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## Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance

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### ABSTRACT

In one of the most extensive analyses to date we show that the balance of diet n-3 and n-6 polyunsaturated fatty acids (PUFA) is the most important determinant of membrane composition in the rat under 'normal' conditions. Young adult male Sprague–Dawley rats were fed one of twelve moderate-fat diets (25% of total energy) for 8 weeks. Diets differed only in fatty acid (FA) profiles, with saturate (SFA) content ranging 8–88% of total FAs, monounsaturate (MUFA) 6–65%, total PUFA 4–81%, n-6 PUFA 3–70% and n-3 PUFA 1–70%. Diet PUFA included only essential FAs 18:2n-6 and 18:3n-3. Balance between n-3 and n-6 PUFA is defined as the PUFA balance (n-3 PUFA as % of total PUFA) and ranged 1–86% in the diets. FA composition was measured for brain, heart, liver, skeletal muscle, erythrocytes and plasma phospholipids, as well as adipose tissue and plasma triglycerides. The conformer–regulator model was used (slope = 1 indicates membrane composition completely conforming to diet). Extensive changes in diet SFA, MUFA and PUFA had minimal effect on membranes (average slopes 0.01, 0.07, 0.07 respectively), but considerable influence on adipose tissue and plasma triglycerides (average slopes 0.27, 0.53, 0.47 respectively). Diet balance between n-3 and n-6 PUFA had a biphasic influence on membrane composition. When n-3 PUFA < 10% of total PUFA, membrane composition completely conformed to diet (average slope 0.95), while diet PUFA balance > 10% had little influence (average slope 0.19). The modern human diet has an average PUFA balance ~10% and this will likely have significant health implications.

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### 1. Introduction

Dietary fat is important for human health, yet it is not fully known how different fats affect various health problems. One potential mechanism is via diet effects on membrane lipid composition. Many previous studies have established that diet affects tissue cell membrane fatty acid composition of humans and other animals, with the role of n-3 polyunsaturated fatty acids (PUFA) in the diet (in particular fish oils) being especially investigated [1–5]. Most studies that have considered dietary fats have been limited, for example by examining the effect of only an individual fatty acid by its presence/absence [e.g. Ref. 6] or considering only a single tissue [e.g. Ref. 3,7] or using a diet with a high content of fat [e.g. Refs. 5,8]. There have been few previous studies in rodents that have quantified the effect of a wide range of diet fat composition on the cell membrane fatty acid composition of tissues under 'normal' conditions. The present study was designed to overcome the limitations of earlier work.

Twelve diets, differing only in their fatty acid profile, were employed to obtain the most comprehensive picture of the effects of diet on membrane lipid composition in the rat. Diet fat profiles were wide ranging for all fat types, including both non-essential fatty acids (saturated and monounsaturated fatty acids; SFA and MUFA respectively) and the essential fatty acids (n-3 PUFA and n-6 PUFA). Furthermore, for each diet we measured the fatty acid composition of phospholipids from six tissues, providing the membrane fat profile for muscle, heart, liver, brain and red blood cells (RBC), as well as the plasma composition. In addition to the membrane lipids the composition of the triglycerides from two sources, including plasma and adipose tissue, was also determined. We recently reported the results for the skeletal muscle [9]. The present manuscript extends these results to all tissues measured and reveals that the muscle membrane lipid results appear to describe a general phenomenon for all tissues.

In the present study rats were used as the model organism with the purpose of determining the relationship for mammals in general. To allow best extrapolation to the human situation a number of 'normal' conditions were implemented. To ensure 'normal' genetic variation the rats were young adults from the outbred Sprague–Dawley strain. To assess the steady state relationship between diet and

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membrane lipid composition the experimental diets were fed to the rats for 8 weeks. This time period is equivalent to approximately one year in humans (in terms of relative metabolic rates; see Ref. [10] for metabolic rate data). To emulate the average human diet all twelve experimental diets contained moderate-fat (25% of total energy to avoid the development of high-fat diet-induced obesity) and the dominant fats were 18-carbon, which is the 'normal' situation for the diet of both rats and humans (see composition of rat chow in Table 2 from Refs. [9] and [11] respectively).

As with the muscle manuscript [9], we have employed the conformer–regulator model to quantify the influence diet fatty acid composition on membrane fatty acid composition in this paper. This model describes the influence of an environmental parameter on an organism by analysing the relationship between the same variable in both the environment and organism [12]. This model has been commonly used to examine physical variables, such as temperature and osmotic pressure, but not so often to examine chemical variables, such as fatty acid composition. In the present context, when the tissue membrane fatty acid composition conforms to diet fatty acid composition then the membrane is conforming to diet (i.e. the slope of the relationship = 1). However, if membrane fatty acid composition is constant irrespective of diet variation the membrane composition is said to be homeostatically regulated (i.e. the slope of the relationship = 0). By applying this analysis in our previous study we found that membrane fatty acid composition of rat skeletal muscle is a highly regulated parameter, with diet SFA, MUFA and total PUFA content exerting very little influence on membrane fat profile [9].

Our previous results also demonstrated, however, that the balance between the essential dietary fatty acids, n–3 PUFA and n–6 PUFA, has the greatest influence on muscle membrane lipid composition [9]. It is important to note that these two fat types are distinct essential dietary components for higher animals, as they are incapable of inter-converting them. These results in muscle membranes further emphasise the importance of considering n–3 PUFA not in isolation, but in association with diet n–6 PUFA levels. The balance between n–3 PUFA and n–6 PUFA will be referred to as "PUFA balance" throughout this manuscript and is defined as "n–3 PUFA as % of total PUFA". PUFA balance is analogous to a proportion (or a ratio where the two values sum to 100%) and this creates a linear scale ranging from 0 to 100, making comparisons between values more simple than the traditionally used n:6/n:3 ratio (see Ref. [9] for a more detailed discussion). PUFA balance can be extremely variable between meals. For example, a recent study of commonly consumed meals demonstrated considerable difference between fish meals; although 'fish and chips' had twice the amount of fat of 'salmon and salad' (14.5% versus 7.4%) it had a PUFA balance of 4% compared to 80% for the salmon meal, due to the high abundance of n–6 PUFA present in the 'fish and chips' [13].

Dietary intake studies have shown that the fat intake of the U.S. diet averages a PUFA balance of ~9.5% [14]. In the present study the diet PUFA balance ranged from 1 to 86% and was found to elicit the greatest response on both membrane lipid and triglyceride fatty acid composition. If the current results in rats also apply to humans, an average PUFA balance of 9.5% in the modern human diet is of considerable concern, as it indicates there are huge numbers of people consuming a diet with a very low and likely inadequate PUFA balance without knowing it.

## 2. Materials and methods

Materials and methods have been described previously [9]. All experiments were approved by the University of Wollongong Animal Ethics Committee and were carried out in accordance with EU Directive 2010/63/EU for animal experiments. A brief description of the methods and any further details are provided below.

### 2.1. Animals and diets

In brief, eight-week-old (young adult) male Sprague–Dawley rats were randomly assigned to either an initial group (n = 6) or one of twelve dietary groups (n = 78). The initial group of rats was euthanised at 8 weeks of age to provide an indication of tissue fatty acid composition prior to dietary manipulation. Rats were fasted for 24 h prior to euthanasia. Following euthanasia tissues were immediately removed, frozen and stored at –80 °C until analysis. Tissues collected included skeletal muscle (*medial gastrocnemius*), heart, liver, brain (cerebral hemisphere), perirenal adipose tissue, RBC and plasma.

