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N–3 polyunsaturated fatty acids impair lifespan but have no role for metabolism

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Summary

Although generally considered as beneficial components of dietary fats, polyunsaturated fatty acids (PUFA) have been suspected to compromise maximum lifespan (MLSP) in mammals. Specifically, high amounts of phospholipid PUFAs are thought to impair lifespan due to an increase in the susceptibility of membranes to lipid peroxidation and its damaging effect on cellular molecules. Also, there is evidence from *in vitro* studies suggesting that highly unsaturated PUFAs elevate basal metabolic rate (BMR). Previous comparative studies in this context were based on small sample sizes, however, and, except for one study, failed to address possible confounding influences of body weight and taxonomic relations between species. Therefore, we determined phospholipid membrane composition in skeletal muscle from 42 mammalian species to test for a relation with published data on MLSP, and with literature data on BMR (30 species). Using statistical models that adjust for the effects of body weight and phylogeny, we found that among mammals, MLSP indeed decreases as the ratio of n–3 to n–6 PUFAs increases. In contrast to previous studies, we found, however, no relation between MLSP and either membrane unsaturation (i.e. PUFA content or number of double bonds) or to the very long-chain, highly unsaturated docosahexaenoic acid (DHA). Similarly, our data set gave no evidence for any notable relation between muscle phospholipid fatty acid composition and BMR, or MLSP and BMR in mammals. These results contradict the 'membrane pacemaker theory of aging', that is, the concept of a direct link between high amounts of membrane PUFAs, elevated BMR, and thus, impaired longevity.

Key words: basal metabolic rate; docosahexaenoic acid; mammals; maximum lifespan; muscle phospholipids; polyunsaturated fatty acids.

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Introduction

Polyunsaturated fatty acids (PUFA) are essential components of dietary fats and have a number of important cellular functions including regulation of enzymes, ion pumps, and immune responses (Stubbs & Smith, 1984; Pond & Mattacks, 1998). In the context of aging, however, there are several arguments suggesting that PUFAs may adversely affect maximum lifespan (MLSP; Barja, 2004; Pamplona *et al*., 2004; Hulbert, 2005). Firstly, because PUFAs are located at the mitochondrial membrane, they are prone to lipid peroxidation, which results in extensive production of radical oxygen species (ROS) (reviewed in Hulbert, 2005). Radical oxygen species readily interact with macromolecules, cause accumulating tissue damage, and eventually lead to death from age according to the 'free radical theory' (Brand, 2000; Hulbert, 2003; Barja, 2004; Speakman, 2005a). Secondly, PUFAs are thought to raise metabolic rate, i.e. one of the factors that seems to be associated with short lifespans (Rubner, 1908; Pearl, 1928; Daan *et al*., 1996; but see Speakman *et al*., 2003, 2004). Species with a high basal metabolic rate (BMR), such as small mammals, also have high membrane PUFA contents (Hulbert, 2005). Furthermore, there is experimental evidence that PUFAs increase the activity of membrane associated metabolically active proteins, such as the sodium pump (Wu *et al*., 2001; Turner *et al*., 2003). Based on these observations, the 'membrane pacemaker theory' of metabolism (Hulbert & Else, 1999, 2000, 2004, 2005; Hulbert, 2003, 2005) suggests that high amounts of membrane PUFAs lead to elevated BMR and increased peroxidation of fatty acids, and thus impair MLSP in mammals.

Among PUFAs, one particular fatty acid, docosahexaenoic acid (DHA), which predominantly occurs in membranes of retina and brain, was shown to significantly increase Na^+-K^+-ATPase molecular activity (Turner *et al*., 2003). Therefore, DHA is thought to act as a particularly important pacemaker of BMR (Hulbert & Else, 1999, 2000, 2004, 2005). Accordingly, numerous studies have demonstrated a negative correlation between DHA content in tissue membranes and MLSP in mammals and birds (Pamplona *et al*., 1998, 1999a; Portero-Otin *et al*., 2001; Hulbert, 2003, 2005).

