INTERPRETIVE SUMMARY

The relationship between fatty acid profiles in milk identified by Fourier transform infrared spectroscopy and onset of luteal activity in Norwegian dairy cattle by Martin *et al.*

Dry film Fourier transform infrared technology can be used to successfully identify the composition of milk fatty acids after freezing. Milk fatty acid analyses in the fourth week of lactation can predict whether onset of luteal activity occurred early (≤21 days in milk) or late (>21 days in milk) in Norwegian Red dairy cows. When these data are made available on farm, this study shows their applicability to improve cow management decision making.

12 The Relationship between fatty acid profiles in milk identified by Fourier transform
13 infrared spectroscopy and onset of luteal activity in Norwegian dairy cattle

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ABSTRACT

In order to investigate the feasibility of milk fatty acids as predictors of onset of luteal activity (OLA) 87 lactations taken from 73 healthy Norwegian Red cattle were surveyed over two winter housing seasons. The feasibility of using frozen milk samples for dry film Fourier transform infrared (FTIR) determination of milk samples was also tested. Morning milk samples were collected thrice weekly (Monday, Wednesday, Friday) for the first 10 WIM. These samples had bronopol (2-bromo-2-nitropropane-1,3-diol) added to them before being frozen at -20°C, thawed, and analysed by enzyme linked immunoassay to determine progesterone concentration and the concentrations of the milk fatty acids C4:0, C14:0, C16:0, C18:0, and C18:1cis9 as a proportion of total milk fatty acid content using dry film FTIR, and averaged by WIM. OLA was defined as the first day that milk progesterone concentrations were >3ng/ml for two successive measurements, the study population was categorised as early (n=47) or late (n=40) OLA, using the median value of 21 DIM as the cut off. Further milk samples were collected 6 times weekly, from morning and afternoon milkings, these were pooled by WIM and one proportional sample was analysed fresh for fat, protein and lactose content by the dairy company TINE SA, using traditional FTIR spectrography in the wet phase of milk. Daily energy balance calculations were performed in 42 lactations and averaged by WIM. Animals experiencing late OLA had a more negative energy balance in WIM 1, 3, 4, and 5 with the greatest differences been seen in WIM 3 and 4. A higher proportion of the fatty acids were medium chained, C14:0 and C16:0, in the early than in the late OLA group from WIM 1. In WIM 4, the proportion of total fatty acid content that was C16:0 predicted late OLA with 74% sensitivity and 80% specificity. The long chain proportion of the fatty acids C18:0 and C18:1cis9 were lower in the early than in the late OLA group. Differences were greatest in WIM 4 and 5. Differences in concentrations of C18:1cis9 were seen between the groups from WIM 1. No relationship was seen between OLA and milk

concentrations of either protein or fat, or between OLA and the milk fat:protein ratio. The differences in milk fatty acid proportions between the two groups are most likely related to differences in EB. The study shows that frozen milk samples can be tested for fatty acids by FTIR spectroscopy, and that FTIR spectroscopy of milk can be used to provide real time information about a cow's reproductive function.

KEY WORDS

Dairy cow, reproduction, fatty acid, infrared.

ABBREVIATION KEY

FTIR Fourier transform infrared; **IR** infrared; **OLA** onset of luteal activity; **LCFA** long chain fatty acid; **MCFA** medium chain fatty acid; **MFPR** milk fat:protein ratio; **NEBAL** negative energy balance; **PLSR** partial least squares regression; **SCFA** short chain fatty acid.

INTRODUCTION

Precision dairy farming has increased in recent years, responding to expansion and efficiency demands (Rutten et al., 2013). Today routine sampling and analysis of milk components is performed using traditional Fourier transform infrared (FTIR) spectrography in the wet phase of milk by dairy companies for price calculations, and management purposes. As precision dairy farming continues to move forward measurements will likely be conducted on-line at every milking in many production systems. The FTIR technique can be used to detect the components of milk, e.g. milk fat fraction, lactose, milk proteins, etc. (Soyeurt et al., 2006,

79 Kaylegian et al., 2009, Rutten et al., 2013). However, an FTIR measurement of minor
80 components in milk samples is limited by the low concentrations of the analytes.
81 Consequently an initial drying step prior to FTIR analysis, i.e. performing FTIR analysis on
82 dried milk films, has been used to decrease detection limits and provide more certain
83 predictions of milk components like fatty acids (Afseth et al., 2010). Dry film FTIR has the
84 potential to be automated and included in high throughput industrial environments, for
85 instance in dairy company laboratories or as part of automated milking systems to provide
86 farmers with real time information about their cows' milk fat composition (Afseth et al.,
87 2010). Previously the use of liquid IR spectroscopy of milk has been used to provide real time
88 in-line analysis of milk composition on farm with promising results (Kawasaki et al., 2008).
89 Conducting large scale trials on the effectivity of FTIR under different farming conditions is
90 difficult as long as the milk samples tested have to be fresh, because the transport to, and
91 capacity of, the laboratory increase the cost and logistical demands of such studies
92 considerably. Therefore establishing whether samples can be frozen and the fatty acid fraction
93 conserved adequately is of importance in developing this technology.