The twelve dietary group rats were fed ad libitum for a further 8 weeks on moderate-fat diets (25% total energy) differing only in fatty acid profile (see Table 1). A wide range of SFA, MUFA, total PUFA, n–3 PUFA, n–6 PUFA and PUFA balance was achieved by mixing various proportions of the following oils: flaxseed oil, safflower oil, olive oil, coconut oil, and beef dripping. No 20-carbon or 22-carbon PUFA were included in the diets. A graphical representation of the fatty acid profile of the experimental and initial diets is presented in Fig. 1. The relative proportions of diet SFA, MUFA, n–6 PUFA and n–3 PUFA are shown in the bar graph and the PUFA balance of each diet displayed in the upper pie graph section. The experimental diets were numbered in order of increasing PUFA balance (1–86%). Fat types were mixed in different combinations to achieve a great degree of variation between diets. For example, diets 6, 7, 8 and 9 all had a PUFA balance of 50%, however, the PUFA content was highly varied in these diets (7–60%). Furthermore, diets 11 and 12 both had very high PUFA balance (84 and 86% respectively), but the total PUFA content varied quite dramatically (25 and 80% respectively).

### 2.2. Fatty acid analysis

Total lipids were extracted using chloroform:methanol (2:1, v:v) and glass/glass homogenizers as previously described [9,15]. The approximate amount of each tissue used was: 200 mg of skeletal muscle, heart, brain and liver, 100 mg for adipose tissue, 1 ml of plasma, and 400 µl for RBC. RBC samples were lysed in 1 ml Milli-Q® water prior to lipid extraction using chloroform:methanol (2:1, v:v). Phospholipids and triglycerides were separated from total lipids using Sep-Pak® Classic Silica Cartridge (Waters, Rydalmere, Australia). The triglyceride fraction was collected for adipose tissue and plasma, using 30 ml and 15 ml ethyl acetate respectively. Phospholipids were eluted with 15 ml hexane for all tissues. Phospholipids and/or triglyceride fractions were transmethylated [16] and fatty acid methyl esters were measured by gas chromatography (Shimadzu GC-17A, Rydalmere, New South Wales, Australia) using a Varian WCOT Fused Silica Column (50 m × 0.25 mm ID, CP7419, Sydney, New South Wales, Australia) with the following temperature programme: 150 °C initial temperature, 17.5 °C/min to 170 °C, 0.5 °C/min to 178 °C, 15 °C/min to 222 °C and 2 °C/min to 232 °C. The split ratio was set at 25:1 for all analyses. Individual fatty acids were identified by comparison with an external standard (FAME Mix C4–C24; Sigma Aldrich, Sydney, Australia) and then expressed as mol % of total fatty acids.

### 2.3. Statistical analysis

Statistical analysis was performed using JMP 5.1 (SAS Institute Inc. NC, USA). See Ref. [9] for details. All results are expressed as means ± S.E.M. with p < 0.05 set as the level of significance. Data for individual rats was used for all linear regression analyses (n = 72 data points for most tissues), however, only the mean and S.E.M. for each diet group are plotted in the figures. Data for the initial group rats is also plotted in some figures (shown as open symbols), but was not included in any linear regression analysis. Significant

**Table 1**Fatty acid composition (% of total fatty acid) of experimental and initial diets; values are mean  $\pm$  S.E.M.; n = 2, except for diets 1, 2, 5, 7 and 12: n = 3 and initial diet: n = 1.

Diet	1	2	3	4	5	6	7	8	9	10	11	12	Initial
8:0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	3.7 $\pm$ 0.2	15.6 $\pm$ 0.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	10.8 $\pm$ 0.1	0.3 $\pm$ 0.0	0.0
10:0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	2.4 $\pm$ 0.1	10.3 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	7.1 $\pm$ 0.0	0.2 $\pm$ 0.0	0.2
12:0	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	12.9 $\pm$ 0.1	30.5 $\pm$ 0.2	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	26.9 $\pm$ 2.4	1.3 $\pm$ 0.2	0.2
14:0	0.1 $\pm$ 0.0	1.9 $\pm$ 0.1	0.1 $\pm$ 0.0	3.0 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	5.0 $\pm$ 0.0	19.8 $\pm$ 0.0	1.3 $\pm$ 0.1	0.1 $\pm$ 0.0	14.4 $\pm$ 0.3	0.5 $\pm$ 0.1	0.2
16:0	10.2 $\pm$ 0.2	18.6 $\pm$ 0.4	7.4 $\pm$ 0.4	26.3 $\pm$ 0.8	13.3 $\pm$ 0.1	8.3 $\pm$ 0.4	6.2 $\pm$ 0.2	8.6 $\pm$ 0.0	13.9 $\pm$ 0.5	5.4 $\pm$ 0.5	6.9 $\pm$ 0.2	4.5 $\pm$ 0.1	16.0
17:0	0.1 $\pm$ 0.0	0.9 $\pm$ 0.0	0.0 $\pm$ 0.0	1.5 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.6 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1
18:0	2.6 $\pm$ 0.0	11.2 $\pm$ 0.0	2.3 $\pm$ 0.1	17.3 $\pm$ 0.0	3.0 $\pm$ 0.0	2.5 $\pm$ 0.1	2.3 $\pm$ 0.0	2.8 $\pm$ 0.0	8.4 $\pm$ 0.2	2.5 $\pm$ 0.2	2.5 $\pm$ 0.1	2.3 $\pm$ 0.0	2.5
16:1n-7	0.8 $\pm$ 0.0	1.9 $\pm$ 0.1	0.5 $\pm$ 0.0	2.8 $\pm$ 0.3	1.1 $\pm$ 0.0	0.5 $\pm$ 0.0	0.2 $\pm$ 0.0	0.0 $\pm$ 0.0	1.3 $\pm$ 0.1	0.2 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1
18:1n-9	34.7 $\pm$ 0.3	28.8 $\pm$ 0.5	12.9 $\pm$ 0.4	41.4 $\pm$ 1.1	62.9 $\pm$ 0.2	27.9 $\pm$ 0.3	9.3 $\pm$ 0.2	5.4 $\pm$ 0.0	21.9 $\pm$ 0.6	10.9 $\pm$ 0.0	6.1 $\pm$ 0.2	9.8 $\pm$ 0.0	24.1
18:1n-7	0.3 $\pm$ 0.3	0.5 $\pm$ 0.3	0.3 $\pm$ 0.1	0.6 $\pm$ 0.6	0.0 $\pm$ 0.0	0.4 $\pm$ 0.4	0.3 $\pm$ 0.0	0.1 $\pm$ 0.0	0.3 $\pm$ 0.3	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0	0.3 $\pm$ 0.0	1.9
18:2n-6	49.9 $\pm$ 0.2	33.2 $\pm$ 0.2	69.8 $\pm$ 0.0	2.9 $\pm$ 0.0	11.0 $\pm$ 0.0	30.2 $\pm$ 0.2	28.9 $\pm$ 0.4	3.4 $\pm$ 0.0	25.4 $\pm$ 0.0	25.0 $\pm$ 0.2	4.0 $\pm$ 0.1	11.0 $\pm$ 0.0	47.8
18:3n-3	0.3 $\pm$ 0.0	1.1 $\pm$ 0.1	5.7 $\pm$ 0.1	1.2 $\pm$ 0.1	7.6 $\pm$ 0.0	29.2 $\pm$ 0.6	28.2 $\pm$ 0.0	3.3 $\pm$ 0.0	25.4 $\pm$ 0.3	54.9 $\pm$ 0.9	20.9 $\pm$ 1.2	69.1 $\pm$ 0.3	5.1
SFA	13.7 $\pm$ 0.2	33.6 $\pm$ 0.5	10.4 $\pm$ 0.3	49.4 $\pm$ 0.9	17.1 $\pm$ 0.3	11.4 $\pm$ 0.3	32.9 $\pm$ 0.6	87.8 $\pm$ 0.1	24.9 $\pm$ 0.7	8.4 $\pm$ 0.6	68.8 $\pm$ 1.6	9.4 $\pm$ 0.3	20.2
MUFA	36.1 $\pm$ 0.1	32.0 $\pm$ 0.4	14.0 $\pm$ 0.3	46.3 $\pm$ 0.9	64.3 $\pm$ 0.3	29.1 $\pm$ 0.1	10.0 $\pm$ 0.2	5.5 $\pm$ 0.0	24.2 $\pm$ 0.4	11.5 $\pm$ 0.1	6.3 $\pm$ 0.2	10.3 $\pm$ 0.0	26.6
PUFA	50.3 $\pm$ 0.2	34.3 $\pm$ 0.2	75.5 $\pm$ 0.0	4.4 $\pm$ 0.0	18.6 $\pm$ 0.0	59.5 $\pm$ 0.4	57.1 $\pm$ 0.4	6.7 $\pm$ 0.1	50.9 $\pm$ 0.3	80.1 $\pm$ 0.7	24.9 $\pm$ 1.4	80.3 $\pm$ 0.3	53.1
n-6 PUFA	49.9 $\pm$ 0.2	33.2 $\pm$ 0.3	69.8 $\pm$ 0.0	3.1 $\pm$ 0.1	11.0 $\pm$ 0.0	30.2 $\pm$ 0.2	28.9 $\pm$ 0.4	3.4 $\pm$ 0.0	25.4 $\pm$ 0.0	25.1 $\pm$ 0.1	4.0 $\pm$ 0.1	11.1 $\pm$ 0.1	47.9
n-3 PUFA	0.3 $\pm$ 0.0	1.1 $\pm$ 0.1	5.7 $\pm$ 0.1	1.3 $\pm$ 0.1	7.6 $\pm$ 0.0	29.3 $\pm$ 0.6	28.2 $\pm$ 0.0	3.3 $\pm$ 0.0	25.5 $\pm$ 0.3	55.1 $\pm$ 0.9	21.0 $\pm$ 1.3	69.2 $\pm$ 0.3	5.3
PUFA Balance	0.7 $\pm$ 0.1	3.2 $\pm$ 0.4	7.5 $\pm$ 0.1	29.9 $\pm$ 2.2	41.0 $\pm$ 0.2	49.2 $\pm$ 0.7	49.4 $\pm$ 0.4	49.6 $\pm$ 0.2	50.1 $\pm$ 0.3	68.7 $\pm$ 0.5	84.0 $\pm$ 0.3	86.2 $\pm$ 0.0	9.9

