Interpretive summary

In vitro ruminal biohydrogenation of EPA, DPA and DHA in cows and ewes: intermediate metabolites and pathways

(by Toral et al.)

This study provided further insight into the biohydrogenation of the major PUFA that are present in the marine lipids used in dairy ruminant feeding, and examined potential differences between bovine and ovine. Results suggested that 20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA) are metabolized through different pathways, with an influence of ruminant species on biohydrogenation kinetics. They also provided the first indication that DPA metabolism may involve the formation of conjugated double-bonds. Higher ruminal production of tentative *trans*-10-containing 20- and 22-carbon intermediates was found in ewes, which was speculated to have some relationship with their susceptibility to marine lipid-induced milk fat depression.

In vitro ruminal biohydrogenation of EPA, DPA and DHA in cows and ewes: intermediate metabolites and pathways

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ABSTRACT

There is still a great deal of uncertainty about intermediate metabolites and pathways explaining the biohydrogenation (BH) of 20- and 22-carbon PUFA. Therefore, this study was conducted to provide further insight into the ruminal metabolism of 20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA), the main n-3 PUFA present in the marine lipids used in dairy ruminant feeding, and to examine potential differences between bovine and ovine. To meet this aim, we investigated the 20- and 22-carbon metabolites accumulated during in vitro incubation of EPA, DPA and DHA with rumen inocula from cows and ewes. The PUFA were added at a dose of 2% incubated dry matter and digesta samples were analyzed after 24 h of incubation,

using complementary gas-liquid chromatography of fatty acid methyl esters and gas chromatography-mass spectrometry of 4,4-dimethyloxazoline derivatives. Results suggested that the main BH pathway of EPA and DPA would proceed via the reduction of the double bond closest to the carboxyl group (*cis*-5 in EPA and *cis*-7 in DPA) but, curiously, this mechanism seemed of much lower importance for DHA. Thus, DPA would not be a major intermediate product of DHA and their BH might actually follow separate pathways, with the accumulation of numerous unique metabolites in each case. A principal component analysis supported this hypothesis, with a clear separation between PUFA treatments in the score and loading plots. Within EPA and DPA groups, cow and ewe samples loaded separately from each other, but not distant. No conjugated 20:5, 22:5 or 22:6 isomer compatible with the initial product of EPA, DPA or DHA metabolism, respectively, was identified in the ruminal digesta, although this would not unequivocally exclude their transient formation. In this regard, results from DPA incubations provided the first indication that the metabolism of this very long-chain PUFA may involve the formation of conjugated double bond structures. The BH of EPA, DPA and DHA resulted in the appearance of several tentative *trans*-10-containing metabolites, showing a general trend to be more abundant in the digesta of ewes than in that of cows. This finding was speculated to have some relationship with the susceptibility of dairy sheep to marine lipid-induced milk fat depression. Differences in the relative proportion of intermediate products would also suggest an influence of ruminant species on BH kinetics, with a process that would likely be slower and less complete in cows than in ewes.

Key words: cattle, mass-spectrometry, PUFA, ruminal lipid metabolism, sheep

INTRODUCTION

The effectiveness of nutritional strategies to enrich milk with very long-chain n-3 PUFA is hindered by rumen microbial biohydrogenation (**BH**; Chilliard et al., 2007; Dewhurst and Moloney, 2013). For this reason, diet supplementation with fish oils or microalgae rich in these fatty acids (**FA**) has often been used to enhance milk CLA content (Offer et al., 1999; Chilliard et al., 2007). On the other hand, these strategies cause milk fat depression, a syndrome often encountered in modern dairy production (Gama et al., 2008; Bichi et al., 2013; Kairenius et al., 2015). Although its origin is as-yet uncertain, some works suggest a putative contribution of very long chain n-3 PUFA or their BH intermediates (Loor et al., 2005; Dallaire et al., 2014; Kairenius et al., 2015).

Numerous studies have demonstrated the extensive ruminal disappearance of major 20- and 22-carbon PUFA from marine lipid supplements [i.e., eicosapentaenoic acid (**EPA**; 20:5n-3) and docosahexaenoic acid (**DHA**, 22:6n-3); Kim et al., 2008; AbuGhazaleh and Jenkins, 2004; Vlaeminck et al., 2014]. However, little is known about the intermediate products of their BH, as very few reports provide further description of this process (Toral et al., 2010; Kairenius et al., 2011; Jeyanathan et al., 2016).

A major constraint for unravelling the rumen metabolic fate of very long-chain n-3 PUFA in in vivo studies is the complex composition of their sources (i.e., fish oils and microalgae; Or-Rashid et al., 2008; Toral et al., 2010; Shingfield et al., 2012). In vitro assays constitute a suitable alternative for characterizing the BH of pure 20- and 22-carbon PUFA but the available literature is still very scant. Jeyanathan et al. (2016) reported a comprehensive description of in vitro DHA metabolism by *Butyrivibrio proteoclasticus* P18, but the role of *Butyrivibrio* species in ruminal BH is probably less relevant than initially thought (Kim et al., 2008; Huws et al., 2011; Shingfield et al., 2012).

By analogy with the BH of 18-carbon PUFA, ruminal EPA and DHA metabolism is considered to involve a first isomerization step that would result in the formation of conjugated 20- and 22-carbon intermediates. Nevertheless, it is only very recently that minor conjugated 22:6 isomers have been detected in digesta (Aldai et al., 2018), whereas other studies pointed to direct saturation as the predominant step in the initial BH of very long-chain PUFA (Toral et al., 2010; Kairenius et al., 2011; Jeyanathan et al., 2016). Docosapentaenoic acid (**DPA**; 22:5n-3) might then be a major metabolite of DHA, which could explain the greater apparent transfer of the former from marine lipids into milk (Offer et al., 1999; Loor et al., 2005; Castañeda-Gutierrez et al., 2007). In line with this, it may also be hypothesized that BH of both PUFA follows common pathways. Yet, a preliminary overview of GC-flame ionization detector (**GC-FID**) chromatograms from a recent in vitro study about ruminal responses to EPA, DPA and DHA (Toral et al., 2017) revealed many unique unidentified peaks in each PUFA treatment, challenging that hypothesis. Complementary identifications would then be needed to confirm this finding.

The study by Toral et al. (2017) also demonstrated specificities in the ruminal response of cows and ewes to EPA, DPA and DHA. Although the reasons for these variations are expected to derive from species differences in rumen microbial composition (Moon et al., 2010; Lee et al., 2012), the influence this may have on the pathways and intermediate products of 20- and 22-carbon PUFA metabolism is as yet unknown.

This study was therefore conducted to provide further insight into the ruminal BH of very long-chain PUFA and to examine potential differences between bovine and ovine. To meet this objective, we investigated the 20- and 22-carbon metabolites accumulated during in vitro incubation of EPA, DPA and DHA with rumen inocula from cows and ewes.

MATERIALS AND METHODS

In Vitro Experiment and Samplings

All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes.

Details of the experimental design and methodology were described in Toral et al. (2017). Briefly, the in vitro trial was conducted in batch cultures following a 2 × 4 factorial arrangement: 2 species (ovine and bovine) and 4 treatments (EPA, DPA and DHA, and a control without FA). In vitro incubations were performed in Hungate tubes using rumen inocula collected (before feeding) from cannulated cows (n = 2) and ewes (n = 2) fed a TMR formulated from alfalfa hay and a concentrate (50:50). They were repeated on 3 days (replicates). The TMR contained, per kg of DM, 187 g of CP, 311 of NDF and 18 of total FA, and the offer was fixed at estimated maintenance energy requirements (INRA, 2007) to work under similar conditions in both species. The three PUFA [10-2005-9 (EPA), 10-2205-9 (DPA) and 10-2206-9 (DHA); Larodan, Solna, Sweden] were dissolved in ethanol, dispersed with an ultrasonic device and added at a dose of 2% substrate (the TMR fed to the animals) just before the incubation started. The control treatment was dosed only the corresponding amount of ethanol. Each tube contained 120 mg DM of the TMR, which were incubated under anaerobic conditions at 39.5 ºC with 12 mL of a mix (1:4) of strained rumen fluid and artificial saliva, as detailed in Toral et al. (2017). After 24 h, the reaction was stopped by placing the vials into ice-water for approximately 5 min. Samples were freeze-dried, and stored at –80 ºC until FA analysis.

Fatty Acid Analysis

Lipid Extraction and Preparation of FAME. The lipids in ruminal digesta were extracted directly in the Hungate tubes, which contained approximately 200 mg of freeze-dried in vitro residue. The extraction procedure was repeated twice using 4 mL of a mixture (3:2, v/v)

of hexane and isopropanol following the adjustment of digesta pH to 2.0 using 2 *M* hydrochloric acid (Shingfield et al., 2003). Organic extracts were combined and dried under nitrogen at 45 ºC. Lipid dissolved in 2 mL of hexane was converted to FAME using a sequential base-acid catalyzed transesterification procedure with freshly prepared 0.5 M sodium methoxide in methanol for 5 min at 20 ºC followed by reaction with 1% (v/v) sulfuric acid in methanol at 50 ºC for 30 min (Toral et al., 2010). Finally, FAME were dissolved in hexane prior to GC-FID analysis.

Preparation of 4,4-Dimethyloxazoline Derivatives. Selected samples of ruminal digesta FAME were converted to 4,4-dimethyloxazoline (**DMOX**) derivatives by incubation overnight with 250 µL of 2-amino-2-methyl-1-propanol under a nitrogen atmosphere (Fay and Richli, 1991) at 170 ºC. Once cooled, the reaction mixture was dissolved with a combination (1:1, v/v) of diethyl ether and hexane and rinsed with distilled water. The organic phase was washed with distilled water, dried over anhydrous sodium sulfate, centrifuged and evaporated to dryness under nitrogen at 30 ºC (http://www.lipidhome.co.uk/ms/basics/derivprep/index.htm). Once prepared, DMOX derivatives were dissolved in hexane and analysed by GC–MS.

GC and GC–MS Analysis. Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA) equipped with a flame-ionization detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2-µm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2 µL sample volume at a split ratio of 1:50 was determined using the temperature gradient program described in Shingfield et al. (2003). The FAME were converted to FA using theoretical relative response factors for GC analysis (Wolff et al., 1995). Some peaks were identified based on retention time comparisons with commercially available standards (GLC463, U-37-M, U-39-M, U-43-M, U-45-M, U-54-M, U-64-M, U-85-M, U-87-M, N-21-M and N-23-M, Nu-Chek Prep., Elysian, MN; 18919-1AMP

Supelco, L6031, L8404, H6389, H6639, T1902, O4129 and O5632, Sigma-Aldrich, Madrid, Spain; and 10-1620, 10-1840, 10-2001-1, 11-1600-8, 20-1606-4, 20-1611, 20-1869-2, 10-2014-5, 20-2024-1, 20-2103-4, 20-2105-4, 20-2210-9, 20-2265-7, 20-2305-1-4, 20-2405-4, 20-2500-1, 20-2500-7, 20-2900-7, 21-1211-7, 21-1413-7, 21-1614-7, 21-1615-7 and BR mixtures 2 and 3, Larodan). Fatty acids not available as commercial standards were identified based on GC-MS analysis of DMOX derivatives.

Mass spectra of FAME and DMOX derivatives were obtained using the same type of gas chromatograph and column used for GC-FID, equipped with a quadrupole mass spectrometer (Agilent 5973N). The mass spectrometer was operated at ion source temperature of 230 °C in positive electron ionization mode and mass spectra were recorded under ionization energy of 70 eV. The injector (split ratio of 1:50) and interface temperatures were maintained at 255 and 240 ºC, respectively. Total FAME and DMOX profiles in a 4 μL sample volume were determined using helium as a carrier gas (142.7 kPa, nominal initial flow 0.7 mL/min) and the same temperature gradient program applied for GC-FID (Kairenius et al., 2011). Electron ionization spectra of DMOX derivatives were used to locate double bonds based on atomic mass unit distances, with an interval of 12 units between the most intense peaks of clusters of ions containing n and n–1 carbon atoms being interpreted as cleavage of the double bond between carbon n and n+1 in the FA moiety. Identification was verified based on comparisons with an online reference library of DMOX derivative electron ionization spectra (http://www.lipidhome.co.uk/ms/dmox/dmox-arch/index.htm) and previous determinations of FA composition in ruminal and omasal digesta of ewes and cows fed FO based on fractionation of FAME by argentation silver-ion thin-layer chromatography and GC-MS analysis of corresponding DMOX derivatives (Toral et al., 2010; Kairenius et al., 2011, 2018). Analysis allowed for unambiguous identification of monoenoic FA. However, as mass spectra of DMOX derivatives does not give indication of double bond geometry, the *cis* or *trans* configuration in

20- and 22-carbon intermediates containing two or more double bonds was inferred based on comparisons of retention times of corresponding FAME determined by GC-FID and the known elution order of authentic geometric isomers of 18:2n-6 and 18:3n-3 methyl esters (L-8404 and L-6031, respectively, Sigma-Aldrich). Partial gas chromatograms indicating the separation of 20- and 22- carbon FAME are shown in Supplementary Fig. 1-3.

