N-6 and n-3 fatty acids in different beef adipose tissues depending on the presence or absence of the gene responsible for double-muscling

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ABSTRACT: Levels of n-6 and n-3 PUFAs, including those with 20 and 22 carbon-chains, in concentrate-fed Asturiana de los Valles (AV) yearling bulls with and without the double muscling gene (*mh/mh* = 24, *mh/+* = 26, +/+ = 25) were measured to examine if this gene influences the pattern of PUFA deposition in different adipose tissues. Fatty acid compositions of muscle tissue (*longissimus thoracis*) and intermuscular and subcutaneous adipose tissues were determined by gas chromatography. The composition of intramuscular fat was unique compared to the other two adipose tissues which were similar in composition. In general, n-6 and n-3 fatty acid elongation and desaturation products were affected by AV genotype and this effect was most evident in n-3 PUFAs of the intramuscular fat of *mh/mh* (n-6/n-3 = 11.8 and 18:2n-6/18:3n-3 = 25.3) compared to *mh/+* and +/+ animals (mean values of n-6/n-3 = 9.86 and 18:2n-6/18:3n-3 = 15.5). PUFA elongation and desaturation end products did not accumulate to any great extent in intermuscular and subcutaneous adipose tissues. Beef from *mh/mh* cattle showed greater deposition rates of n-3 elongation and desaturation products but their absolute content of total n-3 fatty acids was lower (21 mg/100 g meat) in comparison with *mh/+* and +/+ cattle (mean value of 25 mg/100 g meat).

Keywords: polyunsaturated fatty acids; intramuscular; intermuscular; subcutaneous fat; genotype; double-muscling; breed

In Asturias (Northern Spain), the Asturiana de los Valles (AV) beef breed represents more than 90% of calves, heifers and yearling bulls (Pérez, 2005). It is characterised by a high proportion of double-muscling animals, high lean yields, and beef with a reduced fat content (Martínez et al., 2003; Oliván et al., 2004). Due to the lean nature of double-muscled animals, the intramuscular fatty acid composition of this breed and/or genotype differs from the composition of other breeds (Bureš et al., 2006). The unsaturated nature of the intramuscular fat of AV double-muscled bulls could be partly explained by the smaller adipocyte sizes or the low content of mature adipocytes (Harper and Pethick, 2004) and the relatively high ratio of phospholipid to triacylglycerol, while the opposite effect is associated with fatter beef breeds (Aldai et al., 2007a). In contrast, however, the fatty acid composition of tissues composed primarily of adipocytes (i.e. intermuscular and subcutaneous adipose tissues) is less subject to change between carriers and non-carriers of the *mh* (muscular hypertrophy, double muscling) gene, probably due to their lower contribution of polar to total lipid content (Aldai et al., 2007a).

Lipids of animal origin can be significant sources of fatty acids for the human diet. Human nutritionists recommend a low intake of saturated fatty acids (SFA) and higher intakes of polyunsaturated fatty acids (PUFA) (Mataix et al., 2001) especially longer chain n-3 fatty acids, at the expense of n-6 fatty acids, due to their positive effects on heart health (Connor, 1994, 2000; Larsson et al., 2004). North American and Western diets typically have a n-6 to n-3 ratio of > 20-30:1 due largely to an increased consumption of linoleic acid-rich vegetable oils (Stephen and Wald, 1990; Stephen and Sieker, 1994), whereas the ideal ratio is thought to be 4:1 or less (Simopoulos, 1999), and there is a considerable emphasis on trying to correct or improve this imbalance. The main n-6 and n-3 PUFA in cattle feed are linoleic acid (18:2n-6) and linolenic acid (18:3n-3), respectively. If these escape biohydrogenation in the rumen, they can be digested, absorbed and incorporated into body tissues. Once incorporated into tissues, 18:2n-6 and 18:3n-3 can be left intact, oxidized or subjected to elongation and desaturation (Gunstone, 1986). It is known that the n-6 and n-3 families of PUFA use the same enzymes in their elongation and desaturation (Bazan et al., 1982; Sprecher, 2000) and *in vitro* 18:3n-3 is preferred to 18:2n-6 as a substrate (Brenner and Peluffo, 1966, 1969). In addition, elongation and desaturation products can influence enzyme activity (Liou et al., 2007), and there may be competitive interactions between 18- and 20- carbon metabolites with longer chain PUFA biosynthesis (Geiger et al., 1993), but there are only a few reports on how beef genotype may influence these processes (Raes et al., 2001; Aldai et al., 2006a) with 30 and 12 animals, respectively. The aim of the present study was, therefore, to analyse the full series of n-6 and n-3 PUFAs, including those with 20 and 22 carbon-chains, in AV Spanish beef cattle with and without the double muscling gene (*mh/mh*, *mh/+*, *+/+*) to examine its influence on the pattern of PUFA deposition.