differences between the slope values determined for the diet–tissue PUFA balance relationships in Section 3.4 were determined using a Student's t test.

### 3. Results

#### 3.1. Effects of diet on tissue fatty acid composition

The fatty acid composition of each tissue phospholipids and/or triglycerides is presented in the following supplementary tables: heart phospholipids (Table S1), brain phospholipids (Table S2), liver phospholipids (Table S3), RBC phospholipids (Table S4), plasma phospholipids (Table S5), plasma triglycerides (Table S6) and adipose tissue triglycerides (Table S7). For skeletal muscle phospholipid values see Table 2 in Ref. [9]. Comparisons are made between diet groups for all fatty acid types. There are significant differences evident for all the n-6 PUFA and n-3 PUFA in all tissues. The proportion of total PUFA shows significant differences between diet groups for all tissues except brain and RBC phospholipids, while total MUFA shows differences between diet groups for all tissues except brain. For total SFA, the only tissues to show a significant difference are liver phospholipids, as well as plasma and adipose tissue triglycerides. Liver phospholipids and adipose tissue triglycerides are the only tissues to demonstrate significant differences between diet groups for all fat types and individual fatty acids measured.

Within the saturated and monounsaturated fatty acids there are a number of individual fatty acids with no significant differences between diet groups. These include 16:0 (muscle, brain and RBC phospholipids only), 18:0 (muscle, brain, RBC phospholipids and plasma

triglycerides only), 22:0 (RBC only), 24:0 (plasma phospholipids only), 16:1n-7 (RBC and plasma phospholipids only), 20:1n-9 (brain phospholipids only) and 24:1n-9 (brain and plasma phospholipid only).

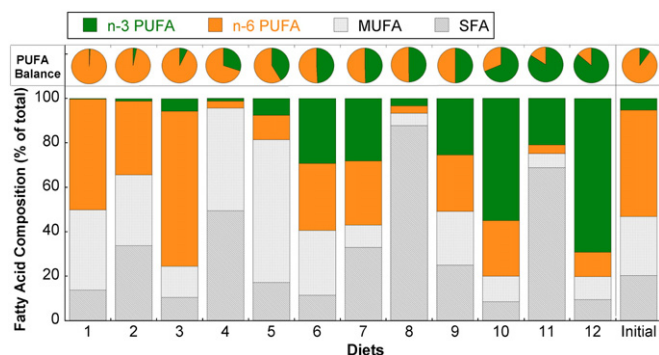
For most tissues the main n-6 PUFA present are 18:2n-6 and 20:4n-6 (including muscle, heart, liver, RBC membrane lipids, as well as plasma phospholipids/triglycerides). However, in the brain membranes 20:4n-6 account for the majority of the n-6 PUFA content. The main contributor to total n-3 PUFA present in the membrane lipids of most tissues is 22:6n-3 (for heart, brain, muscle and liver). Plasma phospholipids contain a mixture of both 18:3n-3 and 22:6n-3, while the predominant n-3 PUFA in plasma triglycerides is 18:3n-3. The n-3 PUFA content of the RBC membrane lipids show far more variation between diet groups, with 22:6n-3 the main membrane n-3 PUFA for diet 1–5 and 8; 22:5n-3 the main contributor for diet 6, 7, 9 and 10; and 20:5n-3 the highest membrane n-3 PUFA for diets 11–12. The PUFA in the adipose tissue triglycerides consists primarily of those provided in the diet; i.e. 18:2n-6 and 18:3n-3.

