains unclear whether the difference in MFG size is due to partitioning of milk fat after secretion or if this is related to inherent changes in the secretion of specific MFG species due to the degree of udder fill, milking frequency, or both. Increases in milk fat synthesis due to more frequent milking in combination with residual milk removal were not accompanied by major alterations in milk fat composition indicating that milking treatments had no influence on the utilisation of fatty acids synthesised de novo relative to preformed fatty acids for triacylglycerol synthesis. Depending on breed, stage of lactation and diet, fatty acid synthesis de novo contributes to proportionately 0·60 on a molar basis or 0·40 by weight total fatty acid secretion in milk (Bauman & Davis, 1974).

Administration of oxytocin or increasing milking frequency from 2× to 4× daily resulted in minor alterations in the relative proportions of specific fatty acids in available and residual milk, with no evidence that the effects of treatment were additive. Even though differences in the composition of milk fat due to treatment were detected, the magnitude of these changes was rather small, suggesting rather limited biological significance. Earlier studies demonstrated that prolonged administration of oxytocin has no influence of neutral lipid composition in available and residual milk in lactating cows (Kernohan et al. 1971), proving further support for the main role of oxytocin in the release of milk from the gland with limited influence on regulating milk fat composition (Dill et al. 1974).

Milk fat composition has been reported to change during the course of milking (Zaks, 1962; Kernohan et al. 1971) and differ between milk collected in morning and afternoon milk in cows milked twice daily (Ferlay et al. 2010). However, increases in the frequency of milking in the present study had relatively small effects on the fatty acid composition of both available and residual milk collected at the end of each treatment period. Increase in MF had no effect on milk yield, which is unexpected based on earlier reports in the literature (Österman & Bertilsson, 2003; Wall & McFadden, 2012; Stelwagen et al. 2013). It is possible that the lack of treatment effects was related to experimental cows being in mid-lactation and therefore less responsive to more frequent milking, since udder fill is not rate limiting for milk secretion after peak lactation. Differences in milk fat composition between milkings have been reported in cows at peak lactation (DIM 61 ± 4 and 76 ± 5; Ferlay et al. 2010), when the effects of milk in the alveoli can be expected to be more influential due to udder fill than is the case for animals used in the present investigation. Further experiments should involve repeated measurements over the course of lactation to determine stage of lactation effects on the response to MF and milk removal treatments. The present study used relatively few cows over a 7 weeks interval such that the inherent variability in milk yield did not allow treatments effects to be determined as significant. It is possible that recruiting a higher number of cows or restricting the evaluation to two treatments and decreasing the length of the study would have improved the sensitivity of the experiment. Nevertheless it was possible to detect an increase in milk fat output to more frequent milking in combination with RMR.

For all treatments the fatty acid composition of available and residual milk was rather similar, which is in agreement with much earlier reports in lactating cows (Kernohan et al. 1971; Dill et al. 1974) and recent observations in lactating ewes (Gómez-Cortés et al. 2011). Nevertheless, the relative proportions of several fatty acids differed between available and residual milk, differences that were more frequent on the 2×RMR treatment. Detailed analysis of milk fat also demonstrated differences in the relative abundance of specific fatty acids between available and residual milk in sheep (Gómez-Cortés et al. 2011). Literature on differences in fatty acid composition between milk fractions in dairy cows is scarce, but in the current study, differences between available and residual milk were found for the treatment 4×RMR. Several of these differences were also

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**Table 4.** Effect of two (2×) or four (4×) times daily milking combined without or with residual milk removal (RMR) on milk C18:2 and C18:3 concentration