MATERIAL AND METHODS

Samples

Animals and management

Seventy-five AV yearling bulls were studied during three consecutive years with similar animal repetition per year. Animals were classified into three groups depending on the presence of the 11-bp deletion in the coding sequence of the myostatin gene causing double-muscling in cattle (Grobet et al., 1998): AV double-muscled (*mh/mh*, *n* = 24), AV heterozygote (*mh/+*, *n* = 26) and AV normal (*+/+*, *n* = 25).

Calves suckled their mothers from birth (winter) to weaning (early autumn). After a postweaning adjustment period of about 15 days, they were fattened by feeding concentrate (84% barley, 10% soybean meal, 3% soybean oil, 3% minerals, vitamins and oligoelements) and barley straw, both *ad libitum* (typical Spanish fattening diet), in the housing facilities of SERIDA Research Institute. Chemical and major fatty acid composition of the concentrate meal was published previously in Aldai et al. (2007b).

Animals were slaughtered when they reached an average live weight of 542 ± 4 kg (age of 460 ± 4 days). Slaughtering was performed in a commercial abattoir according to standard procedures. Yearling bulls were weighed twice (on the day prior to slaughter and on the day of slaughter) to get the final average live weight. After slaughtering and dressing, carcasses were chilled at 3°C for 24 h. The average carcass weight (calculated by subtracting 2% from the hot carcass weight) was 321 ± 2 kg.

Sampling

Twenty-four hours *post-mortem,* the rib joints from the 6th and 9th ribs of the left half-carcasses were removed and transported to the laboratory. From the 8th rib, a *longissimus thoracis* steak and intermuscular and subcutaneous adipose tissue samples were dissected, vacuum packed and frozen at –80°C for subsequent fatty acid analysis.

Methods

Determination of the fatty acids

For n-6 and n-3 fatty acid determination, duplicate 1 g muscle tissue and duplicate 30 mg adipose tissue (intermuscular and subcutaneous independently) were saponified in 6 ml 5M KOH in methanol/water (50:50, v/v) at 60°C for 1 h, and the extracted fatty acids were methylated with trimethylsilyl-diazomethane in methanol:toluene (2:1, v/v) at 40°C for 10 min based on a modification of the method by Elmore et al. (1999) and validated in Aldai et al. (2006b).

Analyses were performed using a Varian Star CX3400 GC equipped with a FID detector and automatic sample injector. Fatty acid methyl esters were separated using a CP-Sil 88 (100 m × 0.25 mm i.d., 0.2 µm film thickness) column (Varian). The temperature program started at 100°C followed by ramping to 170°C at 2°C/min, holding for 15 min, ramping to 180°C at 0.5°C/min and then to 200°C at 10°C/min, holding for 10 min, and then ramping to 230°C at 2°C/min and holding for 10 min. The injector and detector ports were set at 250°C and 300°C, respectively. The carrier gas was helium with a flow rate of 2 ml/min.

Fatty acid methyl esters were identified according to peak retention times using standards (Sigma-Aldrich), and quantified using chromatographic peak area according to the internal standard method using 21:0 as the internal standard with its addition prior to saponification. The response factors of the individual fatty acids were previously calculated.