#### 3.2. Effect of diet SFA, MUFA and PUFA on tissue composition

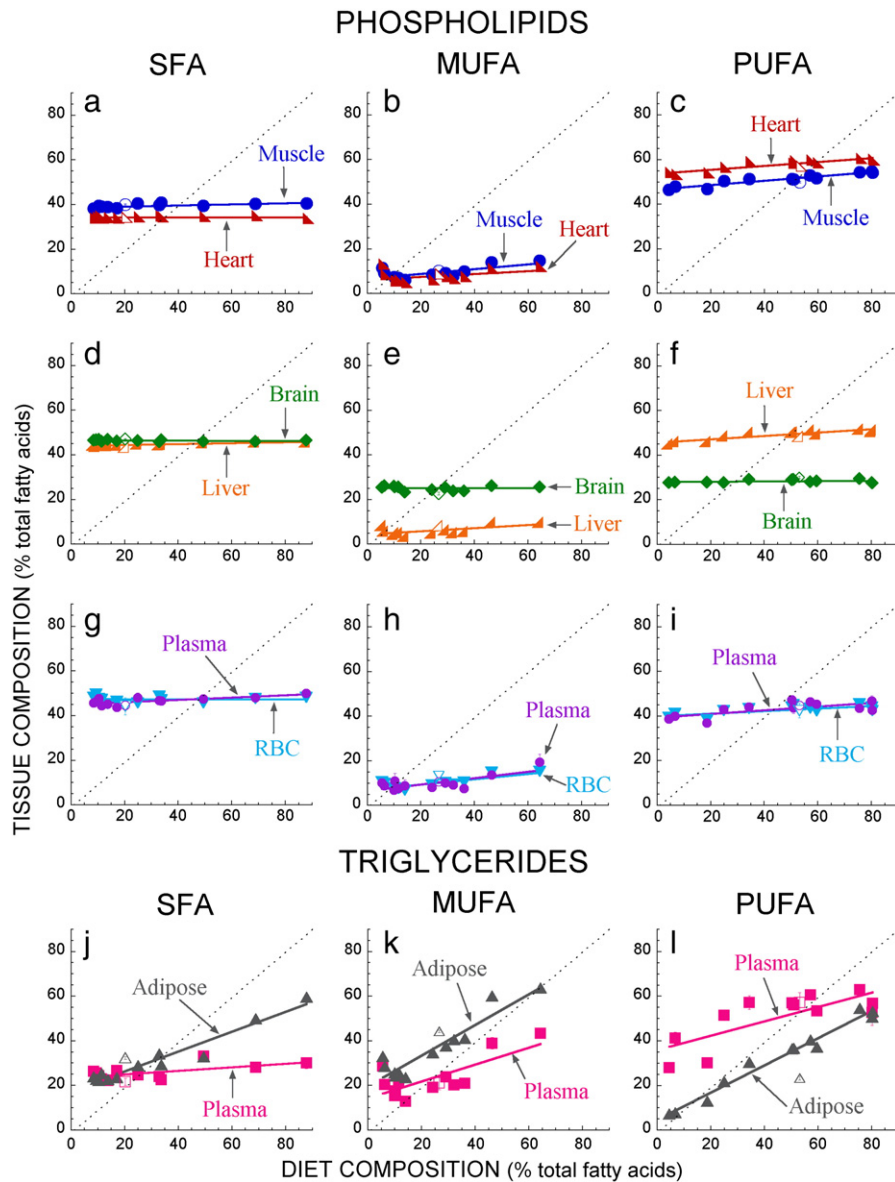
The relationship between diet fat profile (i.e. SFA, MUFA and total PUFA) and tissue fatty acid composition (both phospholipids and triglycerides) was quantified by plotting diet fat type against tissue fatty acid composition (see Fig. 2). The linear regression results for each plot are provided in Supplementary Tables S8–S10.

All tissue phospholipids examined regulate a constant membrane SFA content despite very large changes in diet SFA content (8–88% of total diet fatty acids), with slopes  $\leq$  0.05 for heart, brain, liver, muscle, RBC and plasma (see Fig. 2a, d, g). The plasma triglycerides are similarly unresponsive to diet SFA content (slope 0.08), however, the adipose tissue triglycerides are highly responsive (slope 0.45; see Fig. 2j), indicating that adipose tissue triglyceride SFA changes by 45% for a 100% change in diet SFA. This is reflected in the range of adipose tissue triglyceride SFA content which varies from 22 to 59% of total fatty acids. In contrast, the phospholipid SFA content of the other tissues measured remains fairly constant (varying by only 1–6% over the full diet SFA range of 80%). All tissues maintain a phospholipid SFA content less than 50% of total fatty acids, with brain, liver, RBC and plasma containing very similar amounts of SFA (~45%) and muscle and heart membrane lipids slightly lower amounts (~35%). The SFA levels in the plasma triglycerides are lower again at ~23%.

Most of the tissue phospholipids are only slightly responsive to large changes in diet MUFA content (6–64% of total diet fatty acids),



**Fig. 1.** Fatty acid profile (% of total fatty acids) and PUFA balance (n-3 as % total PUFA) of initial diet (13%en fat) and twelve experimental diets (25%en fat); for values see Table 1.



**Fig. 2.** Fatty acid profile of rat tissues phospholipids and triglycerides relative to fatty acid profile of diet. Includes saturated fatty acids (SFA; left-hand graphs); monounsaturated fatty acids (MUFA; middle graphs) and polyunsaturated fatty acids (PUFA; right-hand graphs). Phospholipids include (a–c) skeletal muscle - ●; heart - ▲; (d–f) brain - ◆; liver - ▲; (g–i) red blood cells (RBC) - ▼; plasma - ●. Triglycerides include (k–l) adipose tissue (perirenal) - ▲; plasma - ■. In each graph the open symbol represents the value for initial rats and all solid coloured symbols represent data from the twelve experimental diets. Error bars show  $\pm$  S.E.M. and if they are not visible they are smaller than symbol. The dotted line in each graph indicates line of perfect diet-membrane conformity. See Supplementary Tables S8–S10 for regression equations and statistical values.

with heart, liver, muscle, RBC and plasma responding similarly (slopes 0.07–0.14; Fig. 2b, e, h). In contrast, the brain MUFA composition is strictly regulated in response to diet MUFA content (slope 0.00; Fig. 2e). The triglycerides are considerably more responsive to diet MUFA content with slopes of 0.37 and 0.69 for plasma and adipose tissue triglycerides respectively (Fig. 2k). This results in increased variation in triglyceride MUFA content for plasma (13–43% of total fatty acids) and adipose tissue (23–64%). The MUFA content of the phospholipids is generally lower than SFA content, with heart, liver, muscle, RBC and plasma phospholipids maintaining similar MUFA levels ( $\leq 19\%$  of total fatty acids). The brain phospholipids contain slightly higher levels of MUFA content than the other tissues ( $\sim 25\%$ ).

Similar to the membrane MUFA content, the total PUFA content of the tissue phospholipids is fairly unresponsive to great changes in diet PUFA content (4–80% of total diet fatty acids), with slopes ranging 0.06–0.10 in RBC, liver, plasma, heart and muscle (Fig. 2c, f, i).

Again, the brain is the most regulated tissue in terms of total PUFA content (slope 0.01) while the triglycerides of the plasma and adipose tissue triglycerides are the most responsive to diet PUFA (slopes 0.32 and 0.61 respectively; see Fig. 2l). PUFA levels are lowest in the brain ( $\sim 30\%$  of total fatty acids), with higher levels seen in the other tissues (heart, muscle and liver  $\sim 50\%$  of total fatty acids and  $\sim 40\%$  in plasma and RBC). PUFA content is highly variable in both plasma triglycerides (28–63%) and adipose tissue triglycerides (7–55%).