As pointed out by Speakman (2005b), there are, however, two major problems with these simple correlations between membrane fatty acid composition, metabolism, and longevity. First, correlations between these variables may be merely due to the fact that all of them are correlated to body weight, a most 'pervasive trait that influences all aspects of organismal biology' (Speakman, 2005b), but may have no actual functional relation to each other. Second, species in comparative data sets may not represent independent replicates, due to phylogenetically

Table 1 Species investigated with body weights, maximum lifespan (MLSP) and fatty acid composition

	Body weight (kg)	MLSP (years)	SFA (%)	MUFA (%)	PUFA (%)	n–3 PUFA (%)	n–6 PUFA (%)
Artiodactyla							
Antilope cervicapra	37	18	24.47	7.65	67.89	11.21	56.69
Axis axis	100	21	25.95	5.23	68.82	11.80	57.10
Bos taurus	369	20	29.66*	34.95*	34.54*	8.90*	25.20*
Capra ibex	40	14	23.01	6.60	70.39	19.74	50.64
Capreolus capreolus	20	15	27.05	7.60	65.40	15.01	50.30
Cervus dama	80	25	28.07	9.50	62.42	11.78	50.60
Cervus elaphus	150	27	24.64	8.51	63.81	14.66	49.15
Cervus nippon	52.5	15	24.60	6.20	69.20	20.30	48.90
Connochaetes taurinus	165	22	22.71	9.45	67.84	5.89	61.95
Giraffa camelopardalis	700	36	25.81	12.90	61.33	6.90	55.40
Ovis ammon	16.1	13	20.9	11.31	67.80	19.60	48.20
Rangifer tarandus	170	15	25.40*	17.30*	46.10*	14.20*	31.90*
Rupicapra rupicapra	35	22	24.41	12.90	62.70	21.60	41.12
Sus domestica	150	27	29.30*	18.15*	52.80*	3.90*	49.70*
Sus scrofa	50	21	27.30	8.06	64.65	6.98	57.66
Sus pekari	30	25	28.44	4.02	67.54	6.43	61.11
Tragelaphus oryx	400	25	27.40	11.37	61.23	16.21	45.02
Tragelaphus angasi	85	16	28.30	17.80	53.90	12.25	41.65
Carnivora							
Acinonyx jubatus	55	15	27.32	22.90	49.79	7.31	42.48
Canis familiaris	20	30	31.65	10.06	58.30	3.56	54.74
Canis lupus	30	16	28.05	13.07	58.88	5.14	53.75
Martes foina	1.58	18	27.61	10.02	62.37	8.33	54.05
Meles meles	8.5	16	27.24	5.40	67.36	7.54	59.82
Mustela putorius	9.1	10	28.99	5.80	65.21	20.83	44.37
Panthera tigris	107	26	29.14	25.61	45.25	7.73	37.53
Vulpes vulpes	5.1	12	28.88	13.34	57.77	6.59	51.18
Insectivora							
Erinacaeus europaeus	1	14	26.22	13.70	60.08	22.95	37.13
Neomys fodiens	0.01	3	32.30*	13.90*	53.80*	37.00*	16.80*
Sorex araneus	0.01	2	34.00*	11.90*	54.10*	41.90*	12.20*
Talpa europaea	0.07	4	27.17	11.26	61.57	46.80	14.77
Lagomorpha							
Lepus europaeus	3.62	12	27.26*	5.66*	66.74*	22.54*	44.28*
Oryctolagus cuniculus	4.10	18	33.34*	15.09*	51.58*	12.65*	38.57*
Perissodactyla							
Equus caballus	500	62	28.31	7.21	64.47	7.19	57.29
Proboscidea							
Loxodonta africana	4000	70	22.65	39.16	38.19	9.55	28.64
Rodentia							
Capromys pilorides	4.5	11	30.90	5.57	63.53	17.86	45.68
Cavia aperea	0.19	8	32.93	6.07	61.00	10.70	50.30
Cricetus cricetus	0.09	4	26.05	15.85	58.10	29.09	29.01
Glis glis	0.09	9	27.18	19.89	52.93	20.29	32.64
Marmota marmota	4	15	30.30	8.38	59.43	20.36	39.07
Mus musculus	0.04	6	33.10*	15.00*	51.90*	20.90*	30.50*
Rattus norvegicus	0.58	3	35.00*	15.10*	49.90*	21.18*	36.50*
Sciurus vulgaris	0.32	12	27.08	6.43	66.50	22.11	44.39

*Data obtained from literature (references are cited in Supplementary Table S1).

caused correlations. Fortunately, both of these problems can be overcome by employing statistical procedures that adjust for body weight and phylogenetic effects. In his reanalysis of relations between DHA, MLSP, and BMR, Speakman (2005b) found that indeed, after statistically adjusting for both body weight effects and phylogeny, there was no significant relation between MLSP and BMR, and only a weak relationship between MLSP and DHA. With regard to membrane fatty acids, this analysis was, however, limited to DHA, and to eight mammalian species only, which may have been one of the reasons for the observed lack of correlations with MLSP.