Statistical analysis

The statistical analysis was conducted using SPSS 12.0 statistical software package for Windows (SPSS, 2003). The effects of genotype (*mh/mh*, *mh/+*, *+/+*) on each n-6 and n-3 fatty acid series and ratios, for each adipose tissue, were studied by ANOVA analysis. Year and genotype per year were also included in the model. When the analysis of variance gave significant differences between genotypes, the LSD *post-hoc* test was applied (multiple comparisons of means).

RESULTS AND DISCUSSION

We previously published the n-6/n-3 ratios for intramuscular fat and intermuscular and subcutaneous adipose tissues (Aldai et al., 2007a) and for comparison purposes we combined these results with 18:2n-6/18:3n-3 ratios in Table 1. In all tissues both ratios were significantly higher in the leanest animals (*mh/mh*) compared to the other genotypes (*mh/+*, *+/+*). Interestingly, however, differences in the overall n-6/n-3 ratio were less marked than in the 18:2n-6/18:3n-3 ratio, particularly for intramuscular fat. In addition, the intramuscular 18:2n-6/18:3n-3 ratio for all animals was similar to other reports (18:2n-6/18:3n-3 = 15.6, concentrate-fed Hereford × Friesian bulls, Enser et al., 1998). The relatively high 18:2n-6/18:3n-3 ratios likely resulted from the high level of 18:2n-6 in the concentrate meal (60% of the total fatty acids, Aldai et al., 2007b). In addition, it may be attributed to a higher polar to neutral lipid ratio in the intramuscular fat (Rule et al., 1995), the preferential deposition of 18:2n-6 in intramuscular phospholipids relative to the more equal partitioning of 18:3n-3 in triacylglycerols and phospholipids (De Smet et al., 2004),

Table 1. Genotype differences in the fatty acid ratios for intramuscular fat, and intermuscular and subcutaneous adipose tissues

	mh/mh	*mh/+*	*+/+*	S.E.M.	Signification
Intramuscular					
n-6/n-3	11.79[a]	10.05[b]	9.68[b]	0.214	***
18:2n-6/18:3n-3	25.32[a]	15.70[b]	15.42[b]	0.525	***
Intermuscular					
n-6/n-3	11.21[a]	10.16[b]	10.24[b]	0.124	***
18:2n-6/18:3n-3	11.63[a]	10.66[b]	10.77[b]	0.140	*
Subcutaneous					
n-6/n-3	10.44[a]	9.76[b]	9.90[b]	0.087	**
18:2n-6/18:3n-3	10.53[a]	9.97[b]	10.06[b]	0.093	*

S.E.M. = standard error of the mean; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; [a,b]means with different superscripts within a row are significantly different at $P < 0.05$;

n-3 = 18:3n-3 + 20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3; n-6 = 18:2n-6 + 18:3n-6 + 20:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6; *mh/mh* = AV double-muscled; *mh/+* = AV heterozygote; *+/+* = AV normal

or the preferential channelling of 18:2n-6 toward acylation and desaturation rather than β-oxidation as seen for 18:3n-3 in human plasma lipids (Chan et al., 1993). Among genotypes, the *mh/mh* animals had a higher 18:2n-6/18:3n-3 ratio (25.3) in intramuscular fat compared to the other genotypes (mean value of 15.5). Genotype differences in the other two adipose tissues were far less striking even though significant differences were found between *mh/mh* (11.6 and 10.5 for intermuscular and subcutaneous adipose tissues, respectively) and the other two genotypes (mean values of 10.7 and 10.0 for intermuscular and subcutaneous adipose tissues, respectively).