94 Dairy cow reproductive performance has declined over the last half century and is considered
95 to be a major bottleneck in the dairy industry (Lucy, 2001). Early detection of subfertile cows
96 could improve both reproductive efficiency, and resource management, by allowing an
97 individual cow to receive targeted nutritional supplementation, treatment, or be managed for
98 an extended lactation (Dobson et al., 2007). Dairy cow reproductive performance can be
99 measured phenotypically, e.g. calving interval, or endocrinologically, e.g. time from calving
100 until onset of ovarian activity (OLA) after calving (Royal et al., 2000). The advantage of the
101 latter measurements is that they are not confounded by on farm management decisions such as
102 voluntary waiting period and oestrus detection methods. Thus OLA provides a clearer
103 illustration of the potential reproductive performance.

104 Precision farming systems have long sought to develop a cow side test to monitor, and
105 predict, dairy cow reproductive performance. This has been partially achieved, and
106 automated, in DeLaval's Herd Navigator system which has the ability to measure
107 progesterone (Herd navigator, DeLaval, Tumba, Sweden). However, the system is expensive
108 and technical challenges exist in reducing the cost of a direct progesterone test. Furthermore,
109 once progesterone is detected OLA has already occurred which limits opportunities to
110 optimise individual cows' treatment and management strategy.

111 Nearly all dairy cows will experience negative energy balance (NEBAL) post-partum
112 (Bauman and Currie, 1980). However, when NEBAL is severe and prolonged subfertility is
113 likely to occur (Butler, 2003). There is an association between NEBAL and reproductive
114 performance (Reksen et al., 2001), and milk composition and NEBAL (Reksen et al., 2002).
115 Early OLA is associated with an increased probability for early AI, shorter interval to
116 pregnancy, and a higher risk of pregnancy (Darwash et al., 1997a, Galvão et al., 2010).

117 Generally, cows suffering from NEBAL have a higher milk fat percentage and a lower milk
118 protein percentage than those in positive energy balance (Grieve et al., 1986, Duffield et al.,
119 1997, de Vries and Veerkamp, 2000). Consequently, milk fat:protein ratios (MFPR) have
120 been used to identify cows at risk of disease and subfertility in the postpartum period (Grieve
121 et al., 1986, Geishauser et al., 1998, Heuer et al., 1999). However, a recent population study
122 found that MFPR were unreliable predictors of fertility (Madouasse et al., 2010a). However,
123 as different milk fatty acids have different origins they are likely to yield more information
124 about the metabolic status of a cow than a generalised milk fat measure (Bauman and
125 Griinari, 2001, Bauman et al., 2006). Therefore they may be more useful in predicting, or
126 diagnosing, subfertile cows. Promising results have been seen using fatty acid profiles to
127 predict subclinical ketosis (Van Haelst et al., 2008). If milk fat composition data can be used
128 to identify cows that are at increased risk of subfertility, or other conditions related to

129 metabolic stress, aberrant results will be able to elicit immediate metaphylactic actions to
130 address the problem at the cow and herd level.

131 The aims of this study were: i) to test the feasibility of performing dry film FTIR on frozen
132 milk samples, and ii) to determine the relationship between OLA and energy balance, MFPR,
133 and milk fatty acid concentration using dry film FTIR.

134

135 ## MATERIALS AND METHODS

136 ### Cows, diets, housing and management

137 Data for this study were collected from 87 lactations (24 first, 15 second, and 48 > second
138 lactations) occurring in 73 Norwegian Red cows over two winter housing seasons between
139 fall 2006 and spring 2008 at the Norwegian University of Life Sciences. The cows were
140 housed in freestalls with rubber mats and concrete slatted floors. Cows were identified
141 electronically using neck collars so that access to feed and recording of experimental data was
142 automated. The cows were fed from automated concentrate feeders and silage bins with
143 vertical feed gates. Grass silage was placed in the bin twice daily and feed remains removed
144 thrice weekly. Feed intake was calculated as the difference between when the gate opened and
145 closed recorded by weigh cells underneath each feed bin (Randby et al., 2012). Concentrates
146 were fed from out of parlour automatic feeders, and the weight of concentrates delivered to
147 each cow was recorded on a daily basis. The cows were milked twice daily, with milking
148 commencing at 06:15 and 15:00. The cows were weighed each time they left the milking
149 parlour.

150

151 ### Milk sampling

Cows were milked twice daily and yields recorded at each milking. Morning milk samples collected on Monday, Wednesday, and Friday from each cow for the first 10 weeks in milk (WIM) were conserved with Bronopol (2-bromo-2-nitropropane-1,3-diol), cooled at 4°C and further stored at -20°C within one hour of collection. These samples were analysed for progesterone by enzyme immunoassay and for milk fatty acid by dry film FTIR. Milk samples from 3 morning and 3 evening milkings from each cow were pooled, based on proportional production, conserved with Bronopol and stored at 4°C. These samples were analysed for fat, protein and lactose with an infrared spectrophotometer (MilkoScan 6000, Foss Electric, Hillerød, Denmark). Energy corrected milk (ECM) yield was calculated from chemical composition of milk (Sjaunja et al., 1991).

Energy Balance

Daily energy balance (EB) calculations were performed on the 42 lactations (26 early OLA and 16 late OLA) that occurred in the first year of the study. Feed sampling and analyses, sheep digestibility trials, and rumen *in sacco* studies are described by (Randby et al., 2010). The contents of ME and NEL in the feed were calculated from feed chemical composition and digestibility values according to (van Es, 1978). Daily EB (MJ of NEL) was calculated as feed energy intake minus energy requirement for maintenance ($0.335 \times BW0.75$), milk yield ($3.036 \times$ kg of ECM + $0.0050321 \times ECM^2$; and for first lactation cows also growth (Ekern, 1991)). Daily EB estimates were averaged per week to provide the EB according to WIM (DIM 0-6 = WIM 1, etc.).