The results show that the phospholipids of all tissues are regulating the composition of the three major fatty acid classes (SFA, MUFA and total PUFA). Heart, liver, muscle, RBC and plasma phospholipids consist of similar amounts of each fat type and respond in a similar manner to changes in diet SFA, MUFA and total PUFA content. Brain phospholipid composition is comparable to the other tissues for SFA, but consists of higher MUFA and lower PUFA contents relative to the phospholipids of the other tissues. The brain membrane lipids are the most regulated for all fatty acid types, while the adipose tissue



triglycerides are the most responsive to changes in diet SFA, MUFA and PUFA content.

### 3.3. Effect of diet n–6 PUFA and n–3 PUFA on tissue composition

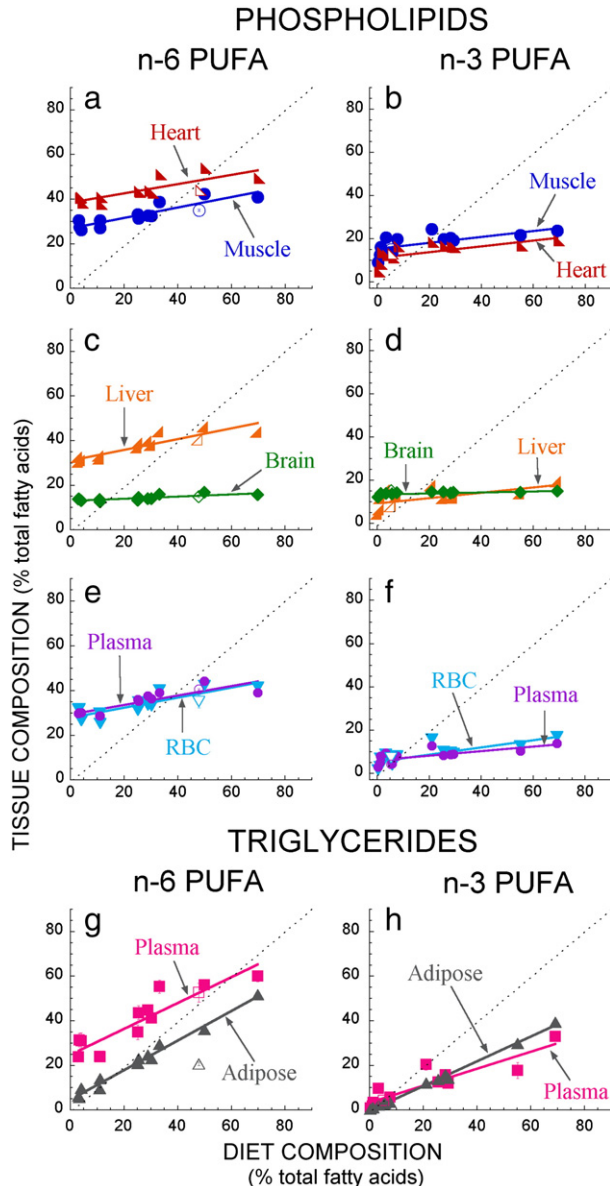
The relationship between each of the diet essential fatty acids (i.e. n–6 PUFA and n–3 PUFA) and tissue fatty acid composition (both phospholipids and triglycerides) is presented in Fig. 3. The linear regression results for each plot are provided in Supplementary Tables S11–S12. The diet n–6 PUFA was present in the form of 18:2n–6 while the tissue n–6 PUFA content included 18 C and 20–22 C n–6

PUFA. Similarly, diet n–3 PUFA contained only 18:3n–3 while the tissue lipids include a mixture of 18 C and 20–22 C n–3 PUFA.

Of all the major fatty acid classes, phospholipids are most responsive to diet n–6 PUFA content. Muscle, heart, liver, RBC and plasma phospholipids respond similarly to diet n–6 PUFA (slopes 0.21–0.24; Fig. 3a, c, e). Brain n–6 PUFA composition is the most constant, but shows a slight increase in response to diet n–6 PUFA (slope 0.05; Fig. 3c). Adipose tissue and plasma triglycerides are highly responsive to diet n–6 PUFA, with slopes of 0.57 and 0.67 respectively (Fig. 3g). The n–6 PUFA content of the phospholipids varies between tissues. The lowest amount of n–6 PUFA is found in the brain (~15% total fatty acids). All other tissues maintain phospholipid n–6 PUFA levels between 25 and 54% (muscle, heart, liver, RBC and plasma). The greatest degree of variation is seen in the triglycerides with n–6 PUFA content ranging 24–60% in plasma triglycerides and 6–52% in adipose tissue triglycerides.

Phospholipid n–3 PUFA content is also quite responsive to diet n–3 PUFA content in most tissues. This is the case for muscle, heart, liver, RBC and plasma phospholipids (slopes 0.10–0.17; Fig. 3b, d, f). The n–3 PUFA content of the brain membranes is more regulated (slope 0.02; Fig. 3d) than the n–6 PUFA content. The phospholipids of all tissues contain similar levels of n–3 PUFA (< 30% of total fatty acids). In accordance with the other fatty acid classes, the triglycerides show the highest response to diet n–3 PUFA content, with slopes of 0.33 for plasma triglycerides and 0.55 for adipose tissue triglycerides (Fig. 3h). The n–3 PUFA content of the triglycerides of plasma and adipose tissue both range similarly between 0 and 39% of total fatty acids.

Although the total PUFA content of the phospholipids is constant, when considered individually the essential fatty acids (n–6 and n–3 PUFA) exert a greater influence on tissue phospholipid composition than total PUFA. In contrast, the triglycerides are similarly highly responsive to all diet fatty acid classes (i.e. SFA, MUFA, PUFA, n–6 PUFA and n–3 PUFA).



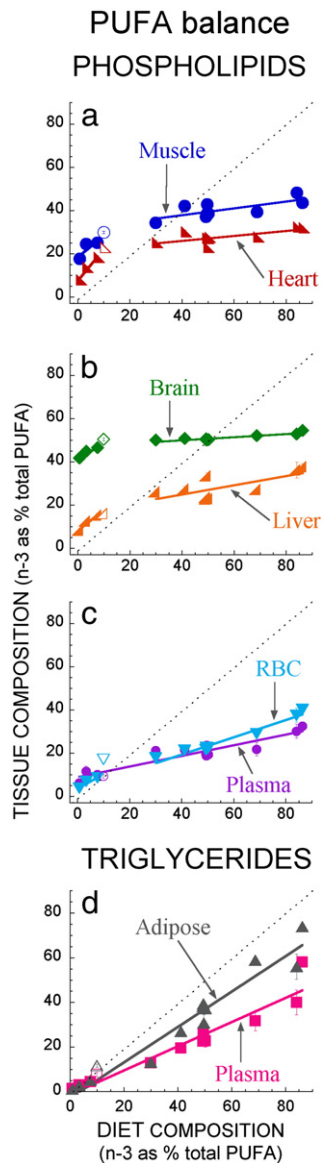
**Fig. 3.** Fatty acid profile of rat tissues phospholipids and triglycerides relative to fatty acid profile of diet. Includes n–6 polyunsaturated fatty acids (n–6 PUFA; left-hand graphs) and n–3 PUFA (right-hand graphs). Phospholipids include (a–b) skeletal muscle - ●; heart - ▲; (c–d) brain - ◆; liver - ▲; (e–f) red blood cells (RBC) - ▼; plasma - ●. Triglycerides include (g–h) adipose tissue (perirenal) - ▲; plasma - ■. In each graph the open symbol represents the value for initial rats and all solid coloured symbols represent data from the twelve experimental diets. Error bars show  $\pm$  S.E.M. and if they are not visible they are smaller than symbol. The dotted line in each graph indicates line of perfect diet-membrane conformity. See Supplementary Table S11–S12 for regression equations and statistical values.