Statistical Analyses

A principal component analysis (**PCA**) was conducted using the 'R-project' software (http://www.r-project.org, version 3.4.1) to explore the relationship among the numerous 20- and 22-carbon FA that were identified in digesta, to try to facilitate the description of the main trends in the dataset and the detection of possible patterns of similarity.

Statistical analyses were also performed using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). Data were analyzed by ANOVA to test the fixed effects of PUFA treatments (**T**; control, EPA, DPA and DHA), species (**Sp**; bovine and ovine), and their interaction. The incubation run and the inoculum nested within the species were designated as random effects. Means were separated through the pairwise differences ("pdiff") option of the least squares means ("lsmeans") statement of the MIXED procedure, and adjusted for multiple comparisons using Bonferroni's method. For FA found exclusively in one of the PUFA treatments, the fixed effects due to T and the interaction Sp × T were removed. Differences were declared significant at $P < 0.05$ and considered a trend towards significance at $0.05 \leq P < 0.10$. Least squares means are reported.

RESULTS

Principal Component Analysis

Two major principal components (**PC**) accounted for more than 70% of total variability in 20- and 22-carbon FA concentrations (38.0 and 32.4% for PC1 and PC2, respectively), whereas the third PC only explained 9.5% of the variation. In the score plot projected on the basis of PC1 and PC2 (Figure 1a), four clusters corresponding to the four PUFA treatments were apparent. Control samples from both species positioned near the origin of the plot, with no relationship with any of these two PC, whereas those from EPA, DPA and DHA incubations, regardless of the species, were grouped separately from each other in three distant positions. Based on PC1, the EPA cluster loaded in the negative range and that of DPA in the positive, whereas DHA only showed slight negative correlation with the first PC. Samples from EPA and DPA were both positively correlated with PC2, and those from DHA negatively correlated. Within EPA and DPA groups, cow and ewe samples loaded separately from each other, although not distant. As shown in the biplot PC1 × PC3 (Figure 1b), the third PC appeared to discriminate on the basis of lipid addition and, within EPA, DPA and DHA treatments, of ruminant species. Thus, all controls clustered together in the negative range and, for incubations with PUFA, cow samples showed no evident correlation with PC3 and ewe samples were positioned in the positive range.

Figure 2 reports the loading plot projected on PC1 and PC2. The vast majority of variables were grouped in three large clusters that loaded in similar positions to those of EPA, DPA and DHA treatments in the score plot. Concentrations of EPA and all 20-carbon FA were negatively correlated with PC1 and, with the exception of *cis*-5 20:1, positively correlated with PC2. The 22:0 was positioned close to this first cluster. The second group of variables included DPA, all 22:1, 22:2 and 22:3 isomers and few 22:4 metabolites, and loaded in the positive range based on both PC1 and PC2. On the contrary, concentrations of all the other 22:4 FA, together

with DHA and 22:5 intermediates, were negatively correlated with PC2 and, to a lower extent, with PC1.

Ruminal 20-carbon fatty acids

As shown in Table 1, numerous 20-carbon FA were identified in ruminal digesta, most of them being unique metabolites exclusively found in EPA incubations. Detailed GC-MS analysis of the digesta did not reveal the occurrence of 20-carbon intermediates containing a conjugated double bond or 20:5 isomers other than EPA.

Differences due to ruminant species did not reach the required level of significance for EPA concentration ($P = 0.112$) or total 20:3 ($P = 0.361$), but the sum of 20:4 FA tended to be more abundant in cow digesta ($P = 0.051$). On the other hand, the greatest amount of total 20:2 and 20:1, and 20:0 was measured in EPA incubations with ovine rumen fluid (interaction Sp × T; $P < 0.01$). Interspecies differences in the proportions of individual isomers usually mirrored those of their respective FA category (i.e., total 20:4, 20:3 or 20:2).

The n-3 PUFA were among the major 20-carbon FA in ruminal digesta, particularly *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:4, which represented 5.60% of total FA in cows and 1.88% in ewes ($P = 0.035$), and *cis*-11 *cis*-14 *cis*-17 20:3. Several tentatively identified metabolites containing at least one *trans* double bond were found in relatively high amounts as well (>1% of total FA), such as *trans*-6 *cis*-11 *cis*-14 *cis*-17 20:4, *trans*-10 *trans*-14 *cis*-17 + *cis*-11 *cis*-14 *trans*-17 20:3 and *trans*-13 *cis*-17 20:2, the proportion of this latter being greater in ovine than bovine ($P = 0.018$). None of these intermediates or other 20:4 and 20:3 isomers were detected in control, DPA or DHA treatments, which were also devoid of 20:2 FA, except the minor *cis*-11 *cis*-14 20:2. On the contrary, *cis*-17 20:1 was the only unique 20:1 in EPA incubations, and was much more abundant in ewes ($P = 0.048$). Concentrations of other individual 20:1 metabolites were also higher in ovine and, generally, in EPA treatment (interaction Sp × T; $P < 0.05$). Addition

of EPA decreased 20:0 in cows, whereas DHA reduced it in ewes, compared with their controls ($P = 0.003$)

Ruminal 22-carbon fatty acids

Table 2 reports ruminal concentrations of 22-carbon FA, which included many unique BH intermediates in both DPA and DHA incubations. Conjugated 22:6 and 22:5 isomers could not be detected in any treatment, whereas DPA chromatograms contained a peak identified as conjugated 22:4. The mass spectrum of its DMOX derivative suggested that it may be a double conjugated intermediate (specifically, Δ11,13,17,19 22:4), but the possible coelution of two mono-conjugated 22:4 isomers (with conjugated double bond structure putatively on Δ11,13 and Δ17,19) cannot be excluded (Figure 3a).

The high variation in DHA concentrations probably resulted in numerical differences between cows and ewes not reaching the required level of significance ($P = 0.176$). This n-3 PUFA was exclusively found in DHA treatment, in which very small amounts of 22:5n-3 were also detected. Several other 22:5 intermediates were tentatively identified in DHA samples, but none was present in DPA treatment, whereas the total amount of 22:4, 22:3, 22:2 and 22:1 isomers was generally higher in DPA than in DHA incubations.

When DPA was added, its abundance remained greater in bovine incubations ($P < 0.01$). Nonetheless, within the group of 22:5 metabolites, interspecies differences were minor, and the most abundant FA (>1% of total FA; i.e., Δ4,7,10,13,17 and Δ4,7,10,14,19 22:5) showed similar proportions in cows and ewes ($P > 0.10$). Figure 3b reports the mass spectrum of the DMOX derivative of Δ4,7,10,14,19 22:5, and its tentative geometry is given in Table 2. Two unique 22:4 isomers were detected in the DHA samples, namely Δ4,7,14,19 and Δ7,10,14,19 22:4, but their concentrations did not differ between ruminant species. The few other 22-carbon intermediates in this treatment were all-*cis* FA (specifically, *cis*-13 *cis*-16 *cis*-19 22:3, *cis*-13

cis-16 22:2 and *cis*-11, *cis*-12 and *cis*-13 22:1) and their very low amounts in digesta were also similar in bovine and ovine ($P > 0.10$ for pairwise comparisons after Bonferroni adjustment).

Interspecies differences in 22-carbon FA were much more frequent in DPA incubations. As a general trend, the lower the number of double bonds in the FA chain, the higher the concentration in ewes. Thus, sheep digesta from the DPA treatment had the greatest proportion of total 22:2 and 22:1 isomers (interaction Sp × T; $P < 0.01$), which also applied to most individual FA within each category, such as *cis*-13 *cis*-16 22:2 or *cis*-12 and *cis*-13 22:1 ($P < 0.01$). The opposite (i.e., a trend toward greater concentrations in cows; $P < 0.10$) was observed, for example, for the unique and abundant Δ10,13,17 and Δ12,16,19 22:3. Regarding 22:4 intermediates, differences between species were only detected for the minor *cis*-7 *cis*-13 *cis*-16 *cis*-19 22:4, whereas the high variation among replicates seem to preclude from reaching the required *P*-level in major isomers, such as *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:4 or Δ11,13,17,19 22:4 ($P = 0.137$ and 0.145, respectively). Finally, 22:0 concentration was negatively affected by DPA and DHA addition in both ruminant species, compared with the control and EPA treatments ($P < 0.001$).

DISCUSSION

Very long-chain n-3 PUFA from marine lipids are extensively metabolized in the rumen, but the intermediates formed and the pathways involved remain largely unknown (Kairenius et al., 2011; Escobar et al., 2016). In this study, we conducted a direct comparison of in vitro ruminal BH of EPA, DPA and DHA in cows and ewes to characterize in detail the 20- and 22-carbon FA profiles of digesta and try to infer potential pathways explaining the metabolic fate of these PUFA in the rumen.

Based on the relatively well known BH of linoleic and α-linolenic acids (Lee and Jenkins, 2011; Alves and Bessa, 2014; Honkanen et al., 2016), it was expected that the

isomerization of a *cis* double bond would also represent the first major step in the ruminal metabolism of other polyenoic FA with different chain length. The transient nature of conjugated 18-carbon FA has not precluded their usual detection in the ruminal digesta (Wallace et al., 2007; Lee and Jenkins, 2011; Honkanen et al., 2016). Nevertheless, detailed chromatographic analyses have failed to identify conjugated 20- and 22-carbon FA of ruminal origin in dairy cows, ewes and goats fed marine lipids (Kairenius et al., 2011; Toral et al., 2012, 2016) or in most in vitro incubations with DHA (Escobar et al., 2016; Jeyanathan et al., 2016). Compared with other data from mixed cultures of rumen microorganisms (Aldai et al., 2012; Vlaeminck et al., 2014; Escobar et al., 2016), the relatively high dose of PUFA in our trial (2% DM of the substrate) would have slowed down the BH process, facilitating the detection of initial intermediates. However, no conjugated 20:5, 22:5 and 22:6 isomers compatible with the first product of EPA, DPA or DHA metabolism, respectively, were found in this study, although this would not unequivocally exclude their transient formation. In this regard, Aldai et al. (2018) has very recently reported that the initial steps of in vitro DHA metabolism involves formation of minor conjugated 22:6 isomers, with predicted increases in their content up to 3.8 h of incubation and decreases afterwards. Thus, longer incubation times (24 h) may have hindered their identification under our conditions, whereas the presence of conjugated 22:4 in DPA treatment provided the first indication that the metabolism of this very long-chain PUFA may involve the formation of conjugated double bond structures. The usually low concentration of DPA in marine lipids could have hampered the detection of its metabolites in previous trials (Lee et al., 2005; Toral et al., 2010; Kairenius et al., 2011). Thus, in spite of the existing literature on the subject (e.g., Shingfield et al., 2012; Toral et al., 2012; Kairenius et al., 2018), further investigations would be advisable to examine the putative formation of 20- and 22-carbon conjugated intermediates under in vivo conditions.

Alternatively, the reduction of the double bond closest to the carboxyl group has been

proposed to represent the main initial step in the BH of 20- and 22-carbon PUFA (Toral et al., 2010; Kairenius et al., 2011; Jeyanathan et al., 2016). Results from our trial might support the relevance of this mechanism for EPA and DPA, suggesting that a major pathway explaining their BH proceeds via the reduction of the *cis*-5 and *cis*-7 double bonds, respectively. Accordingly, the most abundant 20:4 and 22:4 intermediates found in digesta from EPA and DPA incubations were all-*cis* PUFA, namely *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:4 (20:4n-3) and *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:4 (22:4n-3). In the EPA treatment, the subsequent BH step would have preferentially affected the adjacent double bond so the hydrogenation of *cis*-8 in 20:4n-3 might explain the accumulation of *cis*-11 *cis*-14 *cis*-17 20:3, the second most abundant 20-carbon intermediate (Figure 4). Although AbuGhazaleh et al. (2006) suggested that 20:3n-3 would not contribute to the inhibitory effects of marine lipids on *trans*-18:1 saturation, there is some indication that other products of the PUFA metabolism might be effective at modulating this last BH step (Jeyanathan et al., 2016).

The two pathways mentioned above (formation of conjugated intermediates or saturation of the double bond closest to the carboxyl group) may also explain the appearance of other abundant metabolites with tentative *trans* double bond configurations, such as *trans*-6 *cis*-11 *cis*-14 *cis*-17 20:4. First, it could be speculated that the formation of this 20:4 intermediate involved the sequential isomerization and saturation of the *cis*-5 and *cis*-8 double bonds, respectively, in EPA, with *trans*-6 *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:5 as a putative transient product (Figure 4). According to Escobar et al. (2016), this would be possible if the isomerization rate is an order of magnitude smaller than the rate of saturation. A second explanation may be that *trans*-6 *cis*-11 *cis*-14 *cis*-17 20:4 arises from isomerization of *cis*-7 *cis*-11 *cis*-14 *cis*-17 20:4, which in turn might derive from migration of one double bond in *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:4, the major intermediate in EPA incubations. Double bond migration would occur without catalysis by a *cis-trans* isomerase during the saturation of the *cis*-8 bond. As proposed for

linoleic acid (Kitayama et al., 1997), the mechanism would be mediated by the rotation of the C-C bond in the partially-hydrogenated state of the FA. This reaction has recently been suggested to account for the formation of *cis*-7 *cis*-12 *cis*-15 18:3 and Δ8,12,15 18:3 during α-linolenic acid incubations with rumen inoculum from cows (Honkanen et al., 2016).