The relative proportions of the individual n-6 and n-3 fatty acids in intramuscular fat are shown in Figure 1a,b, respectively. The absolute content of n-6 PUFAs was not different between the three genotypes (mean value of 242 mg/100 g meat, $P > 0.05$) although *mh*/+ and +/+ animals consumed significantly more concentrate per day (mean value of 7.2 kg/day) compared to *mh/mh* animals (6.7 kg per day). In addition, only minor differences were noted in the deposition of individual n-6 fatty acids. Most n-6 metabolites (18:3n-6, 20:2n-6 and 20:3n-6) did not accumulate and the main elongation and desaturation product of the n-6 series was 20:4n-6 which is essential for growth, cell signalling, and an eicosanoid precursor (Innis, 2003). Moreover, 20:4n-6 tended to resist further conversion into 22:4n-6 but this may have been also due to retroconversion of 22:4n-6 back to 20:4n-6 as observed in human cells (Rosenthal et al., 1991). Overall, the conversion of 18:2n-6 and accumulation of 20:4n-6 appeared to take place at a similar rate in all genotypes although some significant differences were found for n-6 fatty acid intermediates with lower concentrations (18:3n-6, 20:2n-6, 20:3n-6). This finding was in accordance with Aldai et al. (2006a), however, Raes et al. (2001) found a higher conversion rate from 18:2n-6 to 20:4n-6 in +/+ compared to *mh/mh* in their study using Belgian Blue bulls. These differences might be related to differences between breeds (AV vs. Belgian Blue) or to differences in the n-6 intake of the animals depending on the concentrate composition (feeding diets were not specified in Raes et al., 2001).

The absolute content of total n-3 PUFAs in intramuscular fat was significantly lower in *mh/mh* animals (21 mg/100 g meat, $P < 0.001$) compared to the other genotypes (*mh*/+, +/+) (mean value of 25 mg/100 g meat). Interestingly, more of the n-3 PUFAs in *mh/mh* animals were in the form of elongation and desaturation products (20:5n-3, 22:5n-3 and 22:6n-3; Figure 1b). These findings could be a reflection of differences in enzyme (desaturase and elongase) activities between genotypes and may be related to cell type abundance (i.e. more muscle cells in double muscled animals relative to adipocytes) and differences in their enzyme activity (i.e. possibly higher in muscle cells). Raes et al. (2001) also found that the conversion of 18:3n-3 to longer-chain metabolites occurred preferentially in double-muscle animals (*mh/mh*) compared to other genotypes. It is known that the first enzymatic step (desaturation of 18:2n-6 and 18:3n-3) involves desaturation by the microsomal Δ^6-desaturase and high intakes of 18:2n-6 have been suggested to decrease the conversion of 18:3n-3 to 20:5n-3 and 22:6n-3 and favour higher

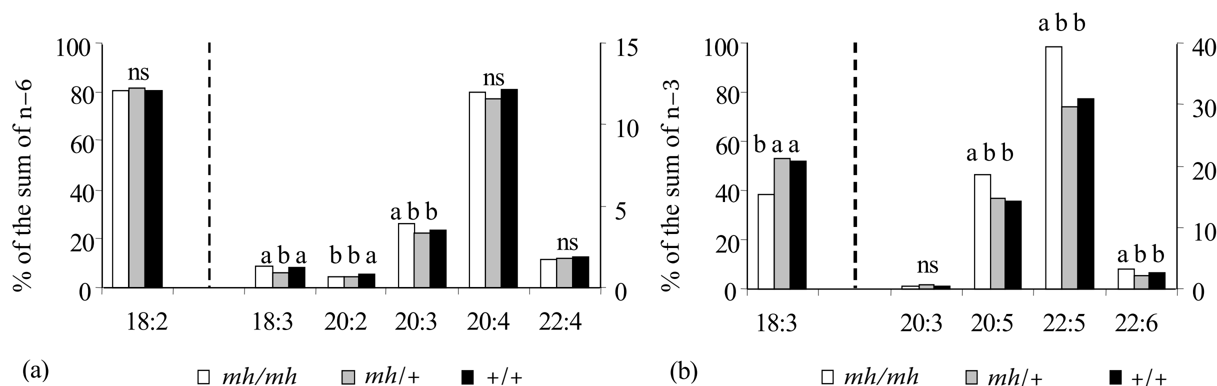

Figure 1. Influence of biological type on the metabolism of (a) n-6 and (b) n-3 fatty acids in intramuscular fat; [a,b,c]significantly different with $P < 0.05$