### 3.4. Effect of the balance between diet n–3 and n–6 PUFA on tissue composition

A single linear relationship does not best describe the diet-tissue PUFA balance relationship for most tissues (see Fig. 4). To determine whether the diet-tissue PUFA balance relationship was biphasic or linear, the slopes of the biphasic relationships were compared to one another, with a significant difference in the slopes for diet PUFA balance 0–10% and PUFA balance 11–100% confirming a biphasic relationship. Significant differences were evident between the slopes of diet PUFA balance 0–10% relative to diet PUFA balance 11–100% for the phospholipids in muscle, heart, brain, liver and RBC (indicating a biphasic response). However, no difference was present for plasma phospholipids, plasma triglycerides and adipose tissue triglycerides (indicating a single linear response). See Supplementary Table S13 for linear regression results.

A biphasic plot with two linear relationships provides a better fit for the muscle, heart, brain, liver and RBC membrane PUFA balance (Fig. 4a, b, c respectively). The results show that for these tissue membranes lipid composition is highly responsive to diet PUFA balance < 10% (slopes 0.65–1.47;  $p \leq 0.0007$  for all tissues) whereas at diet PUFA balances > 10% the membrane lipids are able to maintain a more constant PUFA balance (slopes 0.07–0.40;  $p < 0.0001$  for all tissues).

Interestingly, the plasma phospholipids, plasma triglycerides and the adipose tissue triglycerides do not show the strong biphasic response present in the other tissue membrane lipids, but show a single linear response to diet PUFA balance (Fig. 4c, d). Although not biphasic, these tissue lipids are still highly responsive to diet PUFA balance (slope 0.25, 0.50 and 0.79 respectively).



**Fig. 4.** PUFA balance of rat tissues phospholipids and triglycerides relative to PUFA balance of diet (n–3 PUFA as % of total PUFA). Phospholipids include (a) skeletal muscle - ●; heart - ▲; (b) brain - ◆; liver - ▲; (c) red blood cells (RBC) - ▼; plasma - ●. Triglycerides include (d) adipose tissue (perirenal) - ▲; plasma - ■. In each graph the open symbol represents the value for initial rats and all solid coloured symbols represent data from the twelve experimental diets. Error bars show  $\pm$  S.E.M. and if they are not visible they are smaller than symbol. The dotted line in each graph indicates line of perfect diet-membrane conformity. See Supplementary Table S13 for regression equations and statistical values.

The adipose tissue triglycerides are the most responsive of all the tissue lipids to diet PUFA balance (over the full linear range) with a slope of 0.79. This relationship is closest to the line of perfect diet-membrane conformity (the dotted line in the graph) found for all tissues and fatty acid types. At diet PUFA balance <10% the adipose tissue triglycerides actually fall on the line of perfect conformity. The only higher responsivity to diet occurs in the muscle, heart and liver at diet PUFA balances <10%. However, the adipose tissue triglycerides are highly responsive to diet PUFA balance across the full linear range, resulting in an impressive range of 1–74% in the adipose tissue triglycerides.

Variation in PUFA balance between the tissues is not huge, however, the brain is quite unique in regulating membrane PUFA balance at higher levels than the membrane lipids of other tissues (42–55%). The

PUFA balance of heart, liver, RBC and plasma phospholipids is slightly lower than the brain ranging from ~4 to 40% for all tissues, while muscle levels are maintained at slightly higher levels (18–48%). The PUFA balance of the triglycerides is the most variable, for example, plasma triglycerides ranged 2–58%.

### 3.5. Comparison of response of tissue phospholipids and triglycerides to diet for each fatty acid type

The response of tissue phospholipids and triglycerides to diet fat profile is displayed in Fig. 5, where the slope of each relationship is plotted. The results show that the phospholipid fatty acid composition of muscle, heart, brain, liver, RBC and plasma is regulated relatively constant even over large changes in diet fatty acid profile (see Fig. 5a, b, c, d, e). Phospholipid SFA content is the most highly regulated fatty acid class (average slope 0.02) and phospholipid MUFA and PUFA content only slightly more responsive for all tissues (average slope 0.09 and 0.07 respectively).

The phospholipids are more responsive to diet n–3 PUFA (average slope 0.12) and to an even greater extent to diet n–6 PUFA (average slope 0.20), and this relationship is significant even for brain phospholipids, which are the most regulated membrane lipids (slope 0.02 and 0.05 for n–3 PUFA and n–6 PUFA respectively). The highest slope value of 0.24 for liver n–6 PUFA indicates that a 100% change in diet n–6 PUFA results in only a 24% change in n–6 PUFA content of liver membrane lipids. So although the composition of the essential fatty acids in the membrane lipids can be altered, this can only occur to a small degree.

Adipose tissue “storage lipids” are more responsive to diet fat type than the plasma lipids and also the membrane lipids of all the tissues for all fatty acid types. The adipose tissue triglycerides respond similarly to diet SFA, MUFA, PUFA, n–6 PUFA and n–3 PUFA (slopes 0.45–0.69). The response of plasma triglycerides to the diet fat types is lower than the adipose tissues, but consistently higher than the phospholipids (slopes 0.08–0.57).