Calibration development and dry film FTIR analysis

175 Dry film FTIR spectroscopy was used both to develop the calibration for major fatty acids in
176 frozen stored milk samples, and for the subsequent prediction of fatty acid content in all
177 frozen and stored milk samples of the study. The fatty acid calibration was developed based
178 on 3 different data sets (subsequently denoted data sets A, B, and C) comprising a total of 422
179 samples, exclusively used for FTIR calibration. These data were partly originating from the
180 87 lactations used in the present study, and partly originating from independent samples. Data
181 set A consisted of 219 fresh milk samples obtained from a feeding experiment (Randby et al.,
182 2012). The 219 samples in data set A represent a subset of samples from the 87 lactations in
183 the present study. All samples were measured with FTIR before freezing, and FTIR
184 calibration of fatty acids based on these samples have been previously described (Afseth et
185 al., 2010). Data set B consisted of 102 samples obtained from the same design as data set A.
186 These samples, however, were stored at -20°C before FTIR analysis. Data set B was thus used
187 to expand the fatty acid calibration to account for physical freezing effects, but the reference
188 analysis was the same as used for data set A. Data set C consisted of 101 milk samples
189 obtained from a study conducted within the EU project "True Food: Traditional United
190 Europe Food" (FOOD-CT-2006-016264). The study, which only included Norwegian Red
191 cows, was performed on commercial dairy farms located throughout Norway in collaboration
192 with TINE dairies. The samples were stored at -20°C before FTIR analysis.

193 Milk samples from the calibration data sets A-C were subjected to reference analysis based on
194 gas chromatography for the comparison with dry film FTIR spectroscopy. For data sets A - B,
195 the reference analysis has been previously described (Afseth et al., 2010). For data set C, all
196 milk samples were lyophilized (ThermovacTM-20, Froilabo, Ozoir-la-Ferriere, France). The
197 fatty acids in lyophilized milk samples were methylated directly using a described protocol
198 (Ferlay et al., 2010). The fatty acid methyl esters (FAME) were injected by auto-sampler into
199 a Trace-GC 2000 series gas chromatograph equipped with a flame ionization detector

200 (Thermo Finnigan, Les Ulis, France). The FAME were separated on a 100 m × 0.25 mm i.d.
201 fused silica capillary column (CP-Sil 88, Chrompack, Middelburg, Netherlands). The injector
202 temperature was maintained at 255 °C and the detector temperature at 260 °C. The initial
203 oven temperature was held at 70 °C for 1 min, ramped to 100 °C at 5 °C min_{-1} (held for 2
204 min), and then to 175 °C at 10 °C min_{-1} (held for 42 min), and 5°C min_{-1} to a final
205 temperature of 225 °C (held for 15 min). The carrier gas was hydrogen; pressure was
206 maintained constant during analysis. Peaks were routinely identified by retention time
207 comparisons with commercial authentic standards containing a mixture of FAME (NCP #463,
208 Nu Check Prep, Elysian, USA; Supelco #37, Supelco, Bellefonte, PA, and O5632, Sigma).

209 The concentration of individual fatty acids was expressed in percent of total fatty acids
210 present in the sample (on a fatty acid methyl ester basis). Five fatty acids, namely C4:0,
211 C14:0, C16:0, C18:0, and C18:1cis9, were used for the calibration. The five fatty acids were
212 chosen as they abundant in milk, but the choice was also based on calibration accuracies
213 obtained. In addition, summed fatty acid parameters were calculated directly from the gas
214 chromatography results: SAT (summed saturated fatty acids) and MUFA (summed
215 monounsaturated fatty acids). All milk samples were analyzed with dry film FTIR the same
216 way. All samples, except the samples of data set A, were thawed in a fridge overnight. The
217 samples were shaken in a vortex mixer (Whirlimixer, Scientific Industries) for 10 seconds.
218 The milk samples were diluted with water (75 % milk, 25 % water) and shaken in a vortex
219 mixer (Whirlimixer, Scientific Industries) for 5 additional seconds. Samples (2.5 μL) were
220 then transferred to sample well plates (silicon, 96 wells) and dried in an exicator with silica
221 gel at room temperature for approximately one hour. Dry film FTIR was performed using a
222 high throughput screening eXTension (HTS-XT) unit coupled to a Tensor 27 spectrometer
223 (both Bruker Optik GmbH, Germany), equipped with a DLaTGS detector. Spectra were
224 recorded in transmission mode in the spectral region from 4000 to 500 cm^{-1} with a resolution

225 of 6 cm^{-1} and an aperture of 5.0 mm. Background spectra of the silicon substrate were
226 collected before each sample measurement to account for variation in water vapor and CO_2.
227 All samples were measured in triplicates.

228

229 **Assay of Milk Progesterone**

230 Progesterone concentrations were determined from whole milk by enzyme immunoassay
231 (Waldmann, 1993), using the second antibody coating technique (Waldmann et al., 1999).
232 The intra-assay coefficients of variation for whole milk at progesterone concentrations of 1.5
233 and 19.7 ng/ml were 9.2 and 5.3%, respectively. The limit of sensitivity, using a 20µl sample,
234 was <0.5 ng/ml.