Table 2 The relation between muscle phospholipid fatty acid composition and maximum lifespan in mammals. Regression results from separate models which all included a single fatty acid (class) and body weight as a covariate. Bootstrap results are estimated slopes and 95% confidence intervals of repeated regressions using random picks of individual specimen from each species. Values in bold face indicate significant ($P \leq 0.05$) effects

	Conventional Regression				Phylogenetic regression				Bootstrap results		
	Slope	SE	*t*	*P*	Slope	SE	*t*	*P*	Lower CI	Slope	Upper CI
DHA	−0.015	0.070	−0.222	0.825	0.054	0.067	0.807	0.425	−0.150	−0.018	0.124
Linoleic acid	0.294	0.118	2.480	**0.017**	0.186	0.176	1.056	0.298	**0.068**	**0.323**	**0.771**
Linolenic acid	−0.158	0.055	−2.862	**0.007**	−1.174	0.062	−2.803	**0.008**	**−0.274**	**−0.148**	**−0.040**
UI	−0.149	0.399	−0.374	0.710	−0.003	0.393	−0.007	0.995	−1.160	−0.172	0.664
PI	−0.317	0.264	−1.203	0.236	−0.093	0.271	−0.343	0.734	−0.835	−0.304	0.210
PUFA	0.406	0.352	1.154	0.256	0.082	0.422	0.193	0.848	−0.330	0.408	1.154
n–3 PUFA	−0.273	0.108	−2.534	**0.015**	−0.359	0.155	−2.313	**0.026**	**−0.420**	**−0.252**	**−0.066**
n–6 PUFA	0.433	0.165	2.616	**0.013**	0.395	0.263	1.503	0.141	**0.105**	**0.427**	**0.778**
n–3/n–6 PUFA	−0.368	0.116	−3.173	**0.003**	−0.560	0.188	−2.973	**0.005**	**−0.564**	**−0.348**	**−0.148**
MUFA	−0.126	0.105	−1.200	0.237	0.019	0.129	0.149	0.882	−0.315	−0.114	0.092
UFA	1.186	1.227	0.966	0.340	0.582	1.297	0.449	0.656	−2.020	0.947	4.083
SFA	0.044	0.558	0.080	0.937	0.301	0.655	0.460	0.648	−1.544	0.029	1.174

Therefore, we collected data on DHA and other PUFA muscle phospholipid contents in 42 mammalian species (Fig. 1, Table 1) and re-examined their possible effect on MLSP. In short, we found that after adjusting for the influence of body weight and phylogenetic correlations, MLSP was neither related to DHA content, nor to membrane unsaturation (i.e. PUFA content or number of double bonds). Interestingly, however, MLSP significantly decreased as the class of phospholipid n–3 PUFAs (including DHA) increased, and, consequently n–6 PUFAs decreased. This effect of the n–3/n–6 PUFA ratio appears to be independent from metabolic rate because we found no relation between any characteristic of membrane fatty acid composition and BMR.

Results

Longevity vs. fatty acids

Maximum lifespan was not predicted by fatty acid classes grouped according to their degree of unsaturation, i.e. saturates (SFA), monounsaturates (MUFA) or PUFA ($P > 0.1$). This finding was valid for both conventional and phylogenetic regression models (Table 2). Also, the unsaturation index (UI), or DHA content did not correlate with MLSP of mammals after adjusting for body weight and phylogeny (Fig. 2, Table 2).