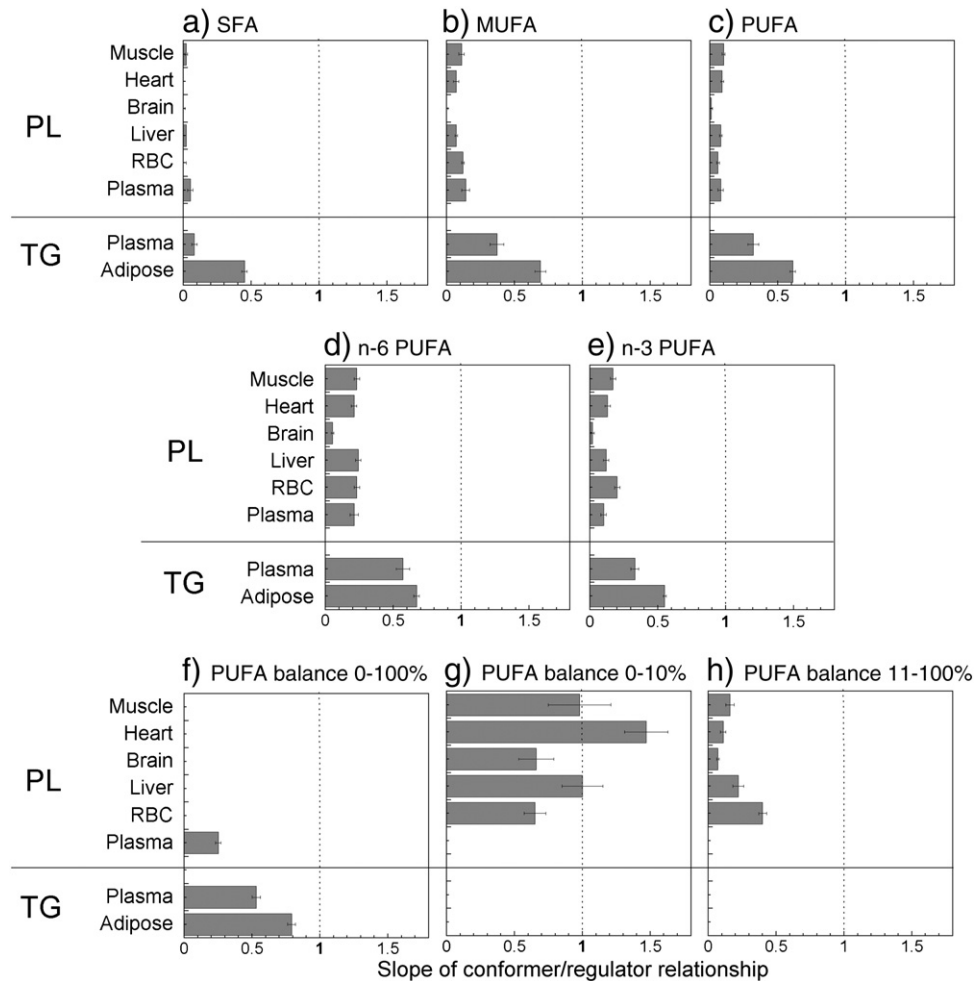
The membrane phospholipids of muscle, heart, brain, liver and RBC respond to diet PUFA balance in a similarly biphasic manner (Fig. 5g, h). The membrane lipids of these tissues are highly responsive to diet PUFA balance <10% (slopes 0.65–1.47), but maintain a more constant level at diet PUFA balances 11–100% (slopes 0.07–0.40). The results show that the diet PUFA balance affects membrane fatty acid composition to a much greater extent than diet SFA, MUFA, total PUFA, n–6 PUFA or n–3 PUFA for all tissues, and this influence is most pronounced at diet PUFA balance <10%.

The tissue most highly responsive to low diet PUFA balance is the heart (slope 1.47). At diet PUFA balances below 10%, heart membrane lipids are gaining n–6 PUFA at the expense of n–3 PUFA to a greater degree than the change in diet PUFA balance (i.e. a 100% change in diet PUFA balance results in a 147% change in membrane PUFA balance). Muscle and liver membrane lipids respond similarly to diet PUFA balance <10%, with slopes ~1.0, indicating that the membrane PUFA balance is changing in perfect balance with PUFA balance of the diet.

The response of plasma phospholipids, plasma triglycerides and adipose tissue triglycerides to diet PUFA balance is a single linear response (Fig. 5f). Although not biphasic, these tissues are still highly responsive to diet PUFA balance (slopes 0.25, 0.50, 0.79 respectively). Examination of the adipose tissue triglycerides indicates that these lipids are more responsive to diet fat, and similar to the membrane lipids, this response is profoundly linked to diet PUFA balance. This shows the expected results that storage fats are highly reflective of the diet fat profile for all fat types.

## 4. Discussion

The tissue fatty acid compositions reported in the present study are comparable to those of equivalent previous studies. For example,



**Fig. 5.** The responsiveness of tissue phospholipids and triglycerides to diet fatty acid composition (% total fatty acids): (a) SFA (b) MUFA (c) PUFA (d) n–6 PUFA (e) n–3 PUFA; and diet PUFA balance (n–3 as % total PUFA), including a single linear relationship for diet PUFA balance ranging from (f) 0–100% and a biphasic relationship using two linear relationships for diet PUFA balance from (g) 0–10% and (h) 11–100%. A value of zero indicates no relationship, while a value of 1 indicates the tissue lipids perfectly conform to diet fat profile. Phospholipids include skeletal muscle, heart, brain, liver, red blood cells (RBC) and plasma. Triglycerides include plasma and adipose tissue. Values are slopes  $\pm$  S.E.M. Significant relationships are evident for all linear regression analyses (i.e. the slope of the line of fit is significantly different from zero;  $p < 0.05$ ), with the following exceptions: muscle, heart, RBC and brain SFA, as well as brain MUFA and PUFA.

the results for liver and RBC membrane lipids, plasma phospholipids, as well as triglycerides from plasma and adipose tissue are similar to those reported by Lands et al. [4], while those for skeletal muscle phospholipids are similar to Soriguer et al. [17], and the fatty acid composition of heart and brain membrane lipids are similar to Barcelo-Coblijn et al. [18].

In membrane lipids from all tissues the relative composition of the major fatty acid classes (SFA, MUFA and PUFA) is strongly regulated over very large variation in diet composition. Diet SFA content has essentially no influence on membrane SFA composition (average slope 0.01). The unsaturated fatty acid (UFA) composition of the membrane lipids was not plotted in the present study, but as fatty acids can only be saturated or unsaturated it can be concluded that diet total UFA also have essentially no influence on membrane UFA content. The membrane response to both diet MUFA and PUFA is slightly greater than the response to SFA, but still regulated, with average slopes 0.07 for both fat types.

Of all tissues the brain membrane lipids consistently show the lowest responsiveness to diet fat profile. Brain membrane fatty acid composition is also distinctive, containing higher MUFA with a corresponding lower total PUFA compared to the other tissues. Between mammalian species comparisons have shown that brain PUFA content is conserved over large changes in body mass, while the PUFA content of the heart and skeletal muscle decreases with body size

[19]. Thus rats have a lower PUFA content in their brain membrane lipids relative to other tissues (muscle, heart, liver, RBC), while the opposite trend is observed in humans [19].

Lipids responsible for transport and energy storage (i.e. plasma lipids and adipose tissue triglycerides) did not show this biphasic response to diet PUFA balance found in all membrane lipids, but instead showed a linear response. The adipose tissue triglycerides are known to be good biomarkers for diet fat profile. In the present study the adipose tissue triglycerides demonstrated the highest response to diet PUFA balance over the full linear range (slope 0.79), and indeed the greatest response to all diet fat types (slopes 0.45–0.67). Furthermore, compared to the other tissues lipids, adipose tissue triglycerides consistently showed the strongest correlation to diet fat profile for all fat classes ( $R^2$ -values 0.86–1.00). Diet fatty acid profile can be difficult to determine in humans and quite often blood or tissue lipid profiles are used as biomarkers for dietary status [see Ref. 20]. The high response values indicate that adipose tissue triglycerides are indeed an extremely useful biomarker of diet fatty acid profile, confirming the results of previous studies in humans [21–23].

In humans the omega–3 index is used as a risk factor for death from coronary heart disease [24]. This index is calculated from RBC membrane 20:5n–3 and 22:6n–3 content only. In the present study we found that the main membrane n–3 PUFA in the RBC of rats are 22:6n–3, 22:5n–3 or 20:5n–3. Thus, if this index is to be



used in rat studies it should include 22:5n–3 as well as 20:5n–3 and 22:6n–3.