A similar mechanism, involving saturation, *cis*-*trans* isomerization and migration of the double bond with no conjugated FA production, might also explain the accumulation of other putative *trans* FA metabolites in our trial, such as *trans*-8 *cis*-13 *cis*-16 *cis*-19 22:4 in DPA incubations. Additional in vitro studies (for example, examining the incorporation of ^{2}H in FA intermediates; Wallace et al., 2007; Honkanen et al., 2016) would be required to confirm this speculation.

Interestingly, the hypothesis of the initiation of the ruminal PUFA metabolism with the saturation of the double bond closest to the carboxyl group (which would mean that DPA may be a major DHA intermediate product) seems indeed of much lower importance for DHA, which contrasts with results reported by Jeyanathan et al. (2016). However, this latter in vitro study was based on pure cultures of *Butyrivibrio proteoclasticus*, a species that may be less relevant in the rumen bacterial community than initially thought (Huws et al., 2011; Shingfield et al., 2012; Toral et al., 2016). The complexity of the microbial community in our mixed cultures would be closer to that of the rumen in the in vivo conditions and, under these circumstances, in line with Aldai et al. (2018), DPA may only be a minor metabolite of DHA. This finding, together with the detection of numerous unique intermediates, would support that the BH of each PUFA may actually follow separate pathways, as shown in the general outcome of PCA, with a clear separation between PUFA treatments in the score and loading plots (Figures 1 and 2, respectively).

The structure of 22:5 intermediates found in DHA incubations might imply an active isomerization and saturation of double bonds at Δ13, Δ16 and Δ19 positions in initial BH steps,

whereas subsequent metabolism was limited compared with those of EPA and DPA, which showed a great array of unique trienoic and dienoic intermediates. On the other hand, most monoenoic FA would also derive from the rumen inocula used for the incubations, as inferred from digesta composition in the control treatment and in cows and ewes fed standard diets (Or-Rashid et al., 2008; Buccioni et al., 2011; Shingfield et al., 2012). The relative importance of this external origin varied among FA. For example, it was particularly high for *cis*-5 20:1, which is consistent with its position in Figure 2, distant from the cluster of 20-carbon FA, and with the lack of other *cis*-5-containing FA in EPA incubations.

The BH of the three incubated PUFA resulted in the appearance of putative *trans*-10-containing 20- and 22-carbon FA, with as-yet-unknown biological activity in ruminants. Increases in the milk concentrations of some of them (e.g., *trans*-10 *trans*-14 *trans*-17 20:3 and *trans*-10 *trans*-16 20:2) have recently been associated with fish oil-induced milk fat depression in dairy cows (Kairenius et al., 2015), but a direct cause and effect has not been demonstrated yet. The accumulation of these FA with a tentative *trans*-10 double bond showed a general trend to be greater in the digesta of ewes than in that of cows, which was unexpected because sheep had long been presumed to be less prone than cows to alterations in ruminal BH related to milk fat depression (Pulina et al., 2006; Mele and Banni, 2010). However, both species are sensitive to the negative impact of marine lipids on milk fat synthesis (Offer et al., 1999; Gama et al., 2008; Bichi et al., 2013). Part of the reason for that detrimental effect on ewe performance might then be related to a high ruminal production of *trans*-10-containing 20- and 22-carbon FA.

Interspecies differences in the microbial community of each inoculum may be at the core of dissimilarities in the lipid composition of in vitro ruminal digesta, even if all donors had received the same diet (Moon et al., 2010; Lee et al., 2012; Toral et al., 2016). Variation in the concentration of some odd- and branched-chain FA (e.g., *iso* 14:0, *iso* 15:0, *anteiso* 15:0 and *iso* 17:0; data reported in Supplementary Table S1), which derive mainly from bacterial

synthesis (Fievez et al., 2012), would be consistent with differences in the microbial composition of inocula from cows and ewes. In spite of this, the same 20- and 22-carbon FA metabolites were found in bovine and ovine incubations, indicating that the BH of very long-chain n-3 PUFA presumably followed similar pathways. However, differences in the relative proportion of these intermediates would suggest an influence of ruminant species on BH kinetics, with a process that was likely slower and less complete in cattle than in sheep, in particular for EPA and DPA. This speculation would be supported by the greater proportions of total 20:4 and 22:3 FA in cow cultures, whereas those of sheep showed higher concentrations of total 20:2, 20:1, 22:2 and 22:1. These results were also consistent with the discrimination of samples in the score plot from PCA (Figure 1), but establishing comparisons with the available literature on this subject is rather complicated (e.g., Kairenius et al., 2011; Toral et al., 2012; Jeyanathan et al., 2016). This is due to the limited number of studies reporting detailed 20- and 22-carbon FA profiles in digesta, and their diversity in terms of experimental approach (in vivo vs. in vitro), very long-chain PUFA source (fish oils, microalgae or pure FA), basal diet composition, and sampling procedures (e.g, collection of ruminal fluid vs. whole content, time post-feeding, etc.). Finally, although we were not able to detect variations between cows and ewes in the extension or rate of BH of 18-carbon FA (Toral et al., 2017), ruminal bacterial species capable of biohydrogenating each type of FA may differ (Maia et al., 2007; Jeyanathan et al., 2016). Therefore, it is possible that interspecies differences in BH kinetics may be accounted for by inherent dissimilarities in the ruminal microbial composition in bovine and ovine (Moon et al., 2010; Lee et al., 2012).

CONCLUSIONS

The main pathway explaining the in vitro BH of EPA and DPA seems to proceed via the reduction of the double bond closest to the carboxyl group (namely *cis*-5 in EPA and *cis*-7 in DPA) but, curiously, this mechanism appears of much lower relevance in the DHA metabolism. Thus, DPA would not be a major intermediate product of DHA and the BH process might actually follow separate pathways, with the accumulation of numerous unique metabolites in each case. Although no conjugated 20:5, 22:5 or 22:6 isomers compatible with the initial product of EPA, DPA or DHA metabolism, respectively, were identified in the ruminal digesta, our results cannot unequivocally exclude their transient formation. In this regard, the detection of conjugated 22:4 provides the first indication that DPA metabolism may involve the formation of a conjugated double bond system. The BH of EPA, DPA and DHA results in the accumulation of tentative *trans*-10-containing FA with as-yet-unknown biological activity, showing a general trend to be more abundant in the digesta of ewes than in that of cows. Although the ruminal microbial metabolism of very long-chain n-3 PUFA presumably follows similar pathways in bovine and ovine, there may be interspecies differences in terms of BH kinetics, with a process that would likely be slower and less complete in cattle than in sheep, in particular for EPA and DPA.

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Table 1. Effect of very long chain n-3 PUFA on the concentration of 20-carbon fatty acids (g/100 g of total fatty acids) after 24-h in vitro incubation with rumen inocula from cows or ewes[1]

		Cow				Ewe					*P*-value[2]		
Fatty acid	Tentative double bond geometry[3]	Control	EPA	DPA	DHA	Control	EPA	DPA	DHA	SED[4]	Sp	T	Sp × T
cis-5 *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:5	-	-	1.003	-	-	-	0.555	-	-	0.2205	0.112	-	-
Σ 20:4 isomers		-	7.826	-	-	-	2.675	-	-	1.2156	0.051	-	-
cis-8 *cis*-11 *cis*-14 *cis*-17 20:4	-	-	5.600	-	-	-	1.875	-	-	0.7167	0.035	-	-
Δ6,11,14,17 20:4	*trans*-6 *cis*-11 *cis*-14 *cis*-17 20:4	-	1.425	-	-	-	0.357	-	-	0.3746	0.104	-	-
Δ7,11,14,17 20:4	*cis*-7 *cis*-11 *cis*-14 *cis*-17 20:4	-	0.115	-	-	-	0.082	-	-	0.0077	0.052	-	-
Δ8,11,14,17 20:4	nd	-	0.242	-	-	-	0.109	-	-	0.0271	0.039	-	-
Σ 20:3 isomers		-	6.111	-	-	-	5.211	-	-	0.8737	0.361	-	-
cis-11 *cis*-14 *cis*-17 20:3	-	-	2.443	-	-	-	1.785	-	-	0.4250	0.197	-	-
Δ8,12,17 + 8,13,17 20:3	*trans*-8 *trans*-12 *trans*-17 + *trans*-8 *trans*-13 *trans*-17 20:3	-	0.580	-	-	-	0.250	-	-	0.1821	0.144	-	-
Δ9,14,17 20:3	nd	-	0.056	-	-	-	0.070	-	-	0.0106	0.259	-	-
Δ9,14,17 20:3	*trans*-9 *trans*-14 *trans*-17 20:3	-	0.239	-	-	-	0.130	-	-	0.0198	0.031	-	-
Δ10,14,17 20:3	*trans*-10 *trans*-14 *trans*-17 20:3	-	0.251	-	-	-	0.298	-	-	0.1058	0.204	-	-
Δ10,14,17 + 11,14,17 20:3	*trans*-10 *trans*-14 *cis*-17 + *cis*-11 *cis*-14 *trans*-17 20:3	-	1.191	-	-	-	1.231	-	-	0.2294	0.869	-	-
Δ11,14,17 20:3	*cis*-11 *trans*-14 *cis*-17 20:3	-	0.248	-	-	-	0.190	-	-	0.0385	0.273	-	-
Δ11,14,17 20:3	*trans*-11 *cis*-14 *cis*-17 20:3	-	0.508	-	-	-	0.663	-	-	0.0995	0.217	-	-
Δ11,14,18 20:3[5]	nd	-	0.103	-	-	-	0.110	-	-	0.0243	0.788	-	-
Σ 20:2 isomers		0.019^{c}	1.669^{b}	0.027^{c}	0.029^{c}	0.058^{c}	3.420^{a}	0.053^{c}	0.104^{c}	0.2599	0.003	<0.001	<0.001
cis-11 *cis*-14 20:2[6]	-	0.019^{e}	0.089^{b}	0.027^{de}	0.029^{cde}	0.058^{c}	0.163^{a}	0.053^{cd}	0.105^{b}	0.0078	<0.001	<0.001	<0.001
Δ8,14 + 14,17 20:2[7]	nd + *cis*-14 *cis*-17 20:2	-	0.493	-	-	-	0.484	-	-	0.0227	0.742	-	-
Δ9,15 20:2	*trans*-9 *trans*-15 20:2	-	0.092	-	-	-	0.186	-	-	0.0096	0.011	-	-
Δ9,17 + 14,17 20:2	nd + *trans*-14 *cis*-17 20:2	-	0.123	-	-	-	0.471	-	-	0.0678	0.036	-	-
Δ10,16 20:2[8]	*trans*-10 *trans*-16 20:2	-	0.135	-	-	-	0.231	-	-	0.0316	0.093	-	-
Δ10,17 20:2	*trans*-10 *cis*-17 20:2	-	0.174	-	-	-	0.362	-	-	0.0392	0.009	-	-
Δ11,17 + 12,17 20:2	nd	-	0.047	-	-	-	0.067	-	-	0.0139	0.290	-	-
Δ12,15 20:2	nd	-	0.138	-	-	-	0.214	-	-	0.0267	0.106	-	-
Δ12,17 + 13,17 20:2	nd	-	0.056	-	-	-	0.125	-	-	0.0118	0.028	-	-

Δ13,17 20:2	*trans*-13 *cis*-17 20:2	-	0.745	-	-	-	1.422	-	-	0.0928	0.018	-	-
Σ 20:1 isomers		0.169^{b}	0.559^{b}	0.164^{b}	0.161^{b}	0.248^{b}	2.330^{a}	0.198^{b}	0.389^{b}	0.3028	0.004	<0.001	0.003
cis-5 20:1	-	0.006^{c}	0.013^{abc}	0.004^{c}	0.002^{c}	0.025^{abc}	0.035^{ab}	0.011^{bc}	0.038^{a}	0.0068	0.009	0.012	0.030
cis-7 + *trans*-13 20:1[9]	-	0.006^{c}	0.058^{bc}	0.009^{bc}	0.008^{bc}	0.056^{bc}	0.293^{a}	0.044^{bc}	0.125^{b}	0.0317	<0.001	<0.001	0.002
cis-9 + *trans*-14 20:1[10]	-	0.007^{b}	0.021^{b}	0.009^{b}	0.012^{b}	0.025^{b}	0.229^{a}	0.021^{b}	0.065^{b}	0.0304	<0.001	<0.001	0.001
cis-11 + *trans*-15+16 20:1	-	0.119^{b}	0.208^{b}	0.105^{b}	0.113^{b}	0.103^{b}	0.758^{a}	0.082^{b}	0.116^{b}	0.1155	0.043	<0.001	0.008
cis-12+13 + *trans*-17 20:1[11]	-	0.019^{b}	0.029^{b}	0.012^{b}	0.015^{b}	0.018^{b}	0.253^{a}	0.013^{b}	0.016^{b}	0.0460	0.029	0.003	0.008
cis-17 20:1	-	-	0.172	-	-	-	0.600	-	-	0.0975	0.048	-	-
trans-9+10 20:1	-	0.002^{b}	0.007^{b}	-	-	0.006^{b}	0.024^{a}	-	-	0.0016	<0.001	<0.001	<0.001
trans-11 20:1	-	0.007^{c}	0.028^{b}	0.013^{c}	-	0.011^{c}	0.064^{a}	0.017^{bc}	0.014^{bc}	0.0040	<0.001	<0.001	<0.001
trans-12 20:1	-	0.006^{b}	0.031^{b}	0.012^{b}	0.011^{b}	0.009^{b}	0.099^{a}	0.010^{b}	0.015^{b}	0.0100	0.003	<0.001	<0.001
20:0	-	0.944^{bc}	0.694^{d}	0.721^{cd}	0.715^{cd}	1.200^{a}	1.253^{a}	1.020^{ab}	0.860^{bcd}	0.0635	<0.001	<0.001	0.003

[a-e]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of species × PUFA treatment.