| | Available milk | | | | | | | | Residual milk | | | | | | | | Available *vs.* residual | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatments† | | | | | *P*-values‡ | | | Treatments† | | | | | *P*-values‡ | | | *P*-values§ | | | |
| mg/100 g fatty acids | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR | 2× | 2×RMR | 4× | 4×RMR | SEM | MF | RMR | MF × RMR | 2× | 2×RMR | 4× | 4×RMR |
| *trans*-11,*trans*-15 18:2 | 134 | 97 | 132 | 118 | 14·3 | 0·325 | **0·028** | 0·242 | 135 | 127 | 150 | 112 | 15·5 | 0·991 | 0·066 | 0·199 | 0·953 | 0·249 | 0·248 | 0·643 |
| *trans*-9,*trans*-12 18:2 + *cis*-11,*trans*-15 18:2 + *trans*-9, *trans*-14 18:2 | 18 | 24 | 17 | 16 | 5·2 | 0·448 | 0·626 | 0·503 | 18 | 12 | 19 | 13 | 1·8 | 0·534 | **0·012** | 0·944 | 0·783 | 0·333 | 0·485 | 0·154 |
| *cis*-9,*trans*-13 18:2 + 11-cyclohexyl-11:0 | 337 | 319 | 335 | 318 | 29·6 | 0·946 | 0·399 | 0·975 | 330 | 308 | 33 | 321 | 26·8 | 0·651 | 0·368 | 0·817 | 0·809 | **0·011** | 0·996 | 0·893 |
| *cis*-9,*trans*-14 18:2 | 103 | 93 | 103 | 91 | 12·9 | 0·853 | 0·148 | 0·853 | 103 | 88 | 99 | 92 | 11·4 | 0·962 | 0·082 | 0·457 | 1·000 | 0·071 | 0·906 | 0·927 |
| *cis*-9,*trans*-12 18:2 | 37 | 32 | 35 | 31 | 4·5 | 0·639 | 0·189 | 0·753 | 34 | 29 | 36 | 29 | 5·2 | 0·588 | 0·071 | 0·784 | 0·563 | 0·134 | 0·887 | 0·787 |
| *trans*-9,*cis*-12 18:2 + *trans*-8, *cis*12 18:2 | 30 | 28 | 30 | 34 | 3·9 | 0·423 | 0·851 | 0·496 | 30 | 28 | 34 | 29 | 3·6 | 0·475 | 0·321 | 0·621 | 1·000 | 0·927 | 0·581 | 0·092 |
| *trans*-11, *cis*-15 18:2 + *trans*-10, *cis*-15 18:2 | 140 | 160 | 151 | 136 | 15·2 | 0·438 | 0·744 | 0·070 | 141 | 152 | 153 | 138 | 17·5 | 0·955 | 0·751 | 0·086 | 0·979 | 0·339 | 0·879 | 0·852 |
| *cis*-9, *cis*-12 18:2+ *cis*-9, *cis*15-18:2 + *cis*7 19:1 | 1478 | 1330 | 1500 | 1399 | 162·2 | 0·746 | 0·388 | 0·867 | 1473 | 1300 | 1500 | 1396 | 155·5 | 0·633 | 0·305 | 0·790 | 0·982 | **0·025** | 0·998 | 0·977 |
| *trans*-12,*cis*-15-18:2 + *cis*-10 19:1 | 38 | 45 | 40 | 38 | 4·7 | 0·398 | 0·489 | 0·160 | 40 | 44 | 40 | 40 | 6·3 | 0·721 | 0·680 | 0·641 | 0·911 | 0·553 | 0·977 | 0·670 |
| *cis*-12,*cis*-15 18:2 | 15 | 16 | 16 | 16 | 2·3 | 0·873 | 0·873 | 0·790 | 14 | 17 | 15 | 14 | 1·8 | 0·667 | 0·447 | 0·284 | 0·319 | 0·789 | 0·744 | 0·162 |
| *trans*-9,*trans*-11 CLA + *trans*-8,*trans*-10 CLA + *trans*-7,*trans*-9 CLA | 34 | 32 | 32 | 32 | 2·1 | 0·747 | 0·653 | 0·566 | 33 | 34 | 33 | 36 | 1·0 | 0·124 | **0·007** | 0·406 | 0·239 | 0·489 | 0·774 | 0·133 |
| *trans*-11,*trans*-13 CLA | 23 | 19 | 27 | 18 | 2·3 | 0·607 | **0·025** | 0·252 | 23 | 24 | 24 | 20 | 2·3 | 0·400 | 0·594 | 0·255 | 1·000 | 0·092 | 0·266 | 0·473 |
| *trans*-11,*cis*-13 CLA + *trans*-12,*trans*-14 CLA | 63 | 56 | 69 | 53 | 7·1 | 0·747 | 0·056 | 0·424 | 62 | 62 | 70 | 56 | 7·0 | 0·723 | 0·071 | 0·071 | 0·909 | 0·193 | 0·857 | 0·745 |
| *trans*-9,*cis*-11 CLA | 11 | 8 | 9 | 8 | 1·1 | 0·190 | **0·049** | 0·294 | 12 | 10 | 13 | 9 | 1·8 | 0·934 | 0·096 | 0·683 | 0·604 | 0·295 | 0·292 | 0·215 |
| *trans*-10, *cis*-12 CLA | 3·8 | 0·8 | 3·3 | 1·8 | 0·80 | 0·759 | **0·028** | 0·372 | 3·8 | 2·8 | 2·5 | 2·8 | 0·60 | 0·327 | 0·549 | 0·327 | 1·000 | 0·139 | 0·215 | 0·546 |
| *cis*-9,*trans*-11 CLA¶ | 548 | 530 | 551 | 500 | 34·2 | 0·566 | 0·187 | 0·501 | 549 | 524 | 559 | 510 | 35·2 | 0·934 | 0·156 | 0·635 | 0·983 | 0·187 | 0·871 | 0·761 |
| 18:3 n-3 | 820 | 770 | 820 | 730 | 52·4 | 0·698 | 0·207 | 0·698 | 830 | 760 | 820 | 750 | 51·8 | 0·881 | 0·247 | 0·966 | 0·928 | 0·205 | 0·937 | 0·194 |
| 18:3 n-6 | 19 | 18 | 19 | 23 | 1·8 | 0·098 | 0·311 | 0·157 | 20 | 17 | 20 | 22 | 2·0 | 0·082 | 0·691 | 0·110 | 0·641 | 0·861 | 0·683 | 0·664 |
| *trans*-9,*trans*-12,*cis*-15 18:3 + *cis*-9,*cis*-12, *trans*-15 18:3 | 9·3 | 6·5 | 10·3 | 9·0 | 0·92 | 0·083 | 0·055 | 0·407 | 9·5 | 6·5 | 10·3 | 7·8 | 1·14 | 0·402 | **0·048** | 0·829 | 0·861 | 1·000 | 1·000 | 0·342 |
| *cis*-9,*trans*-11,*trans*-15 18:3 | 15 | 17 | 17 | 14 | 1·9 | 0·601 | 0·895 | 0·180 | 14 | 15 | 16 | 16 | 1·0 | 0·140 | 0·522 | 0·348 | 0·699 | 0·153 | 0·689 | 0·201 |
| *cis*-9,*trans*-11,*cis*-15 18:3 | 40 | 37 | 44 | 37 | 6·9 | 0·526 | 0·143 | 0·477 | 41 | 38 | 42 | 42 | 7·4 | 0·498 | 0·548 | 0·657 | 0·940 | 0·444 | 0·845 | 0·384 |