235

236 **Onset of Luteal Activity**

237 The day of OLA after calving was determined using milk progesterone data. This was defined
238 as the first day that milk progesterone concentrations were >3ng/ml for two successive
239 measurements (Garmo et al., 2009). The median value was calculated for OLA and used to
240 categorise lactations according to early (on or before median day of OLA) or late OLA (after
241 the median day of OLA). In one lactation accurate determination of OLA was not possible, as
242 sampling stopped after 55 DIM, this lactation was omitted from the calculation of descriptive
243 statistics, but included in analyses where OLA was categorised. Consequently, accurate
244 assessment of the time from calving until OLA was possible in 86 lactations (23 first, 15
245 second, and 48 > second lactations) in 72 individual cows.

246

247 **Statistical analyses**

248 *a) Calibration of frozen milk samples for dry film Fourier transform infrared (FTIR)*
249 *determination of fatty acids:* FTIR spectra obtained from the calibration and the prediction
250 data sets were pre-processed identically during data analysis: FTIR spectra were subjected to
251 a standard quality check according to absorbance and noise levels. All spectra were subjected
252 to second derivative by the Savitzky-Golay algorithm (Savitzky, 1964) using a polynomial of
253 degree two and a window size of 9 points in total, followed by normalization by extended
254 multiplicative signal correction (Martens and Stark, 1991). The spectral regions of 600 cm^{-1} –
255 1800 cm^{-1} and 2800 cm^{-1} – 3200 cm^{-1} were selected for subsequent data analysis. Pre-
256 processed spectra of the calibration data sets were used to develop multivariate regression
257 models based on Partial Least-Squares Regression (PLSR) (Wold, 1983). Chemical reference
258 values of fatty acid composition (all fatty acids expressed as percentage by weight of total
259 fatty acid content) were used in the PLSR modeling. The optimal number of PLSR factors of
260 the calibration models was determined using segmented cross-validation using 20 segments.
261 Samples for all segments were chosen in such a way that samples having a biological link, i.e.
262 coming from the same animal on the same DIM and represented in data sets A and B, were
263 clustered in the same segment. The prediction errors of the cross-validated PLSR models,
264 expressed as root mean square error of cross validation, and the correlation coefficients (R^2)
265 were used as the major figures of merit to evaluate the developed calibration equations. The
266 Unscrambler X 10.3 (CAMO PROCESS AS, Oslo, Norway), and Matlab, V. 8.1 (The
267 Mathworks Inc., Massachusetts), was used for all data analysis.

268 *b) Associations between milk yield and components, EB, and OLA*: Data were collected from
269 on farm data recording systems (feed intake weights, milk recording data, laboratory results,
270 and merged into a single Excel file for validation before they were transferred to Stata 12
271 (Stata Corp., College Station, Texas, USA) for statistical analysis. Statistical significance was

272 determined by a *P*-value <0.05. Statistical analyses were performed at lactation level. Initial
273 descriptive and distributive analyses were performed in STATA. Subsequently the lactations
274 were categorised according to time to OLA with the description early OLA and late OLA.
275 The Student's T test was used to determine whether differences existed between EB and the
276 milk characteristics (ECM yield, milk protein, milk fat, milk fat:protein, C4:0, C14:0, C16:0,
277 C18:0, C18:1cis 9) of animals with early versus late OLA. Logistic regression with the
278 categorized outcome was used to determine the sensitivity and specificity of each of the
279 following milk parameters individually as a predictor for late OLA. The dichotomous
280 statistical analyses involving OLA were based on all 87 lactations, with the exception of
281 calculations involving EB which were based on 42 lactations. In this paper the study unit is
282 lactation, 14 cows have contributed two lactations to the 87 lactations available to study. The
283 lack of independence between these lactations has not been corrected for in the statistical
284 analysis.

285

286 **RESULTS**

287 **Onset of luteal activity**

288 The mean, standard deviation and median OLA for the lactations were calculated to be 24.9,
289 13.4, and 21 days, respectively. The range of OLA seen in this study was between 7 and 84
290 DIM. When the lactations were categorised 47 lactations had early OLA and 40 late OLA.

291

292 **Energy balance**

293 Cows which experienced late OLA had, on average, deeper and more prolonged NEBAL than
294 animals which experienced early OLA (Table 1, Figure 1). The largest difference between the

two groups was in the third week of lactation. In both groups the trend in EB was positive from the first WIM. However, on average animals with early OLA entered positive EB before those in the late OLA group. The change between positive and negative EB occurred in the third and sixth WIM for the early OLA and late OLA groups, respectively.

FTIR calibration development

The FTIR calibration was developed based on 3 different data sets, comprising a total of 422 samples. The data sets B and C were added in order to take into account physical variation related to freezing of milk. The calibration results from frozen samples compared with gas chromatography from fresh samples are provided in Table 2. The table shows that calibration models of unsaturated fatty acids features are generally modelled better than saturated fatty acid features. This is in accordance with previous results (Afseth et al., 2010). In addition, the correlation coefficients are generally lower and the estimation errors are higher than as previously shown for corresponding measurements of fresh milk samples (Afseth et al., 2010). However, the estimation errors as provided in the table shows that these models are undoubtedly feasible for screening of fatty acid features as performed in the present study.