[1]The incubated substrate was a TMR containing no additional PUFA (control) or supplemented with 2% DM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) or docosahexaenoic acid (DHA).

[2]Probability of significant effects due to species (Sp), PUFA treatment (T), and their interaction (Sp × T).

[3]Tentative geometry of double bonds as inferred based on retention times and elution order of FAME during GC analysis; nd = not determined.

[4]Standard error of the difference.

[5]Coelutes with a 20:3 isomer of indeterminate double bond position.

[6]In EPA treatment, coelutes with a 20:2 isomer of indeterminate double bond position.

[7]Coelute with Δ6,12,17 20:3 (tentatively identified as *trans*-6 *trans*-12 *trans*-17 20:3).

[8]Coelutes with *trans*-11 *cis*-13 18:2.

[9]In DPA and DHA treatments, no traces of *trans*-13 20:1 were detected.

[10]In DPA and DHA treatments, no traces of *trans*-14 20:1 were detected.

[11]In the control, DPA and DHA treatments, no traces of *cis*-12 and *trans*-17 20:1 were detected. In the EPA treatment, *trans*-17 20:1 was the major isomer.

Table 2. Effect of very long chain n-3 PUFA on the concentration of 22-carbon fatty acids (g/100 g of total fatty acids) after 24-h in vitro incubation with rumen inocula from cows or ewes[1]

		Cow				Ewe					*P*-value[2]		
Fatty acid	Tentative double bond geometry[3]	Control	EPA	DPA	DHA	Control	EPA	DPA	DHA	SED[4]	Sp	T	Sp × T
cis-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:6	-	-	-	-	6.109	-	-	-	3.263	1.3813	0.176	-	-
cis-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:5[5]	-	-	-	1.708^{a}	0.020^{c}	-	-	1.109^{b}	0.018^{c}	0.1101	0.016	<0.001	0.003
Σ 22:5 isomers (excluding 22:5n-3)		-	-	-	7.428	-	-	-	7.382	0.2345	0.861	-	-
Δ4,7,10,13,17 22:5	*cis*-4,*cis*-7,*cis*-10,*cis*-13,*trans*-17 22:5	-	-	-	1.273	-	-	-	1.307	0.0740	0.690	-	-
Δ4,7,10,14,17 22:5[6]	*cis*-4,*cis*-7,*cis*-10,*trans*-14,*trans*-17 22:5	-	-	-	0.897	-	-	-	0.992	0.0293	0.083	-	-
Δ4,7,10,14,19 22:5	*cis*-4,*cis*-7,*cis*-10,*trans*-14,*cis*-19 22:5	-	-	-	2.707	-	-	-	2.599	0.1668	0.584	-	-
Δ4,7,10,16,19 22:5	*cis*-4,*cis*-7,*cis*-10,*cis*-16,*cis*-19 22:5	-	-	-	0.594	-	-	-	0.494	0.0023	<0.001	-	-
Δ5,10,13,16,19 22:5	*trans*-5,*cis*-10,*cis*-13,*cis*-16,*cis*-19 22:5	-	-	-	0.465	-	-	-	0.359	0.0824	0.328	-	-
Σ 22:4 isomers		-	-	5.479^{A}	0.645^{B}	-	-	1.543^{AB}	0.834^{B}	1.1466	0.124	0.002	0.064
cis-10 *cis*-13 *cis*-16 *cis*-19 22:4[7]	-	-	-	4.470	0.403	-	-	1.265	0.220	1.0749	0.137	0.005	0.140
Δ4,7,14,19 22:4	nd	-	-	-	0.130	-	-	-	0.187	0.0351	0.248	-	-
Δ7,10,14,19 22:4	nd	-	-	-	0.350	-	-	-	0.393	0.0539	0.508	-	-
Δ7,13,16,19 22:4	*cis*-7 *cis*-13 *cis*-16 *cis*-19 22:4	-	-	0.084	-	-	-	0.026	-	0.0181	0.085	-	-
Δ8,13,16,19 + 9,13,16,19 22:4	*trans*-8 *cis*-13 *cis*-16 *cis*-19 + *trans*-9 *cis*-13 *cis*-16 *cis*-19 22:4	-	-	0.307	-	-	-	0.119	-	0.0739	0.126	-	-
Δ10,13,16,19 22:4	*trans*-10 *trans*-13 *cis*-16 *cis*-19 22:4	-	-	0.175	-	-	-	0.103	-	0.0453	0.252	-	-
Δ11,13,17,19 22:4[8]	nd	-	-	0.617	-	-	-	0.133	-	0.2076	0.145	-	-
Σ 22:3 isomers		-	-	3.913	0.071	-	-	2.988	0.059	0.3700	0.227	<0.001	0.246
cis-13 *cis*-16 *cis*-19 22:3	-	-	-	0.494	0.071	-	-	0.605	0.059	0.0908	0.587	<0.001	0.748
Δ9,16,19 22:3	*cis*-9 *cis*-16 *cis*-19 22:3	-	-	0.056	-	-	-	0.088	-	0.0184	0.157	-	-
Δ10,13,17 22:3[9]	*cis*-10 *cis*-13 *trans*-17 22:3	-	-	1.144	-	-	-	0.890	-	0.0512	0.038	-	-
Δ10,14,19 + 12,15,19 22:3	*trans*-10 *trans*-14 *cis*-19 + *trans*-12 *trans*-15 *cis*-19 22:3	-	-	0.762	-	-	-	0.305	-	0.1332	0.075	-	-
Δ11,14,17 22 :3	*trans*-11 *trans*-14 *trans*-17 22 :3	-	-	0.385	-	-	-	0.174	-	0.0537	0.059	-	-
Δ11,14,19 + 13,16,19 + 13,16,19 22:3	*trans*-11 *trans*-14 *cis*-19 + *trans*-13 *cis*-16 *trans*-19 + *cis*-13 *cis*-16 *trans*-19 22:3	-	-	0.143	-	-	-	0.094	-	0.0158	0.090	-	-
Δ11,16,19 + 13,16,19 + 13,16,19 22:3	*cis*-11 *cis*-16 *cis*-19 + *trans*-13 *cis*-16 *cis*-	-	-	0.337	-	-	-	0.367	-	0.0526	0.632	-	-

	19 + *cis*-13 *trans*-16 *cis*-19 22:3												
Δ12,16,19 22:3	*trans*-12 *cis*-16 *cis*-19 22:3	-	-	1.276	-	-	-	0.961	-	0.0789	0.058	-	-
Δ13,16,19 22:3[10]	*cis*-13 *trans*-16 *trans*-19 22:3	-	-	0.150	-	-	-	0.142	-	0.0297	0.809	-	-
Σ 22:2 isomers		-	-	2.929^{b}	0.022^{c}	-	-	4.834^{a}	0.035^{c}	0.3432	0.049	<0.001	0.004
cis-13 *cis*-16 22:2	-	-	-	0.172^{b}	0.022^{c}	-	-	0.250^{a}	0.035^{c}	0.0123	0.002	<0.001	<0.001
Δ11,17 + 13,18 22:2	*trans*-11 *trans*-17 + *trans*-13 *trans*-18 22:2	-	-	0.260	-	-	-	0.320	-	0.0336	0.148	-	-
Δ12,17 22:2	*cis*-12 *trans*-17 22:2	-	-	0.260	-	-	-	0.487	-	0.0532	0.051	-	-
Δ12,17 22:2	*trans*-12 *trans*-17 22:2	-	-	0.065	-	-	-	0.151	-	0.0144	0.027	-	-
Δ12,19 + 14,19 22:2	*trans*-12 *cis*-19 + *cis*-14 *trans*-19 22:2	-	-	0.084	-	-	-	0.210	-	0.0164	0.044	-	-
Δ14,17 22:2	*trans*-14 *cis*-17 22:2	-	-	0.755	-	-	-	1.078	-	0.1856	0.157	-	-
Δ15,19 22:2[11]	*trans*-15 *cis*-19 22:2	-	-	0.888	-	-	-	1.410	-	0.2622	0.117	-	-
Σ 22:1 isomers		0.048^{c}	0.121^{bc}	0.763^{b}	0.076^{c}	0.067^{c}	0.050^{c}	1.995^{a}	0.072^{c}	0.1784	0.005	<0.001	<0.001
cis-7 22:1	-	-	-	0.034	-	-	-	0.104	-	0.0078	0.012	-	-
cis-9 22:1	-	-	-	0.063	-	-	-	0.198	-	0.0112	0.007	-	-
cis-11 + *trans*-15 22:1[12]	-	0.009^{c}	-	0.104^{b}	0.012^{c}	0.019^{bc}	-	0.261^{a}	0.026^{bc}	0.0224	0.001	<0.001	<0.001
cis-12 22:1	-	0.017^{b}	-	0.065^{b}	0.011^{b}	0.011^{b}	-	0.235^{a}	0.010^{b}	0.0187	<0.001	<0.001	<0.001
cis-13 22:1	-	0.023^{c}	0.101^{bc}	0.384^{b}	0.053^{c}	0.037^{c}	0.037^{c}	0.861^{a}	0.035^{c}	0.0860	0.032	<0.001	0.002
cis-15 22:1	-	-	0.020^{b}	0.072^{b}	-	-	0.013^{b}	0.219^{a}	-	0.0184	0.002	<0.001	<0.001
cis-17 22:1	-	-	-	0.015	-	-	-	0.034	-	0.0018	0.009	-	-
cis-19 22:1	-	-	-	0.017	-	-	-	0.055	-	0.0028	0.006	-	-
trans-13 22:1	-	-	-	0.009	-	-	-	0.029	-	0.0033	0.027	-	-
22:0	-	0.617	0.615	0.535	0.514	0.595	0.640	0.566	0.505	0.0290	0.809	<0.001	0.265

[a-c]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of species × PUFA treatment.

[A-B]Within a row, different superscripts indicate a trend towards significance ($P < 0.10$) due to the effect of species × PUFA treatment.

[1]The incubated substrate was a TMR containing no additional PUFA (control) or supplemented with 2% DM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) or docosahexaenoic acid (DHA).

[2]Probability of significant effects due to species (Sp), PUFA treatment (T), and their interaction (Sp × T).

[3]Tentative geometry of double bonds as inferred based on retention times and elution order of FAME during GC analysis; nd = not determined.

[4]Standard error of the difference.

[5]In DHA treatment, mass spectrum was not informative and, therefore, identification was only based on retention time comparison with the standards.

[6]Coelutes with Δ10,13,16,19 22:4 (tentatively identified as *trans*-10 *trans*-13 *cis*-16 *cis*-19 22:4).

[7]Coelutes with a 22:5 isomer of indeterminate double bond positions in DHA treatment.

[8]An alternative identification as the putative coelution of two mono-conjugated 22:4 isomers (with conjugated double bond structure on Δ11,13 and Δ17,19) cannot be unequivocally excluded. Coelutes with a 22:4 isomer of indeterminate double bond position only in cows.

[9]Coelutes with Δ15,20 22:2 (tentatively identified as *cis*-15 *cis*-20 22:2).

[10]Coelutes with *cis*-16 *cis*-19 22:2.

[11]Coelutes with an unidentified fatty acid.

[12]In control and DHA treatments, no traces of *trans*-15 22:1 were detected.