CLA, Conjugated Linoleic Acid.

†Values represent least square means of measurements made for samples collected from four cows at the end of a 96 h treatment period.

‡Probability of effects due to milking frequency (MF), residual milk removal (RMR) and their interaction (MF × RMR). Bold typeface indicates significant differences ($P \leq 0{\cdot}05$).

§Probability of differences between available and residual milk collected on the last milking during each treatment period. Bold typeface indicates significant differences ($P \leq 0{\cdot}05$).

¶Contains trans-7, cis-9 CLA and trans-8, cis-10 CLA as minor components.

reflected in an interaction between residual milk removal and milking frequency. Since available milk accounted for such a small fraction of the total milk on the 4×RMR treatment and similar differences were not found between the milk fractions on other treatments, it is unlikely that this is a reflection of true differences between the milk fractions. Also, the biological significance of these differences remains unclear. Several fatty acids formed during incomplete biohydrogenation of unsaturated fatty acids in the rumen are known to regulate mammary lipogenesis in lactating cows (Harvatine et al. 2009; Shingfield et al. 2010). The lower proportions of *trans*-10, *cis*-12 CLA and *trans*-9, *cis*-11 CLA in available milk following residual milk removal over a 96 h interval is intriguing and may indicate that milk fat composition is potentially influenced by the degree of milk removal, even though RMR in the present study had no major role in the regulation of mammary lipogenesis in the mid lactating cows.

The increase in the proportion of residual milk in response to RMR treatments highlighted a rapid adaptation of the mammary tissue to endogenous oxytocin injection. Much earlier reports highlighted a decline in the lower sensitivity of the mammary gland to the high amounts of oxytocin (Donker et al. 1954), while the decrease over the course of RMR treatment in the present study demonstrated a clear effect over consecutive days. A shift in the distribution of available and residual milk fractions is an important consideration in the design of experiments investigating the effects of RMR on milk production and evaluating differences in the fatty acid composition of available and residual milk. Several studies have reported a residual effect on milk composition during short milking intervals of up to 6 h (Stelwagen et al. 1996, 2008; Dutreuil et al. 2016). The residual effect has been described as the dilution by the high fat content in residual milk, leading to a decrease in lactose and protein content and elevated fat content after a short milking interval. In the present experiment, fat content appeared to be higher on the first two milkings with 6 h intervals (data not presented). It is rather surprising that this effect was also observed on the 4×RMR treatment, since residual milk was removed and could not have caused the changes in milk composition. Such findings indicate that the residual effect reported in literature may also arise due to an increase in milk fat secretion in addition to the effects of residual milk retention in the gland.

## Conclusions

Increases in milking frequency and residual milk removal alone or in combination had no effect on milk yield or on the secretion of lactose and protein in milk. However, residual milk removal during more frequent milking increased milk fat synthesis. Milking treatments had no major influence on the fat composition of available milk, but resulted in rather small, but not systemic changes in the relative abundance of specific fatty acids, with no evidence that the additive effects of treatments were due to higher utilisation of preformed fatty acids relative to fatty acid synthesis de novo. For all treatments, fat composition of available and residual milk was rather similar indicating a highly uniform fatty acid composition of milk fat within the mammary gland.

## Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000681.

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