Maximum lifespan did significantly decrease, however, with the total proportion of all n–3 PUFAs in muscle phospholipids ($F_{1,39} = 6.271$; $P = 0.015$, Table 2), and correspondingly increased as n–6 PUFA content increased ($F_{1,39} = 6.851$; $P = 0.013$, Table 2). This finding was also reflected by a significant relation between the n–3/n–6 PUFA ratio and MLSP ($F_{1,39} = 10.074$; $P = 0.003$, Fig. 3). Differential effects of n–3 and n–6 PUFAs were also detectable for the most common single fatty acid of each of these classes: linolenic acid (C18:3 n–3) had a negative impact on longevity ($F_{1,39} = 7.799$; $P = 0.007$), while increases in linoleic acid (C18:2 n–6) were associated with increased MLSP ($F_{1,39} = 6.167$; $P = 0.017$, Table 2).

We found no evidence for the possibility that these results could have been caused, or substantially biased, by the inclusion of species with low numbers of replicates and hence increased uncertainty in the predictor variables: 95% confidence intervals of the regression slopes computed from a bootstrap procedure with random picks of single specimens from each species (see Experimental procedures) did not overlap with zero in all cases in which the conventional regression indicated a significant statistical effect ($P < 0.05$), and vice versa (Table 2).

Our results from conventional regression models were further confirmed by very similar effect sizes and *P* values from phylogenetically corrected regressions (Table 2). Only the positive association between MLSP and n–6 PUFAs (as well as their dominant component, linoleic acid) was reduced to a non-significant trend after phylogenetic correction. The negative relation between MLSP and the n–3/n–6 PUFA ratio, however, remained highly significant in the phylogenetic regression model (Table 2).

Basal metabolic rate vs. fatty acids

Expectedly, BMR was strongly correlated with body weight (partial effect: $F_{1,27} = 721.137$; $P < 0.0001$; Fig. 4). However, we found no significant change of body weight-adjusted BMR with either total muscle phospholipid n–3 PUFA content or DHA (Table 3, Fig. 4). Also, BMR was neither predicted by the fatty acid classes SFA, MUFA, PUFA, and UFA nor by any other membrane fatty acid characteristic in conventional regression models (Table 3). These results from conventional multiple regression were confirmed by phylogenetically corrected regressions (Table 3), except that in one case, phylogenetic regression indicated a decrease of BMR with increasing peroxidizability of membrane fatty acids. However, given the multiplicity of *P* values computed, we would consider this partial correlation ($P = 0.035$) a tendency only.