Figure 1. Principal component analysis of 20- and 22-carbon fatty acids after 24-h in vitro incubation with rumen inocula from cows or ewes. Score plot projected on the basis of (a) the first and second principal components (PC1 and PC2) and (b) the first and third principal components (PC1 and PC3). The incubated substrate was a TMR containing no additional PUFA (control) or supplemented with 2% DM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) or docosahexaenoic acid (DHA)

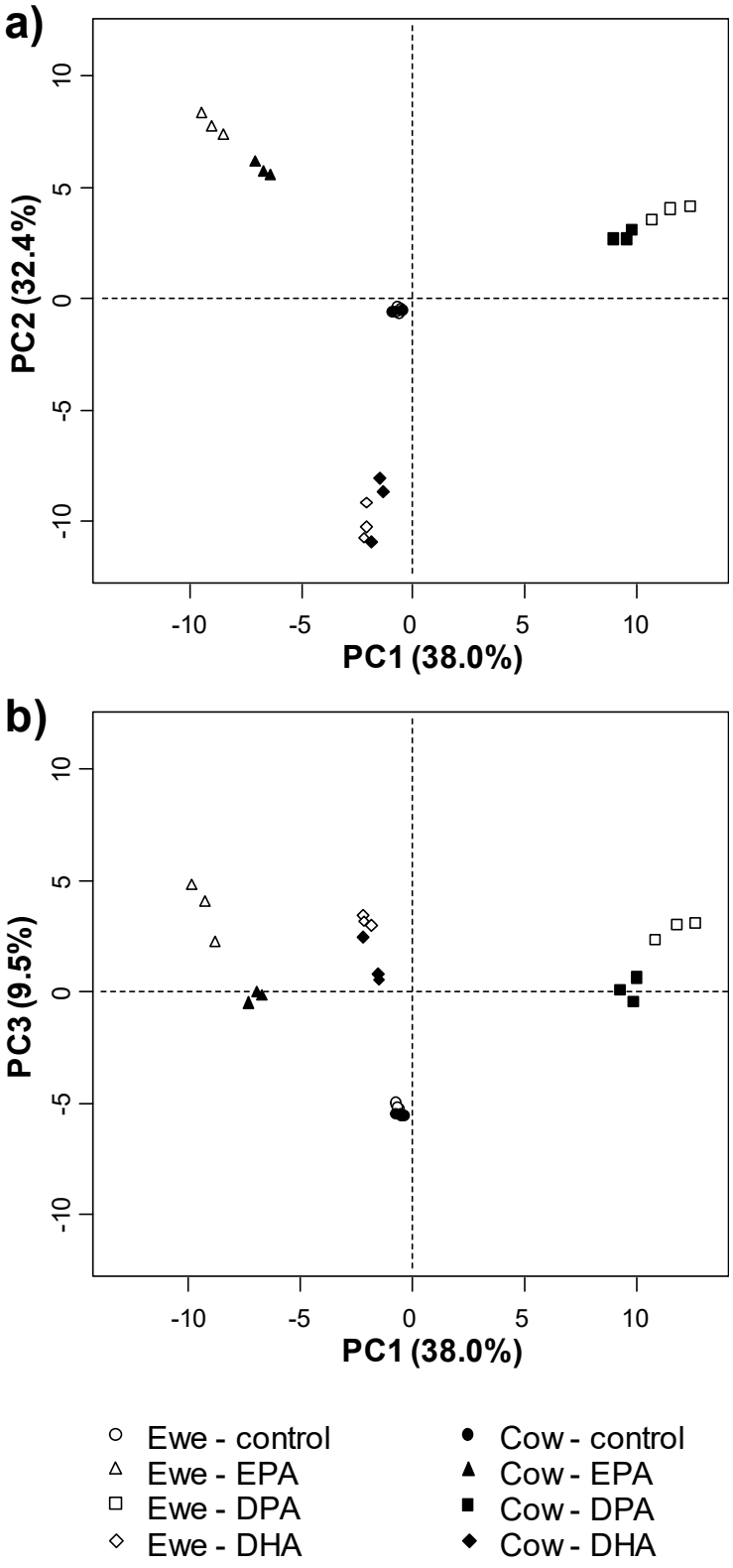
Figure 2. Principal component analysis of 20- and 22-carbon fatty acids after 24-h in vitro incubation with rumen inocula from cows or ewes. Loading plot projected on the basis of the two principal components (PC1 and PC2) and enlarged view of the three major clusters of variables. The incubated substrate was a TMR containing no additional PUFA (control) or supplemented with 2% DM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) or docosahexaenoic acid (DHA). For clarity purposes, abbreviated names of fatty acids are reported in the figure, including tentative double bond geometry (*c*, *cis*; *t*, *trans*). The suffix "-in" denotes that isomers have indeterminate double bond position.

Figure 3. Gas chromatography-electron ionization mass spectrum of the 4,4-dimethyloxaline (DMOX) derivative of (a) Δ11,13,17,19 22:4 and (b) Δ4,7,10,14,19 22:5 detected in digesta after 24-h in vitro incubation with rumen inocula from cows or ewes. For Δ11,13,17,19 22:4, an alternative identification as the coelution of two mono-conjugated 22:4 isomers (with conjugated double bond structure putatively on Δ11,13 and Δ17,19) cannot be unequivocally excluded. For Δ4,7,10,14,19 22:5, the tentative double bond geometry (*cis*, *trans*) is reported in the structural formula.

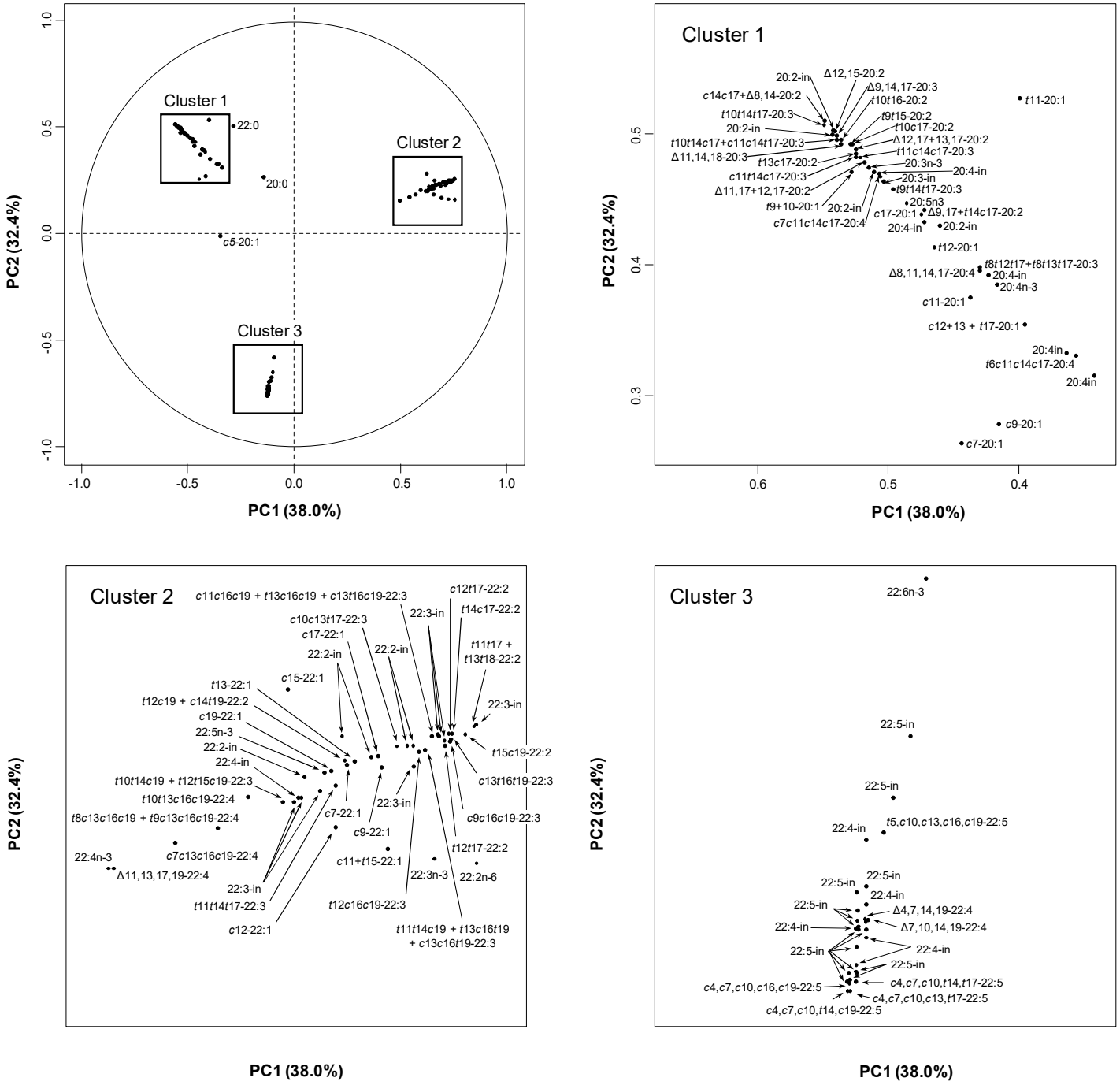
Figure 4. Putative pathways describing initial 20:5n-3 biohydrogenation. For clarity purposes,

abbreviated names of fatty acids are reported in the figure (*c*, *cis*; *t*, *trans*). Thick arrows highlight the potentially major pathway; grey arrows and text represent a hypothetical pathway involving the formation of a conjugated 20:5 intermediate (not identified in digesta yet).

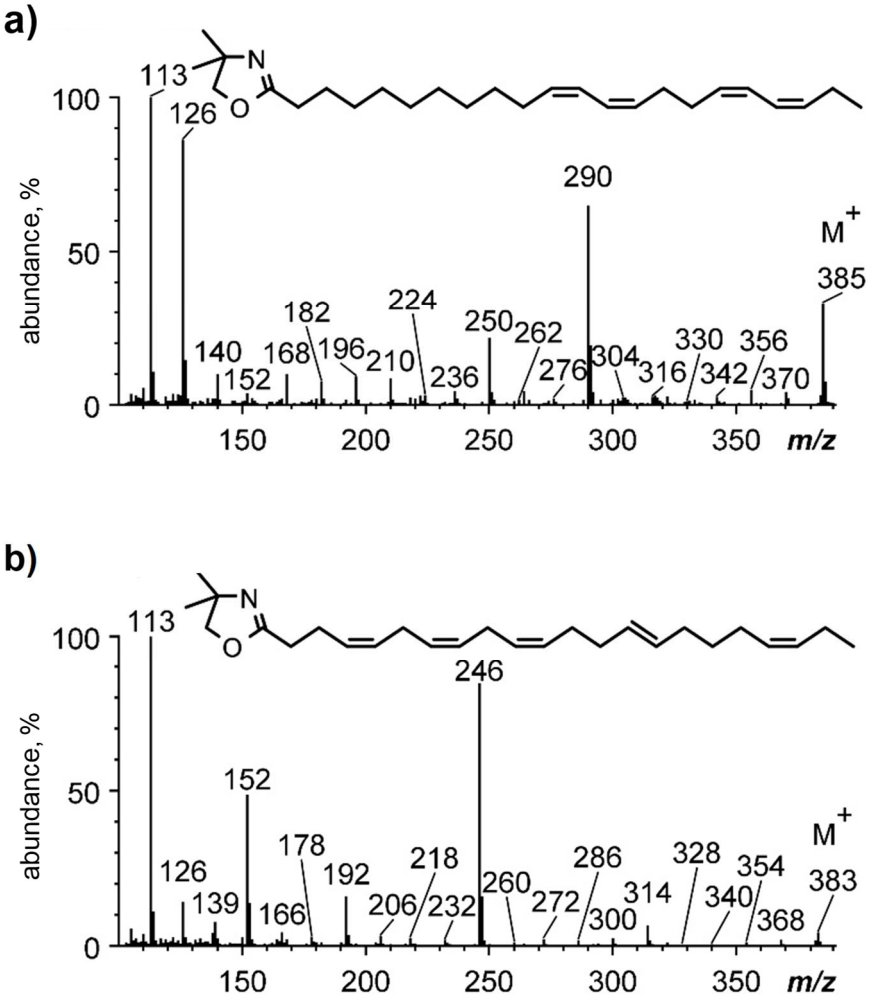
(Figure 1)


a)
PC2 (32.4%)
PC1 (38.0%)
b)
PC3 (9.5%)
PC1 (38.0%)
Ewe - control
Ewe - EPA
Ewe - DPA
Ewe - DHA
Cow - control
Cow - EPA
Cow - DPA
Cow - DHA