For each particular species membrane fatty acid composition is regulated, most likely to maintain optimal fluidity, and this is generally achieved by altering the degree of unsaturation in the fatty acyl chains in a process known as ‘homeoviscous adaptation’ in ectothermic vertebrates [25]. This regulatory process is important for the proper functioning of membrane-associated processes [26] and means that it is only within the limits of homeoviscous adaptation that external factors, such as diet fat profile, can influence membrane lipid profile. Membrane fatty acid composition appears to be specific for each species, suggesting that it is genetically regulated, and this specificity may even exist at the sub-species level. For example, in a recent study three mice strains were shown to have significantly different membrane fatty acid profiles even though they were maintained on identical diets and housing conditions for their entire lives [27,28]. Little is known about the precise mechanisms involved in the regulation of membrane fatty acid composition at the species level, however, the variety of enzymes involved in membrane remodelling (especially acyltransferases) are likely important.

The earlier work by William Lands established that acyltransferases catalyse the incorporation of n–3 and n–6 PUFA into the sn-2 position of the membrane phospholipids during membrane remodelling, and although they have a very high preference for PUFA, they do not discriminate between n–3 PUFA and n–6 PUFA [29]. The relative abundance of these two types of polyunsaturates in the diet will likely therefore strongly influence the balance between them in membrane lipids. This may provide an explanation as to why the balance between the n–3 and n–6 PUFA in the diet (i.e. PUFA balance) has the greatest influence on membrane fatty acid composition.

Although the membrane lipids are relatively unresponsive to total PUFA content of the diet (average slope 0.07) they show a higher individual responsiveness to the two types of PUFA (average slopes for n–3 PUFA = 0.12 and n–6 PUFA = 0.19). The essential requirement of rats for polyunsaturates was first shown by Burr and Burr [30]. Collins et al. demonstrated that n–6 PUFA was an essential fatty acid for humans in 1971 [31], but it was another 11 years before it was definitively shown that n–3 PUFA was also an essential component of the human diet [32]. Thus, n–3 PUFA and n–6 PUFA have been shown to be independent essential fats, and in some ways it is misleading to consider them combined in the category of ‘total PUFA’ in the diet.

Possibly the most important finding reported here is the biphasic nature of the PUFA balance relationship between diet and membrane lipid for all tissues (see Fig. 4). When the PUFA balance of the diet is below 10% the membrane composition is essentially conforming to diet. Such a conclusion is supported by the fact that the average slope is 0.95 for membrane lipids from heart, liver, muscle, brain and RBC. Above 10% PUFA balance, diet has much less influence on membrane composition and the diet-membrane lipid relationship for PUFA balance (average slope 0.19) is similar to that for n–3 and n–6 PUFA separately. The strong influence of low PUFA balance in the diet (i.e. <10%) represents the greatest diet influence on membrane composition observed in this study. Furthermore, the conformity of the membranes to low diet PUFA balance (<10%) is greater than the response of the adipose tissue triglycerides to diet PUFA balance over the full linear range (slope 0.79).

This biphasic relationship between diet PUFA balance and membrane PUFA balance is likely not solely dependent on the amount of fat in the diet. For example, using a low-fat diet (~10% energy) Bourre et al. have determined the requirement of rat brain and other tissues for diet 18:3n–3 during development [33,34]. Although the conditions of these studies are not identical to the current study, we have calculated and plotted the PUFA balance of both diets and membrane lipids (for liver and brain) from Ref. [33], revealing a biphasic pattern with a break at a diet PUFA balance 6% for liver and 4% for brain [33]. Furthermore, the diets in Bourre’s study contained 20 and 22-carbon

n–3 PUFA [33], so it would seem that the biphasic pattern for PUFA balance may also apply to diets containing longer-chained PUFA.

The findings of the present study on rats have relevance for humans. In any extrapolation from findings of a small mammal (such as laboratory rodents) to a large mammal (such as humans) the relationship between body size and the rate of metabolic processes (and thus time) needs to be taken into account. It has been known for a long time that metabolic processes are slower in larger animals [10]. The initial conclusion that humans, unlike rats, did not have an essential requirement for PUFA was based on the error of not taking this ‘relative time’ into account. The symptoms of EFA deficiency take ~3 months to become manifest in rats and thus the original six months of testing period in humans was likely inadequate [see Ref. 35]. We now know that rats and humans essentially have the same dietary requirements for n–6 and n–3 PUFA (% of total energy). As mentioned earlier, the 8 week feeding period in rats used in the present study is analogous to approximately one year in humans.

There are important implications of our findings for the current human diet. As mentioned in the **Introduction**, the modern human diet has an average PUFA balance of 9.5% [14]. If the current results in rats also apply to humans, this low diet PUFA balance is of grave concern, with an imbalance between diet n–3 and n–6 PUFA associated with a number of diseases which have become prevalent in today’s society, such as dyslipidaemia, hypertension, inflammation, depression, abdominal obesity, type 2 diabetes and cardiovascular disease [7,36–38]. In particular, chronic inflammation is known to be the driving force behind many diseases that have increased in recent time, including insulin resistance, obesity, atherosclerosis, cancer and neurodegenerative diseases, such as Alzheimer’s disease [39,40]. Diet fatty acid composition is known to influence inflammation through changes in cell membrane fatty acid composition, with changes in the substrate availability for pro-inflammatory eicosanoids (from 20:4n–6) and anti-inflammatory resolvins and protectins (from 20:5n–3 and 22:6n–3) [40]. So an imbalance towards too much n–6 PUFA in the modern human diet may result in increased membrane 20:4n–6 levels and thus increased chronic inflammation in many humans. This very important aspect of our study will be discussed in much greater detail in a future publication.

Recommendations by the American Heart Association to increase n–6 PUFA intake have been met with considerable concern from some researchers [see Ref. 41] and it has since been shown that in fact, increasing linoleic acid intake without combined increases in n–3 PUFA intake may actually lead to higher risks of coronary heart disease and death [42]. This further emphasises the importance of improving the balance between n–3 and n–6 PUFA in the modern human diet.

Diet SFA content had no influence whatsoever on membrane composition in the present study, so any potential health effects are not likely to be associated with membrane fatty acid composition. Diet SFA content may be exerting greater influence through changes in food intake and body composition, which we will report on in an upcoming paper.

For people to improve their diet they need to know what they are eating and especially the balance between n–3 and n–6 PUFA in their food. An immediate step may be for the Food Databases (e.g. [www.nal.usda.gov/fnic/foodcomp/search](http://www.nal.usda.gov/fnic/foodcomp/search) and [www.foodstandards.gov.au/consumerinformation/nuttab2006](http://www.foodstandards.gov.au/consumerinformation/nuttab2006); both accessed 10/01/11), which currently provide information about the content of individual fatty acids in various foods (including the total PUFA content) to also include the total n–3 PUFA and total n–6 PUFA data. Similarly, food labelling only provides the total SFA, MUFA and PUFA content of food products in most countries. The different physiological activities of n–3 PUFA and n–6 PUFA mean that these fats need to be identified separately on food labels or better still, the balance between these n–3 and n–6 PUFA [see Ref. 13] so that we can more effectively increase our diet PUFA balance.



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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2012.01.011.

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