Temporal pattern of milk characteristics

When the milk fat fractions as determined by dry film FTIR were analysed no relationship was seen between the proportion of milk fatty acids that were short chained (C4:0) and the dichotomous variable OLA. A relationship was seen between proportion of the MCFA C14:0 and C16:0 and the dichotomous variable OLA. Animals with early OLA had higher concentrations of MCFA in the milk fat fraction than animals with late OLA. This

318 relationship was seen in each of the first seven WIM for both C14:0 and C16:0, and between
319 the seventh and tenth WIM for C14:0 (Table 3). The difference between the concentrations of
320 these two MCFA between the two groups of cows is greatest in the first month after calving.
321 A statistically significant relationship was seen in the first 6 WIM whereby the early OLA
322 group had lower proportion of the long chain fatty acid (LCFA) C18:1cis9 (oleic acid) in the
323 milk fatty acid fraction than the late OLA group. Statistically significant differences were only
324 detected between the LCFA C18:0 and the dichotomous variable OLA in WIM 4, although
325 the early group had numerically lower concentrations of the fatty acid in the fatty acid fraction
326 throughout the study period

327 The variability in the diagnostic properties of a test system using milk fatty acids to predict
328 early or late OLA varied between the individual fatty acids. Table 4 shows the sensitivity,
329 specificity, positive predictive value and negative predictive values for the three fatty acids
330 shown to have a significant relationship with OLA; C14:0, C16:0 and C18:1cis9, in the
331 second third and fourth WIM. The table shows that whilst the sensitivity varies between the
332 different variables, between 35 and 74%, the specificity ranges from 74 - 85%. Of the three
333 fatty acids tested C16:0 in WIM 4 gave the most accurate predictions; sensitivity, specificity,
334 positive predictive value and negative predictive value of 74%, 80%, 76% and 78%,
335 respectively.

336 Cows with late OLA produced more ECM than those with early OLA in the third and fourth
337 weeks of lactation. Outside of these weeks no statistically significant relationship existed
338 between ECM yield and OLA (Table 3). No statistically significant relationship was seen
339 between milk protein, milk fat or milk fat: protein ratio and the dichotomous variable OLA in
340 the first ten weeks of lactation with one exception. In WIM 8 cows with late OLA had
341 statistically lower milk fat concentration that those with early OLA. There was a numerical

342 trend for animals with early OLA to have higher concentrations of both milk fat and milk
343 protein than those with late OLA (Table 3).

344

345 **DISCUSSION**

346 This study shows that in a population of moderate yielding Norwegian Red cattle it is possible
347 to use dry film FTIR technology to accurately analyse the milk fat fraction after milk samples
348 have been stored frozen. This is of great importance for upcoming studies on relationships
349 among milk components, metabolic status and reproduction in dairy cattle. The proportion of
350 a single fatty acid in the milk fat fraction, C16:0, in the fourth WIM measured in this way can
351 be used to determine, with 74% sensitivity and 80% specificity whether OLA occurred after
352 21 DIM. For this specific fatty acid fraction, four times out of five, a cow identified as been at
353 risk of having late OLA would go on to have it. Also the predictive properties of other fatty
354 acids were high and motivate further investigations of relationships between metabolic
355 function and single milk components. Further studies of such relationships in cows exposed to
356 different feed rations, and the exploration of amino acids in relation to OLA will be aims for
357 further studies. Furthermore, the possibility of developing an extended model for the
358 prediction of metabolic function and OLA after correction for milk yield, age, feed intake and
359 other relevant covariates will be explored in a planned experiment, as it probably offers a
360 cheap and effective tool to help steer herd reproductive performance.

361 Fatty acids enter milk in one of two ways: they can be manufactured in the mammary gland
362 *de novo*, or they can be absorbed from circulating blood (Bauman et al., 2006). The fatty acids
363 which are absorbed from blood can originate directly from the diet, be formed in the rumen by
364 biohydration or degeneration, or can be released from body fat stores (MacGibbon, 2006).

365 Previous work has shown that milk fat composition alters according to the cow's metabolic
366 state, which has been used to identify cows with subclinical ketosis (Van Haelst et al., 2008).

367 In this study consistent relationships were seen between higher concentrations of the MCFA
368 measured and early OLA. The difference between the milk MCFA content between the early
369 and late OLA groups peaked in the third and fourth WIM, the same time that the EB
370 difference in the two groups was greatest. Broadly speaking MCFA (those containing 10-16
371 carbon atoms) are synthesised almost exclusively *de novo* (Bauman et al., 2006).
372 Consequently animals with more NEBAL reduce *de novo* milk fat synthesis to conserve
373 energy, thus having lower MCFA concentrations and late OLA.

374 Conversely, LCFA which contain more than 16 carbon atoms, are almost exclusively
375 absorbed into milk from circulating blood (Bauman et al., 2006). Cows in NEBAL mobilize
376 fat reserves which increase blood and milk concentrations of LCFA (Stoop et al., 2009). This
377 explains why the LCFA measured in this study, C18:0 (stearic acid) and C18:1cis9 (oleic
378 acid) were consistently higher in the group of cows experiencing late OLA. The difference
379 peaked in the fourth and fifth WIM and was apparent for oleic acid from the first WIM.
380 Whilst clear numerical trends were seen throughout the early lactation period for both stearic
381 and oleic acid a statistically relationship was present for many more weeks for oleic acid.
382 Ruminant adipose tissues are very rich in C18:0 and C18:1 fatty acids, and the transition from
383 pregnancy to lactation is accompanied by increases in the concentration of C18:0 fatty acid
384 and decreases in C18:1 fatty acids in adipose tissue (Smith, 2009). Studies on plasma and
385 hepatic lipid fatty acid composition led to the hypothesis of a preferential release of oleic acid
386 from the adipose tissue when ruminants experience a negative energy balance (Chilliard,
387 1977) which likely explains the relative importance of C18:1cis9 over C18:0 in this study.