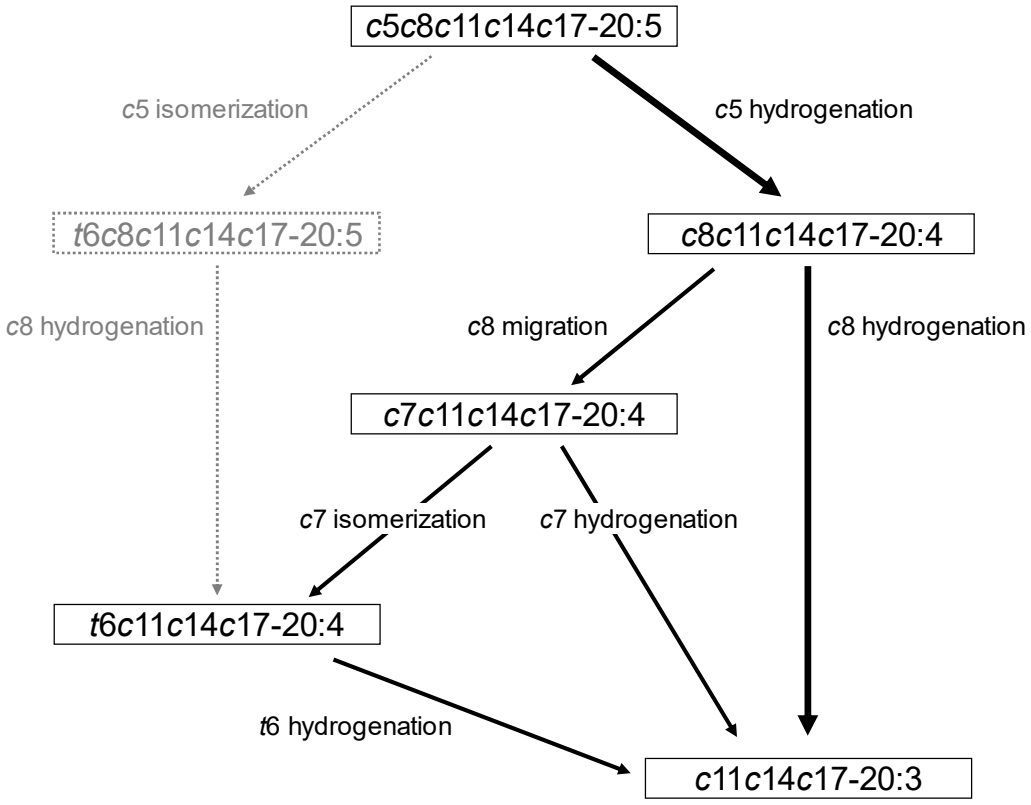
(Figure 2)


Cluster 1
Cluster 2
Cluster 3
22:0
20:0
c5-20:1
PC1 (38.0%)
PC2 (32.4%)
Cluster 1
20:2-in
Δ12,15-20:2
Δ9,14,17-20:3
c14c17+Δ8,14-20:2
t10t16-20:2
t11-20:1
t10t14t17-20:3
t9t15-20:2
20:2-in
t10c17-20:2
t10t14c17+c11c14t17-20:3
Δ12,17+13,17-20:2
Δ11,14,18-20:3
t11c14c17-20:3
t13c17-20:2
20:3n-3
20:4-in
c11t14c17-20:3
20:3-in
Δ11,17+12,17-20:2
t9t14t17-20:3
20:5n3
t9+10-20:1
20:2-in
c17-20:1
Δ9,17+t14c17-20:2
c7c11c14c17-20:4
20:4-in
20:2-in
t12-20:1
t8t12t17+t8t13t17-20:3
Δ8,11,14,17-20:4
20:4-in
20:4n-3
c11-20:1
c12+13 + t17-20:1
20:4in
t6c11c14c17-20:4
20:4in
c9-20:1
c7-20:1
PC1 (38.0%)
PC2 (32.4%)
Cluster 2
c11c16c19 + t13c16c19 + c13t16c19-22:3
c12t17-22:2
22:3-in
t14c17-22:2
c10c13t17-22:3
c17-22:1
t11t17 +
t13t18-22:2
22:2-in
22:2-in
c15-22:1
t13-22:1
t12c19 + c14t19-22:2
22:3-in
c19-22:1
22:5n-3
t15c19-22:2
22:2-in
22:4-in
c13t16t19-22:3
t10t14c19 + t12t15c19-22:3
t10t13c16c19-22:4
22:3-in
c7-22:1
c9c16c19-22:3
t8c13c16c19 + t9c13c16c19-22:4
c9-22:1
22:4n-3
c7c13c16c19-22:4
t12t17-22:2
c11+t15-22:1
Δ11,13,17,19-22:4
22:3n-3
22:2n-6
22:3-in
t12c16c19-22:3
t11t14t17-22:3
c12-22:1
t11t14c19 + t13c16t19
+ c13c16t19-22:3
PC1 (38.0%)
PC2 (32.4%)
Cluster 3
22:6n-3
22:5-in
22:5-in
22:4-in
t5,c10,c13,c16,c19-22:5
22:5-in
22:5-in
22:4-in
22:5-in
Δ4,7,14,19-22:4
22:4-in
Δ7,10,14,19-22:4
22:5-in
22:4-in
22:5-in
c4,c7,c10,c16,c19-22:5
c4,c7,c10,t14,t17-22:5
c4,c7,c10,c13,t17-22:5
c4,c7,c10,t14,c19-22:5
PC1 (38.0%)
PC2 (32.4%)

(Figure 3)


a)
113
126
290
M+
385
182
224
250
262
140
168
196
210
236
276
304
316
330
342
356
370
152
100
50
0
abundance, %
150
200
250
300
350
m/z
b)
113
246
152
126
178
192
218
260
286
328
354
383
139
166
206
232
272
300
314
340
368
M+
100
50
0
abundance, %
150
200
250
300
350
m/z

(Figure 4)


c5c8c11c14c17-20:5
c5 isomerization
c5 hydrogenation
t6c8c11c14c17-20:5
c8c11c14c17-20:4
c8 hydrogenation
c8 migration
c8 hydrogenation
c7c11c14c17-20:4
c7 isomerization
c7 hydrogenation
t6c11c14c17-20:4
t6 hydrogenation
c11c14c17-20:3

Supplementary Table 1. Effect of very long chain n-3 PUFA on the concentration of other fatty acids (g/100 g of total fatty acids) after 24-h in vitro incubation with rumen inocula from cows or ewes[1,2]

	Cow				Ewe					*P*-value[3]		
	Control	EPA	DPA	DHA	Control	EPA	DPA	DHA	SED[4]	Sp	T	Sp × T
12:0	0.117	0.091	0.094	0.093	0.098	0.081	0.100	0.092	0.0157	0.656	0.068	0.373
13:0	0.048	0.042	0.041	0.042	0.109	0.095	0.087	0.103	0.0053	0.004	0.006	0.126
iso 13:0	0.076	0.079	0.064	0.080	0.082	0.084	0.072	0.093	0.0060	0.223	<0.001	0.596
anteiso 13:0	0.009	0.011	0.008	0.009	0.008	0.012	0.010	0.008	0.0044	0.975	0.155	0.567
14:0	1.386	1.214	1.209	1.201	1.463	1.276	1.277	1.351	0.0435	0.001	<0.001	0.466
iso 14:0	0.177^{A}	0.150^{AB}	0.138^{B}	0.141^{B}	0.094^{C}	0.086^{C}	0.075^{C}	0.092^{C}	0.0087	0.006	0.003	0.071
15:0	1.302	1.144	1.112	1.021	1.219	1.071	1.037	0.966	0.0561	0.226	<0.001	0.913
iso 15:0	0.282	0.292	0.211	0.258	0.325	0.326	0.276	0.355	0.0259	<0.001	0.008	0.364
anteiso 15:0	0.966^{a}	0.939^{ab}	0.755^{bc}	0.911^{ab}	0.644^{cd}	0.611^{cd}	0.529^{d}	0.698^{cd}	0.0516	<0.001	0.001	<0.001
16:0	17.354	14.497	14.184	14.710	17.170	14.661	14.183	14.917	0.4910	0.903	<0.001	0.887
iso 16:0	0.292	0.240	0.206	0.237	0.249	0.213	0.193	0.234	0.0137	0.007	<0.001	0.215
cis-6 16:1	0.035^{cd}	0.050^{bcd}	0.031^{d}	0.059^{bcd}	0.078^{bc}	0.083^{b}	0.048^{bcd}	0.185^{a}	0.0113	0.011	<0.001	<0.001
cis-9 16:1	0.030	0.059	0.035	0.062	0.046	0.035	0.024	0.044	0.0131	0.416	0.046	0.096
trans-12 16:1	0.036	0.037	0.029	0.061	0.038	0.036	0.029	0.059	0.0067	0.990	<0.001	0.965
trans-13 16:1	0.028	0.017	0.017	0.021	0.017	0.014	0.016	0.018	0.0040	0.047	0.116	0.377
17:0	0.863^{a}	0.691^{b}	0.714^{b}	0.701^{b}	0.849^{a}	0.749^{b}	0.740^{b}	0.723^{b}	0.0204	0.319	<0.001	0.011
iso 17:0	0.308^{C}	0.323^{C}	0.302^{C}	0.313^{C}	0.530^{A}	0.540^{A}	0.503^{B}	0.541^{A}	0.0313	0.019	<0.001	0.082
anteiso 17:0	0.376	0.361	0.309	0.362	0.463	0.429	0.383	0.450	0.0320	0.104	<0.001	0.828
iso 18:0	0.069	0.059	0.059	0.064	0.094	0.077	0.081	0.080	0.0044	0.029	<0.001	0.227
19:0	0.134^{e}	0.269^{bc}	0.166^{de}	0.220^{cd}	0.314^{b}	0.416^{a}	0.314^{b}	0.254^{bc}	0.0185	0.006	<0.001	<0.001
cis-11 19:1	0.028^{d}	0.107^{a}	0.038^{cd}	0.069^{b}	0.031^{cd}	0.074^{b}	0.026^{d}	0.052^{bc}	0.0062	<0.001	<0.001	0.009
S3,R7,R11,15-tetramethyl 16:0	0.134	0.192	0.181	0.176	0.244	0.307	0.298	0.304	0.0125	0.007	<0.001	0.547
R3,R7,R11,15-tetramethyl 16:0	0.330	0.228	0.232	0.232	0.372	0.277	0.300	0.268	0.0227	0.081	<0.001	0.220
21:0	0.075^{cd}	0.083^{cd}	0.060^{d}	0.056^{d}	0.119^{b}	0.189^{a}	0.101^{bc}	0.076^{cd}	0.0093	<0.001	<0.001	<0.001
cis-12 21:1	-	0.036^{b}	-	-	0.035^{b}	0.066^{a}	0.042^{b}	0.043^{b}	0.0061	0.023	<0.001	0.011

cis-6 *cis*-9 *cis*-12 *cis*-15 *cis*-18 21:5	-	-	0.072	-	-	-	0.045	-	0.0173	0.261	-	-
23:0[5]	0.259	0.427	0.613	0.226	0.398	0.319	0.748	0.184	0.1007	0.545	<0.001	0.246
cis-14 23:1[6]	0.020^{c}	0.186^{bc}	0.314^{b}	0.021^{c}	0.030^{c}	0.042^{c}	0.547^{a}	0.023^{c}	0.0584	0.400	<0.001	0.003
cis-15 24:1	0.059	0.082	0.080	0.047	0.058	0.079	0.089	0.053	0.0067	0.442	<0.001	0.556
25:0[7]	0.144	0.119	0.229	0.149	0.151	0.119	0.180	0.184	0.0230	0.908	0.002	0.132
cis-16 25:1	0.055	0.050	0.057	0.000	0.058	0.044	0.036	0.000	0.0101	0.471	<0.001	0.288
26:0	0.547	0.485	0.506	0.481	0.682	0.612	0.594	0.572	0.0340	0.010	0.004	0.524
cis-17 26:1	-	-	0.095	-	-	-	0.049	-	0.0232	0.115	-	-
27:0	0.004^{B}	0.004^{B}	0.058^{A}	0.030^{AB}	0.049^{A}	0.042^{AB}	0.063^{A}	0.060^{A}	0.0113	0.015	<0.001	0.066
28:0	0.250	0.231	0.240	0.253	0.276	0.266	0.271	0.269	0.0219	0.183	0.583	0.872
cis-9 28:1	-	0.021	0.039	0.038	-	0.025	0.033	0.040	0.0052	0.925	<0.001	0.612
29:0	0.057	0.040	0.045	0.041	0.054	0.050	0.052	0.045	0.0055	0.118	0.028	0.458
30:0	0.540^{BCD}	0.439^{D}	0.455^{CD}	0.448^{CD}	0.676^{AB}	0.609^{AB}	0.696^{A}	0.594^{ABC}	0.0406	0.006	0.003	0.087

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$) due to the effect of species × PUFA treatment.

[A-D]Within a row, different superscripts indicate a trend towards significance ($P < 0.10$) due to the effect of species × PUFA treatment.

[1]The incubated substrate was a TMR containing no additional PUFA (control) or supplemented with 2% DM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DPA) or docosahexaenoic acid (DHA).

[2]Data on 18-carbon fatty acid profile were reported in Toral et al. (2017). J. Dairy Sci. 100:6187-6198.