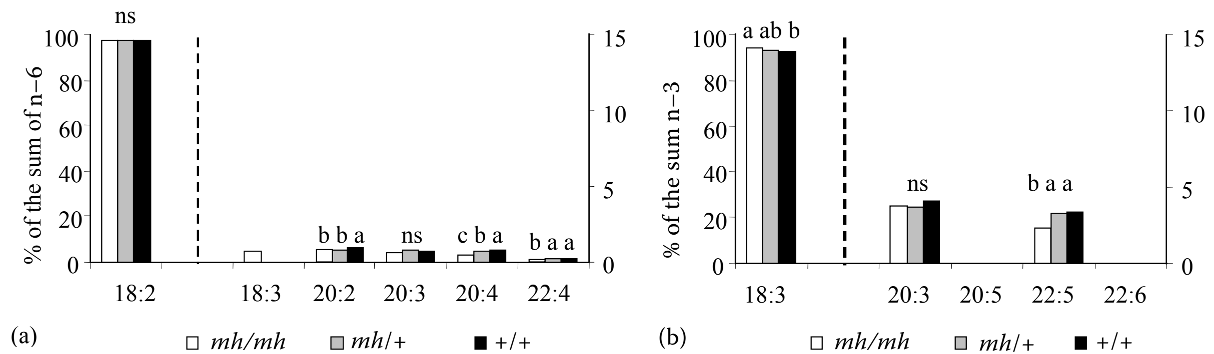

Figure 2. Influence of biological type on the metabolism of (a) n-6 and (b) n-3 fatty acids in intermuscular fat; [a,b,c]significantly different with $P < 0.05$. Note that 18:3n-6 (in *mh/+* and *+/+*) and 20:5n-3 and 22:6n-3 (in all genotypes) were not quantifiable according to the method used

20:4n-6 production through competition for the Δ^6-desaturase as found in humans (Raederstorff and Moser, 1992; Kris-Etherton et al., 2004). Hence, desaturation and elongation of 18:3n-3 could be lower in *mh/+*, *+/+* animals due to their higher intake of concentrate rich in 18:2n-6. Looking at the conversion of 20:5n-3 to 22:5n-3 and 20:4n-6 to 22:4n-6, both governed by the Δ^5-elongase, it seemed that this enzyme preferentially acted on n-3 fatty acids as postulated by Raes et al. (2001) or that the retroconversion of 22:5n-3 to 20:5n-3 could be less extensive as found by Rosenthal et al. (1991). Some other studies in humans have shown that supplementation with 18:3n-3 increases blood lipid 20:5n-3 with small or no increase of 22:6n-3 (Li et al., 1999). They also showed that n-3 fatty acid metabolism is limited beyond 20:5n-3. Moreover, no evidence was found that the high intake of 18:2n-6 interferes with 22:6n-3 synthesis in animals (Sheaff et al., 1995). In humans, adiposity and insulin action have been related to the muscle membrane lipid composition, with greater fatness being related to a reduced Δ^5-desaturase activity but this resulted in reductions in accumulations in both long chain n-6 and n-3 fatty acids (Pan et al., 1995). According to our study, however, the results suggest that genotype effects in cattle were more visible in n-3 than n-6 PUFA end products.

The relative proportions of the individual n-6 and n-3 PUFAs for intermuscular adipose tissue are shown in Figure 2a,b, respectively. For n-6 fatty acids, differences in the metabolism between the studied genotypes were noted. The *mh/mh* animals generally had lower levels of elongation and desaturation products (20:2n-6, 20:4n-6 and 22:4n-6), but overall, the n-6 elongation and desaturation products were not found to accumulate to any great extent. In intermuscular adipose tissue, deposition