388 Over the first ten WIM the overall milk protein and fat content of animals in this study
389 reduced, as is expected and well described (Fox, 1998). However, the temporal pattern of
390 specific milk fatty acid concentrations varied. The concentration of milk LCFA included in
391 this study fell over the ten week period. These are derived either from nutritional intakes or
392 from body fat breakdown and are almost exclusively absorbed from circulating blood
393 (Bauman et al., 2006). Consequently if the diet is relatively stable and EB is becoming more
394 positive mobilization of body fat reserves reduces, resulting in less LCFA in blood and milk.
395 Milk concentrations of MCFA increased in the first 10 WIM of this study, as has been
396 previously described in another study (Karijord et al., 1982). The MCFA are primarily
397 synthesised *de novo* in the mammary gland and their increasing concentration in milk is
398 associated with an improved EB (Garnsworthy et al., 2006, van Knegsel et al., 2007). The
399 positive correlation between MCFA and EB and negative correlation between LCFA and EB
400 seen in this study was described in a recent review of the literature (Moate et al., 2007) and
401 can partially explain why MFPR are unreliable predictors of fertility. As one fat fraction
402 decreases in line with falling EB, the other fat fraction increases meaning that overall milk fat
403 concentrations do not necessarily alter. Furthermore milk fat content is influenced by a wide
404 array of factors including breed, genetic variation within breed, parity, lactation stage, season,
405 disease, EB, rumen function, feeding management, and nutrition which complicates a
406 simplistic ratio. This study could not verify the reported negative linear relationship between
407 milk protein and EB (Coulon and Remond, 1991).

408 In this study no relationship between MFPR and OLA was found. Previous studies have
409 shown that OLA is closely related to the reproductive performance of individual cows at the
410 lactation level and that it is a more accurate predictor of potential reproductive performance
411 than traditional measure of fertility (Royal et al., 2000). This supports the findings of
412 population based studies which found MFPR to be poor lactation level predictors for

413 reproductive performance (Madouasse et al., 2010a, Madouasse et al., 2010b). However, it
414 disagrees with a number of smaller studies which found MFPR to be linked with reproductive
415 performance. In one study noted that first insemination pregnancy rates were 29% and 49%
416 lower in animals with high-normal and abnormally high butterfat percentages respectively
417 when compared with their normal counterparts (Kristula and Uhlinger, 1995). A further study
418 found negative effects on fertility in animals with a first test day MFPR >1.5 (Heuer et al.,
419 1999). Similarly, MFPR were found to be a useful predictor of whether cows were likely to
420 exceed certain calving to conception intervals (Podpecan et al., 2008). Delayed return to
421 cyclicity, reduced pregnancy rates and decreased risk of pregnancy in animals with high
422 MFPR ratios have also been shown (Loeffler et al., 1999, Gabor et al., 2008, Samarutel et al.,
423 2008).

424 The animals in this study had a moderate ECM yield (averaging 2008L for the first 70 DIM).
425 The effects of milk yield on OLA are difficult to determine in isolation, as high milk yields
426 often result in larger NEBAL with subsequent delayed OLA (Petersson et al., 2006). This
427 finding is supported by this study which showed that animals with early OLA had lower ECM
428 yields in the third and fourth WIM than those with later OLA. Although other studies have
429 shown that milk yield *per se* has no effect on OLA (Darwash et al., 1997b, Lopez et al., 2005,
430 Pedernera et al., 2008).

431 Norwegian Red cattle generally have earlier OLA than other dairy breeds (Royal et al., 2000,
432 Petersson et al., 2006, Garmo et al., 2009). It is therefore surprising that such clear differences
433 in OLA can be seen between fatty acid profiles in this study. Furthermore the distribution of
434 OLA is more consistent in the Norwegian Red than in the Holstein breed, which has a higher
435 proportion of cows experiencing both an early OLA and a very late OLA (Friggens et al.,
436 2010). The bimodal distribution of OLA in Holstein cows may enhance the differences seen
437 in milk fatty acid profiles and OLA when compared to the study population, although this

438 requires further research. In this study the outcome variable has been dichotomized, as either
439 early or late OLA, due to the size and distribution of the study material. The functionality of
440 the information would be improved if more than two outcomes were accounted for, and this
441 requires further study.

442 Technically this study was challenging to perform as the milk samples were frozen prior to
443 analysis. Alterations in the physical stability of the milk, e.g. emulsion properties or protein
444 denaturation, may occur upon thawing, affecting the physical properties of the milk. Thus, in
445 this study, a calibration for prediction of fatty acid features using FTIR was established,
446 taking into account the possible physical changes in frozen stored samples. The standard
447 liquid FTIR analysis, which is performed in narrow capillary cells of sizes in the 10-50 μm
448 range, can also easily be clogged by particles in the liquids, like frequently encountered in the
449 analysis of frozen stored milk samples. The dry film FTIR technique is not affected in the
450 same way by particulate liquids, and in the present study, a calibration for fatty acid
451 composition taking into account physical changes in milk related to freeze-storage was
452 developed. Table 2 shows the calibration results are not as accurate as obtained by fresh milk
453 only (Afseth et al., 2010), but the estimation errors are still feasible for screening of fatty acid
454 features as performed in the present study. This opens the opportunity for rapid progression in
455 this field as historically frozen samples can be analysed and compared with complete datasets
456 rather than waiting for new field trials to be performed. It also allows for samples to be
457 gathered from farms in different areas to build a robust understanding of how milk fatty acid
458 composition may be used in precision farming.