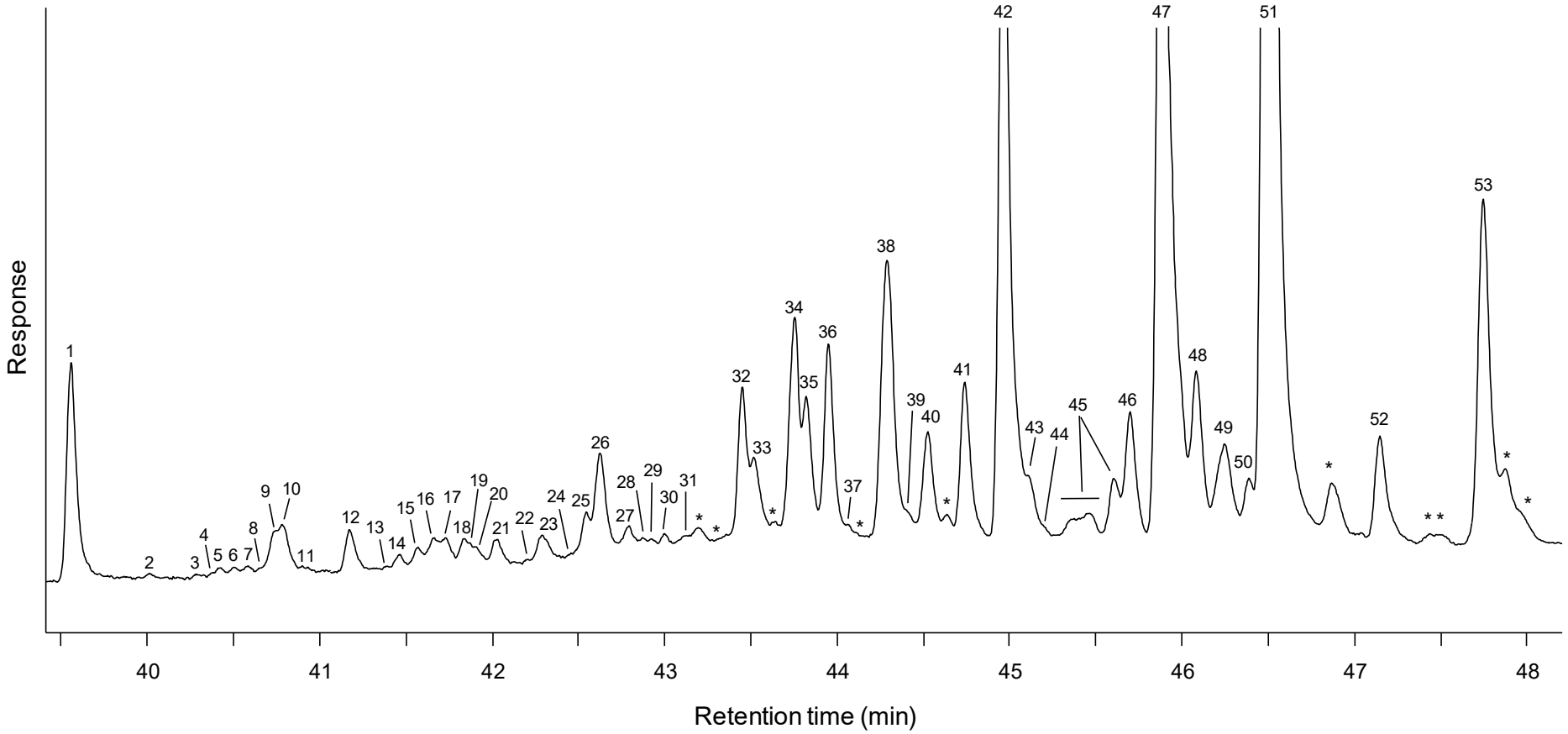
[3]Probability of significant effects due to species (Sp), PUFA treatment (T), and their interaction (Sp × T).

[4]Standard error of the difference.

[5]Coelutes with Δ7,11,14,17 + Δ8,11,14,17 20:4 in EPA treatment and *trans*-14 *cis*-19 22:2 in DPA treatment.

[6]Coelutes with Δ10,20 22:2 and Δ10,13,17 22:3 (tentatively identified as *cis*-10 *cis*-20 22:2 and *trans*-10 *trans*-13 *trans*-17 22:3, respectively) in DPA treatment.

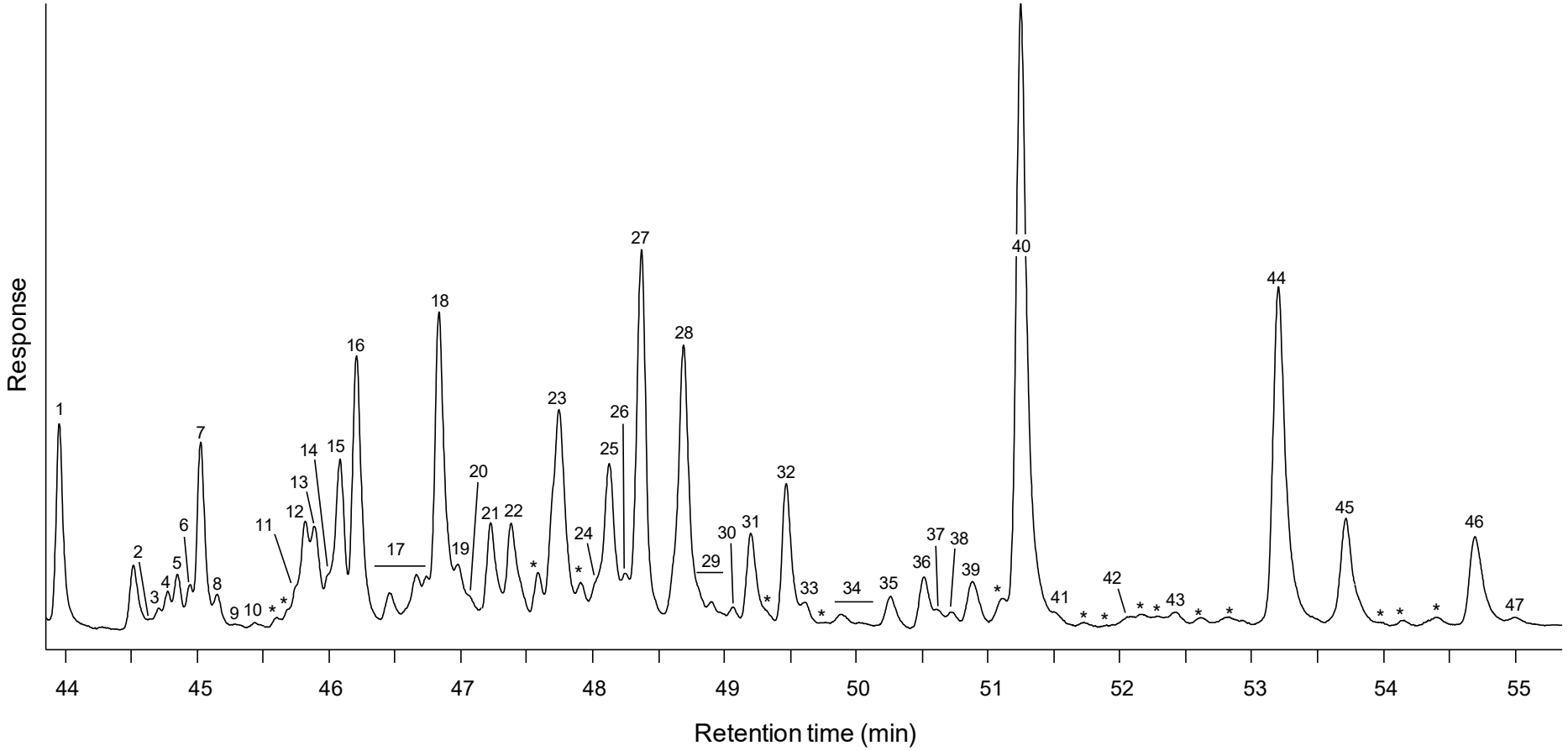
[7]Coelutes with 22:2 of indeterminate double bond position in DHA treatment.



Supplementary Figure 1. Partial gas chromatogram showing the separation of 20-carbon FAME prepared from lipids in digesta samples after 24-h in vitro incubation with rumen inocula from cows. The incubated substrate was a TMR supplemented with 2% DM of eicosapentaenoic acid (EPA). The y-axis represents arbitrary response units.

Peak identification (includes tentative geometry of double bonds as inferred based on retention times and elution order of FAME during gas chromatography analysis): 1 = 20:0; 2 = *trans*-9 *trans*-12 *cis*-15 + *cis*-9 *cis*-12 *trans*-15 18:3; 3 = *trans*-9+10 20:1; 4 = *cis*-5 20:1; 5 = *trans*-11 20:1; 6 = *trans*-12 20:1; 7 = *cis*-7 + *trans*-13 20:1; 8 = *cis*-9 + *trans*-14 20:1; 9 = *cis*-9 *cis*-12 *cis*-15 18:3; 10 = *cis*-11 + *trans*-15+16 20:1; 11 = *cis*-

12+13 + *trans*-17 20:1; 12 = *cis*-9 *trans*-11 18:2 (contains *cis*-14 20:1 as a minor component); 13 = *cis*-11 *trans*-13 18:2 + *cis*-16 20:1; 14 = *trans*-10 *cis*-12 18:2; 15 = *trans*-9 *trans*-15 20:2; 16 = *cis*-17 20:1; 17 = *trans*-10 *trans*-16 20:2 + *trans*-11 *cis*-13 18:2; 18 = 21:0; 19 = Δ11,17 + 12,17 20:2; 20 = Δ12,17 + 13,17 20:2; 21 = Δ12,15 20:2; 22 = *trans*-11 *trans*-13 18:2; 23 = *trans*-7 *trans*-9 + *trans*-8 *trans*-10 + *trans*-9 *trans*-11 18:2; 24 = 20:2 isomer of indeterminate double bond position; 25 = *trans*-10 *cis*-17 20:2; 26 = *trans*-13 *cis*-17 20:2; 27 = *cis*-11 *cis*-14 20:2 + 20:2 isomer of indeterminate double bond position; 28 = 20:2 isomer of indeterminate double bond position; 29 = *cis*-12 21:1; 30 = Δ9,17 + *trans*-14 *cis*-17 20:2; 31 = 20:2 isomer of indeterminate double bond position; 32 = *cis*-14 *cis*-17 20:2 + Δ8,14 20:2 + *trans*-6 *trans*-12 *trans*-17 20:3; 33 = *trans*-9 *trans*-14 *trans*-17 20:3; 34 = *trans*-10 *trans*-14 *trans*-17 20:3; 35 = *trans*-8 *trans*-12 *trans*-17 + *trans*-8 *trans*-13 *trans*-17 20:3; 36 = 22:0; 37 = Δ9,14,17 20:3; 38 = *trans*-10 *trans*-14 *cis*-17 + *cis*-11 *cis*-14 *trans*-17 20:3; 39 = Δ11,14,18 20:3 + 20:3 isomer of indeterminate double bond position; 40 = *cis*-11 *trans*-14 *cis*-17 20:3; 41 = *trans*-11 *cis*-14 *cis*-17 20:3; 42 = *cis*-11 *cis*-14 *cis*-17 20:3; 43 = *cis*-13 22:1; 44 = *cis*-15 22:1; 45 = 20:4 isomers of indeterminate double bond position; 46 = Δ8,11,14,17 20:4; 47 = *trans*-6 *cis*-11 *cis*-14 *cis*-17 20:4; 48 = 23:0 + Δ7,11,14,17 20:4 + Δ8,11,14,17 20:4; 49 = 20:4 isomer of indeterminate double bond position; 50 = *cis*-7 *cis*-11 *cis*-14 *cis*-17 20:4; 51 = *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:4; 52 = *cis*-14 23:1; 53 = *cis*-5 *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:5; * = unidentified peaks.