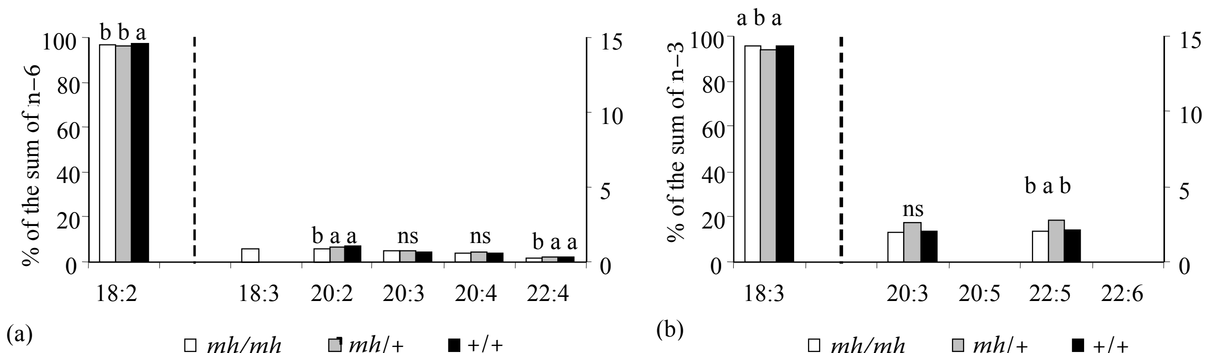

Figure 3. Influence of biological type on the metabolism of (a) n-6 and (b) n-3 fatty acids in subcutaneous fat; [a,b,c]significantly different with $P < 0.05$. Note that 18:3n-6 (in *mh/+* and *+/+*) and 20:5n-3 and 22:6n-3 (in all genotypes) were not quantifiable according to the method used

of n-3 PUFAs was also genotype-dependent (Figure 2b). While the 18:3n-3 was significantly higher in *mh/mh*, intermediate in *mh/*+ and lower in +/+, the 18:3n-3 elongation and desaturation products were higher in *mh/*+ and +/+ animals (22:5n-3, $P < 0.05$). Interestingly, the effects of genotype on n-3 PUFA elongation and desaturation were opposite to the effects seen in intramuscular fat but they were also less dramatic. The results again suggest, however, that the genotype effect was slightly more evident in n-3 than in n-6 fatty acid metabolism.

In subcutaneous adipose tissue, the relative proportions of the individual n-6 and n-3 PUFAs in their corresponding series are shown in Figure 3a,b, respectively. As seen for the intermuscular adipose tissue, no n-6 PUFA elongation and desaturation end products accumulated in the subcutaneous adipose tissue. Minor differences in the metabolism of n-6 fatty acids were found between the studied genotypes. It seemed that the conversion of 18:2n-6 to other metabolites took place at a similar rate in *mh/mh* and +/+, and at a slightly higher rate in *mh*/+. For the n-3 PUFAs, the pattern was similar to intermuscular adipose tissue and the 18:3n-3 elongation and desaturation products were not found to accumulate (Figure 3b) although minor differences were found for which explanations are not immediately evident. As for the n-6 PUFA, *mh/*+ animals seemed have a higher rate of conversion to other metabolites (20:3n-3, $P > 0.05$ and 22:5n-3, $P < 0.05$) compared to other genotypes (*mh/mh*, +/+) and again the explanation for this is not immediately evident.

In summary, the composition of intramuscular fat was the most different compared to the other two adipose tissues (intermuscular and subcutaneous) which were similar in composition. It remains to be determined whether there are tissue-specific differences related to the expression of enzymes for elongation and desaturation. In general, n-6 and n-3 fatty acid elongation and desaturation products were affected by genotype and this effect was most evident in n-3 PUFAs of the intramuscular fat of double-muscled animals (*mh/mh*) compared to *mh/*+ and +/+ AV animals. In this sense, the meat from *mh/mh* animals produced more long-chain n-3 fatty acid end products but in contrast, they have a lower absolute amount of 18:3n-3. Consequently, if they were fed higher levels of n-3 PUFA, they might achieve greater accumulations of long-chain n-3 PUFA together with a lower n-6/n-3 ratio which may translate into health benefits for the consumer (Finnegan et al., 2003a,b). In the present experiment, only a single diet was fed and it had a relatively high content of 18:2n-6. Future experiments should, therefore, focus on feeding different diets (concentrate meal with different components, grass plus concentrate, grass) and different feed intakes to further elucidate any interactions between genotype and diet. In addition, this should be combined with investigations at the biochemical and molecular level to unravel the mechanisms underlying differences between genotypes in the metabolism and incorporation of fatty acids into specific adipose tissues.

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