459 The potential practical application of this work in the future is the incorporation of dry film
460 FTIR analysis into automated milking systems to provide the producer with real time
461 information about individual cows and herd performance. This study has focused on a link
462 between milk metabolites and OLA, which is most likely, explained by NEBAL. Precision

463 farming tools such as this are most likely to be applicable to high input, intensive farming
464 systems and in this context individual dietary changes can be difficult to initiate, as cows dry
465 matter intakes are often maximised. However, the knowledge that an individual cow is more
466 likely to be subfertile would enable an active management decision to be made to place her in
467 a group, or on a programme, whereby her lactation length was voluntarily, and deliberately
468 extended, thus avoid many of the costs associated with failed 365 day calving intervals
469 (Dobson et al., 2007). Cow level management has been recently shown to be economically
470 beneficial to dairy farmers (Inchaisri et al., 2011). Changes in milk fat composition can also
471 signal signs of metabolic stress before they are detectable by other means, e.g. blood BHBA
472 measurements, this will allow early metaphylactic changes to be made to herd rations or
473 management before clinical disease is seen (Van Haelst et al., 2008). Currently many dairy
474 herds already engage in regular, systematic sampling of blood and milk metabolites and use
475 this information to make management changes at the herd level (Caraviello et al., 2006), and
476 on line FTIR fatty acid concentration analysis could replace this labour intensive, relatively
477 expensive exercise by allowing herd management decisions to be made on the basis of real
478 time data.

479

480 **CONCLUSIONS**

481 This study shows that OLA is related to the proportion of some specific fatty acids found in
482 the milk fat fraction. These relationships can be seen as early as the first WIM. When OLA is
483 dichotomised as early or late univariate analysis indicated that prediction accuracy was
484 highest in the fourth WIM. The study also shows that dry film FTIR performed on previously
485 frozen milk samples can determine milk fatty acid composition.

486

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643

644 **Table 1.** Mean daily energy balance (MJ NEL) by weeks in milk grouped by early or late

645 onset of luteal activity.

646

Onset of Luteal Activity	Weeks in milk									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Early	-34.8	-7.1	7.5	9.4	10.6	12.9	12.4	12.9	14.9	14.3
Late	-48.8*	-17.2	-11.6**	-3.9*	-1.3*	3.5	7.0	8.4	12.5	11.8

647 ttest performed comparing late and early groups *denotes $0.01 \geq p < 0.05$, **denotes $0.001 \geq p < 0.01$

648

649 **Table 2. Statistics and FTIR calibration results for prediction of fatty acids in frozen**
650 **milk samples compared with gas chromatography.***

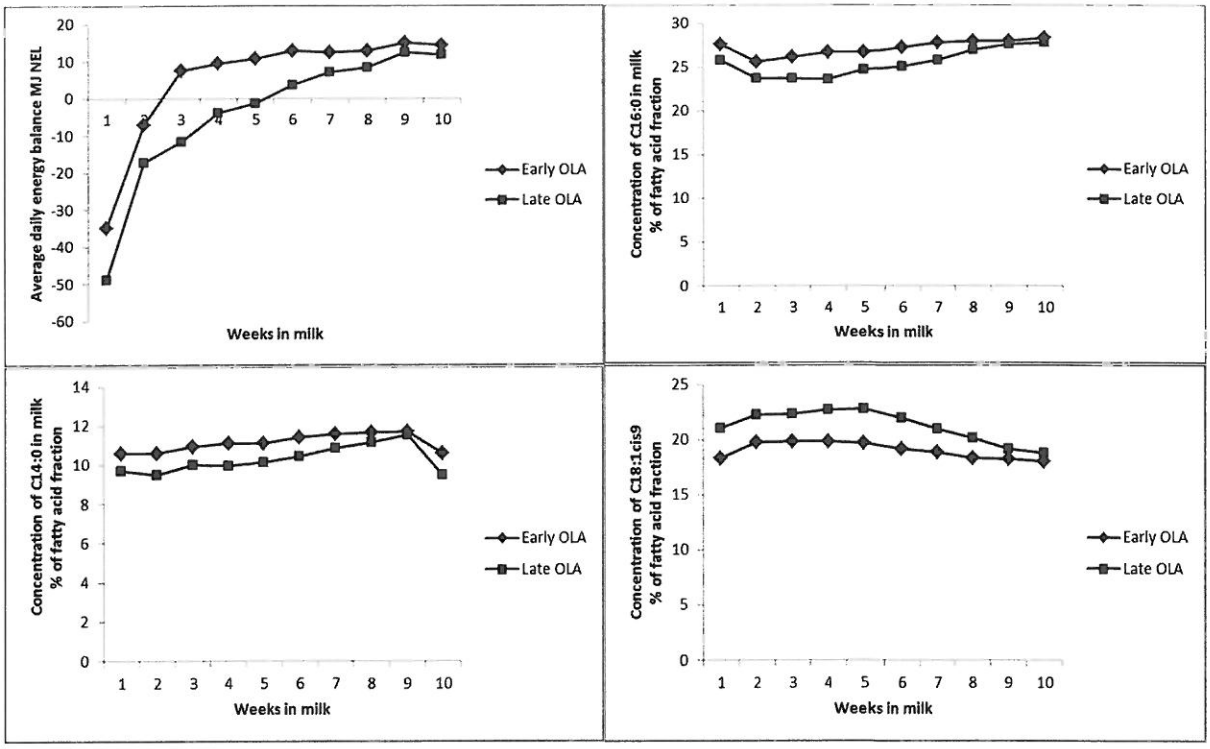
	Sample statistics (n=422)				FTIR calibration results	
Fatty acid	**Min**	**Max**	**Mean**	**STD**	**R^2**	**RMSECV**
C4:0	1.5	5.6	4.0	0.8	0.82	0.3
C14:0	6.0	15.1	12.1	1.1	0.82	0.5
C16:0	19.1	34.3	27.7	2.6	0.81	1.1
C18:0	6.0	15.2	10.8	1.5	0.79	0.7
C18:1cis9	12.2	35.4	18.5	2.9	0.92	0.8
Saturated fatty acids	46.5	75.1	68.1	3.4	0.88	1.2
Monounsaturated fatty acids	16.3	42.8	23.8	3.8	0.93	1.0