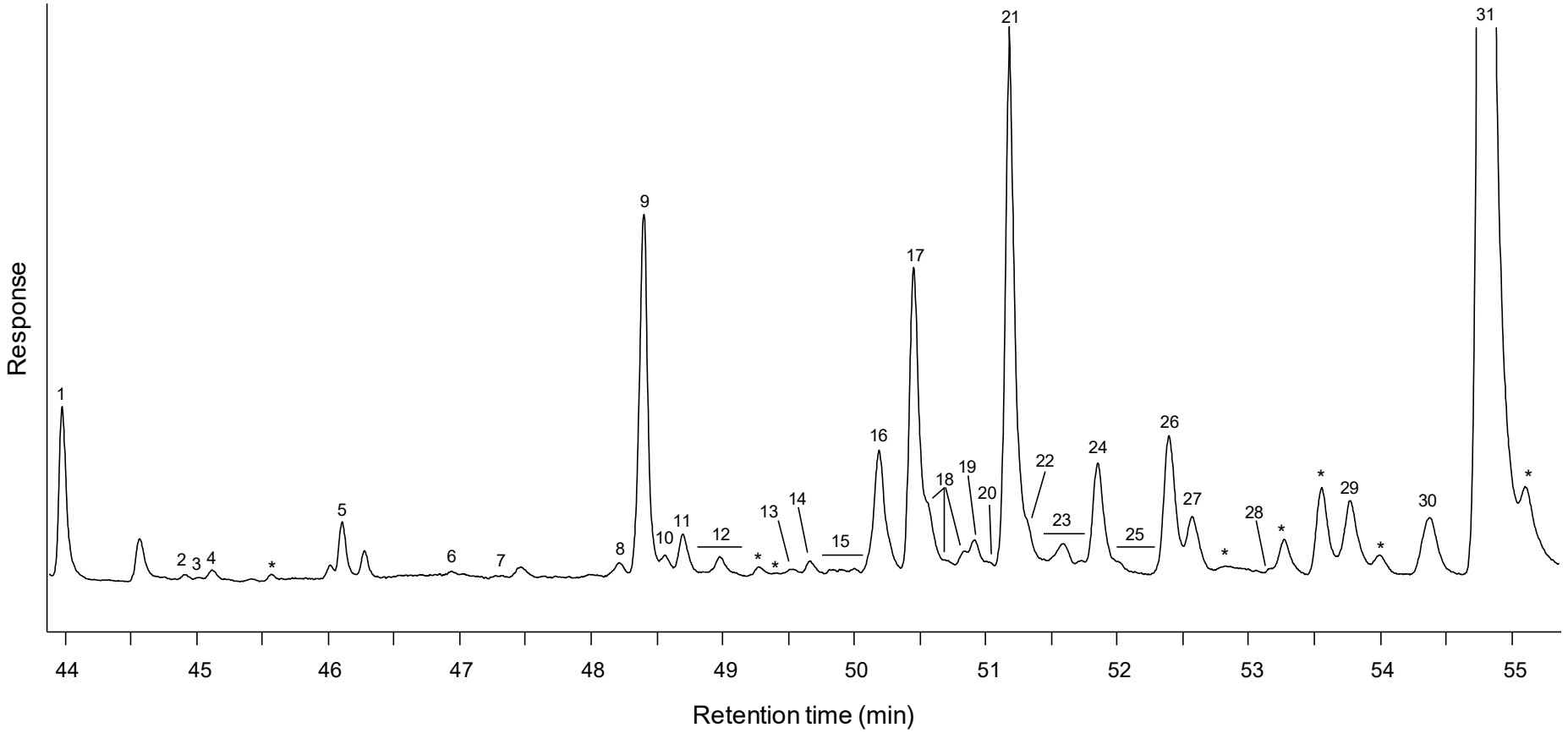


Supplementary Figure 2. Partial gas chromatogram showing the separation of 22-carbon FAME prepared from lipids in digesta samples after 24-h in vitro incubation with rumen inocula from cows. The incubated substrate was a TMR supplemented with 2% DM of docosapentaenoic acid (DPA). The y-axis represents arbitrary response units.

Peak identification (includes tentative geometry of double bonds as inferred based on retention times and elution order of FAME during gas chromatography analysis): 1 = 22:0; 2 = *trans*-13 22:1; 3 = *cis*-7 22:1; 4 = *cis*-9 22:1; 5 = *cis*-11 + *trans*-15 22:1; 6 = *cis*-12 22:1; 7 = *cis*-13 22:1; 8 = *cis*-15 22:1; 9 = *cis*-17 22:1; 10 = *cis*-19 22:1; 11 = *trans*-12 *trans*-17 22:2; 12 = *trans*-11 *trans*-17 + *trans*-13 *trans*-18 22:2; 13 = *cis*-12 *trans*-

17 22:2; 14 = *trans*-12 *cis*-19 + *cis*-14 *trans*-19 22:2; 15 = 23:0 + *trans*-14 *cis*-19 22:2; 16 = *trans*-14 *cis*-17 22:2; 17 = 22:2 isomers of indeterminate double bond position; 18 = *trans*-15 *cis*-19 22:2 + unidentified fatty acid; 19 = *cis*-13 *cis*-16 22:2; 20 = 22:2 isomer of indeterminate double bond position; 21 = *cis*-14 23:1 + *cis*-10 *cis*-20 22:2 + *trans*-10 *trans*-13 *trans*-17 22:3; 22 = *trans*-11 *trans*-14 *trans*-17 22:3; 23 = *cis*-10 *cis*-13 *trans*-17 22:3 + *cis*-15 *cis*-20 22:2; 24 = *cis*-13 *trans*-16 *trans*-19 22:3 + *cis*-16 *cis*-19 22:2; 25 = *trans*-10 *trans*-14 *cis*-19 + *trans*-12 *trans*-15 *cis*-19 22:3; 26 = *trans*-11 *trans*-14 *cis*-19 + *trans*-13 *cis*-16 *trans*-19 + *cis*-13 *cis*-16 *trans*-19 22:3; 27 = 24:0 + dimethylacetal; 28 = *trans*-12 *cis*-16 *cis*-19 22:3; 29 = 22:3 isomers of indeterminate double bond position; 30 = *cis*-9 *cis*-16 *cis*-19 22:3; 31 = *cis*-11 *cis*-16 *cis*-19 + *trans*-13 *cis*-16 *cis*-19 + *cis*-13 *trans*-16 *cis*-19 22:3; 32 = *cis*-13 *cis*-16 *cis*-19 22:3; 33 = *cis*-15 24:1; 34 = 22:3 isomers of indeterminate double bond position; 35 = *trans*-10 *trans*-13 *cis*-16 *cis*-19 22:4; 36 = *trans*-8 *cis*-13 *cis*-16 *cis*-19 + *trans*-9 *cis*-13 *cis*-16 *cis*-19 22:4; 37 = *cis*-6 *cis*-9 *cis*-12 *cis*-15 *cis*-18 21:5; 38 = *cis*-7 *cis*-13 *cis*-16 *cis*-19 22:4; 39 = 25:0; 40 = *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:4; 41 = 22:3 + 22:4 isomers of indeterminate double bond position; 42 = *cis*-16 25:1; 43 = 22:3 isomer of indeterminate double bond position; 44 = *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:5; 45 = 26:0; 46 = Δ11,13,17,19 22:4 + 22:4 isomer of indeterminate double bond position[1]; 47 = *cis*-17 26:1; * = unidentified peaks.

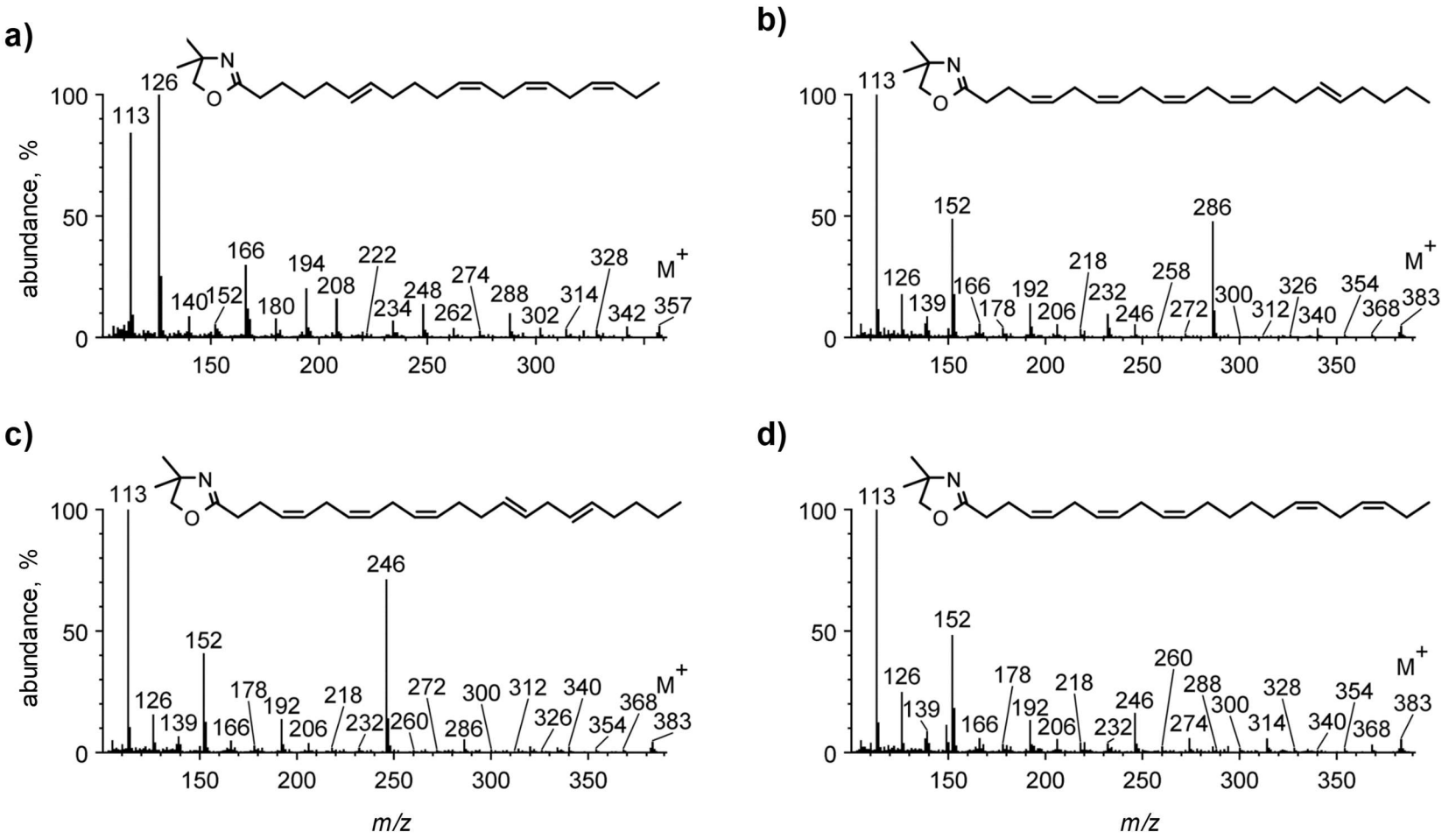
[1]This coelution was not detected in ewes. An alternative identification as the coelution of two mono-conjugated 22:4 isomers (with conjugated double bond structure putatively on Δ11,13 and Δ17,19) cannot be unequivocally excluded.



Supplementary Figure 3. Partial gas chromatogram showing the separation of 22-carbon FAME prepared from lipids in digesta samples after 24-h in vitro incubation with rumen inocula from cows. The incubated substrate was a TMR supplemented with 2% DM of docosahexaenoic acid (DHA). The y-axis represents arbitrary response units.

Peak identification (includes tentative geometry of double bonds as inferred based on retention times and elution order of FAME during gas chromatography analysis): 1 = 22:0; 2 = *cis*-11 22:1; 3 = *cis*-12 22:1; 4 = *cis*-13 22:1; 5 = 23:0; 6 = *cis*-13 *cis*-16 22:2; 7 = *cis*-14 23:1; 8 = Δ4,7,14,19-22:4; 9 = 24:0 + dimethylacetal; 10 = 22:4 isomer of indeterminate double bond position; 11 = Δ7,10,14,19-22:4; 12 = 22:4 isomers of

indeterminate double bond position; 13 = *cis*-13 *cis*-16 *cis*-19 22:3; 14 = *cis*-15 24:1; 15 = 22:5 isomers of indeterminate double bond position; 16 = *cis*-4 *cis*-7 *cis*-10 *trans*-14 *trans*-17 22:5 + *trans*-10 *trans*-13 *cis*-16 *cis*-19 22:4; 17 = *cis*-4 *cis*-7 *cis*-10 *cis*-13 *trans*-17 22:5; 18 = 22:5 isomers of indeterminate double bond position; 19 = 25:0; 20 = 22:5 isomer of indeterminate double bond position; 21 = *cis*-4 *cis*-7 *cis*-10 *trans*-14 *cis*-19 22:5; 22 = *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:4 + 22:5 isomer of indeterminate double bond position; 23 = 22:5 isomers of indeterminate double bond position; 24 = *cis*-4 *cis*-7 *cis*-10 *cis*-16 *cis*-19 22:5; 25 = 22:5 isomers of indeterminate double bond position; 26 = *trans*-5 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:5; 27 = 22:5 isomer of indeterminate double bond position; 28 = *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:5; 29 = 26:0; 30 = 22:5 isomer of indeterminate double bond position; 31 = *cis*-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:6; * = unidentified peaks.



Supplementary Figure 4. Gas chromatography-electron ionization mass spectrum of the 4,4-dimethyloxaline (DMOX) derivative of (a) Δ6,11,14,17 20:4, (b) Δ4,7,10,13,17 22:5, (c) Δ4,7,10,14,17 22:5, and (d) Δ4,7,10,16,19 22:5 detected in digesta after 24-h in vitro incubation with rumen inocula from cows or ewes. Tentative double bond geometry (*cis*, *trans*) is reported in structural formula.