651 * All values, except R^2, expressed as percentage by weight of total fatty acid content. Abbreviations: STD – Standard deviation; R^2 –
652 correlation coefficient; RMSECV – Root mean square error of cross-validation.

653

654 **Figure 1.** Graphs displaying mean values of (clockwise from top left): a) daily energy
655 balance, b) concentration of C16:0 in milk, c) concentration of C18:1cis9, d) concentration of
656 C14:0 in milk, by weeks in milk grouped according to early or late onset of luteal activity.
657 Parameters b), c) and d) were estimated using dry film FTIR.

658



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662

663 **Table 3.** Selected milk parameters measured by dry film FTIR by weeks in milk and grouped

664 according to early or late onset of luteal activity.

665

Milk component	Onset of luteal activity	Weeks in milk (WIM)									
		1	2	3	4	5	6	7	8	9	10
Energy corrected milk yield (L)	Early	23.77	27.07	27.93	28.39	29.05	29.04	29.33	29.11	28.99	29.14
	Late	23.38	27.79	29.74*	30.64*	30.50	30.80	30.25	29.80	30.08	29.67
Milk protein (g/L)	Early	4.65	3.74	3.37	3.22	3.17	3.16	3.13	3.14	3.15	4.55
	Late	4.96	3.69	3.34	3.17	3.07*	3.05	3.06	3.07	3.09	4.89
Milk fat (g/L)	Early	4.38	4.38	4.17	4.13	4.09	4.05	4.04	4.03	4.00	3.95
	Late	4.37	4.37	4.09	4.08	3.99	3.89	3.90	3.86*	3.87	3.88
Milk fat:protein	Early	1.01	1.18	1.24	1.29	1.29	1.29	1.29	1.29	1.28	1.26
	Late	1.01	1.19	1.23	1.27	1.30	1.28	1.28	1.26	1.26	1.26
C4:0 Butyric†	Early	4.60	4.79	4.63	4.53	4.67	4.62	4.52	4.50	4.50	4.42
	Late	4.12	4.73	4.64	4.64	4.58	4.57	4.56	4.59	4.69	4.63
C14:0 Myristic†	Early	10.60	10.60	10.93	11.10	11.11	11.38	11.57	11.66	11.71	10.60
	Late	9.69*	9.51**	10.02*	9.95**	10.11**	10.43*	10.83*	11.15*	11.52**	9.51*
C16:0 Palmitic†	Early	27.57	25.60	26.12	26.69	26.67	27.24	27.77	27.93	27.94	28.34
	Late	25.76*	23.73**	23.70***	23.61***	24.69**	25.05**	25.77**	26.96	27.62	27.81
C18:0 Steric†	Early	12.13	13.07	12.14	11.52	11.46	11.01	10.48	10.49	10.30	10.10
	Late	12.71	13.97	13.25	12.86*	12.15	11.85	11.42	10.86	10.42	10.15
C18:1cis 9 Oleic†	Early	18.34	19.74	19.85	19.86	19.70	19.16	18.84	18.28	18.22	18.00
	Late	21.07*	22.22*	22.29*	22.73**	22.75**	21.98**	20.96	20.11	19.15	18.76

666 ttest performed comparing late and early OLA groups *denotes $0.01 \geq p < 0.05$, **denotes $0.001 \geq p < 0.01$, *** denotes $p < 0.001$

667 † expressed as percentage by weight of total fatty acid content

668

669 **Table 4.** Sensitivity, specificity, positive and negative predictive values for the ability of a
670 univariate milk component measured by dry film FTIR to predict/detect late onset of luteal
671 activity by weeks in milk.

672

Fatty Acid	**C14:0**			**C16:0**			**C18:1cis9**		
WIM	2	3	4	2	3	4	2	3	4
Sensitivity (%)	41.67	41.03	52.63	52.78	56.41	73.68	36.11	35.90	50.00
Specificity (%)	84.78	74.47	75.00	78.26	74.47	79.55	82.22	76.60	79.55
Positive Predictive Value (%)	68.18	57.14	64.52	65.52	64.71	75.68	61.90	56.00	67.86
Negative Predictive Value (%)	65.00	60.34	64.71	67.92	67.37	77.78	61.67	59.02	64.81

673

674