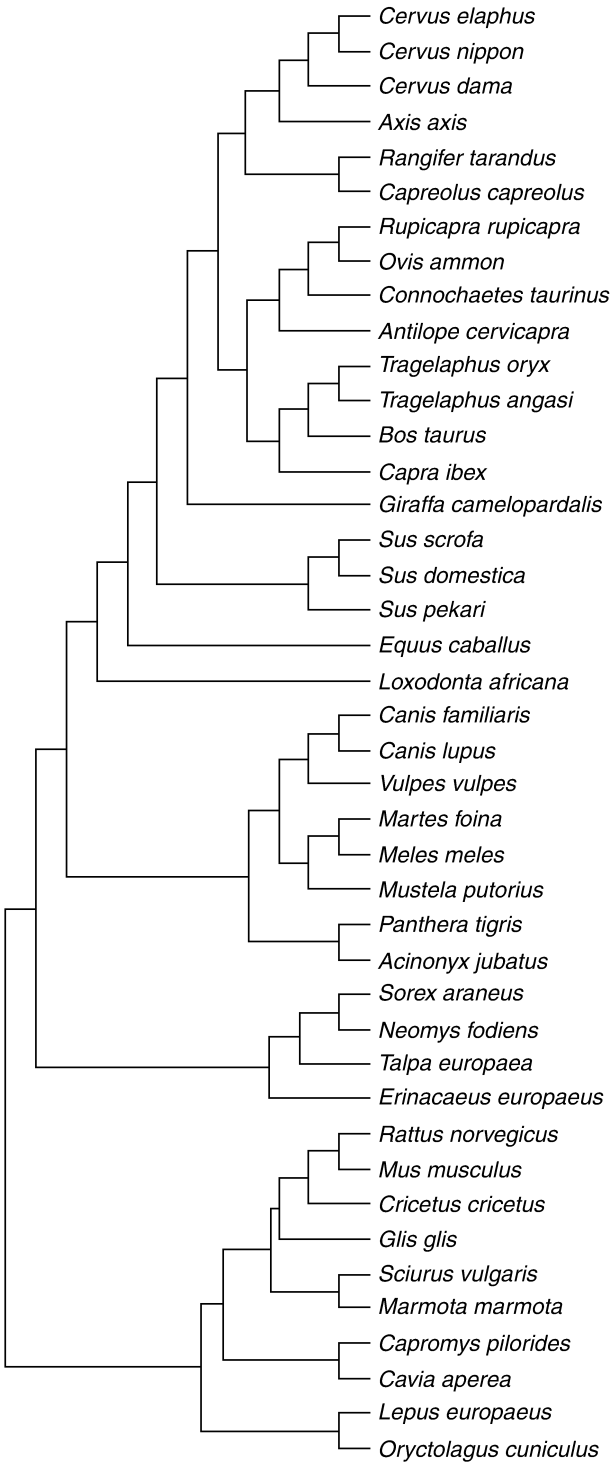

Fig. 1 Phylogeny of the 42 mammalian species for which phospholipid muscle fatty acid composition was determined. Branch lengths are arbitrary. This dendrogram was constructed using the statistical package R for Windows (version 2.3.1, R-Project, 2005).

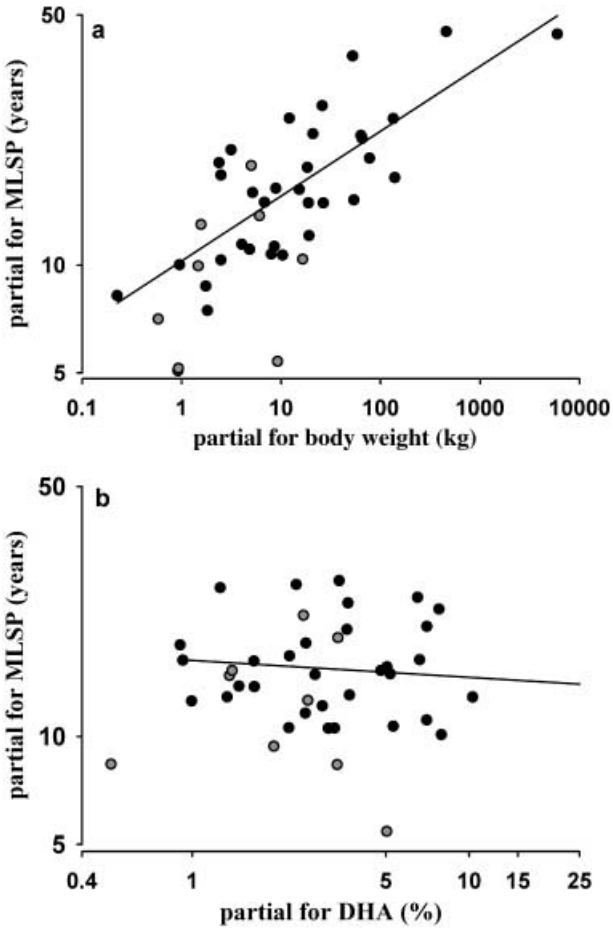

Fig. 2 Body weight, docosahexaenoic acid (DHA), and maximum lifespan (MLSP) in mammals. (a) Partial regression plot of the relation between body weight and MLSP. (b) Partial correlation between DHA in muscle phospholipids and MLSP. All traits were log transformed. Maximum lifespan was significantly related to body weight ($F_{1,39}$ = 138.3; P < 0.0005, panel a), but DHA content in muscle phospholipids was not related to MLSP ($F_{1,39}$ = 0.018; P = 0.825, panel b). Grey dots represent data obtained from the literature, whereas black dots represent values from this study. We found no systematic difference between literature data and results from our lab.

Longevity vs. basal metabolic rate

Finally, we tested whether longevity was predicted by metabolic rate in our data set and found no significant association with BMR ($F_{1,27}$ = 2.474; P = 0.127, Fig. 5), after adjusting for the significant influence of body weight ($F_{1,27}$ = 152.01; P < 0.0005).

Discussion

Longevity and phospholipid fatty acids

Our analysis has clearly demonstrated that, even after statistically eliminating the influence of body size, longevity across mammals was significantly predicted by the ratio of n–3 to n–6 PUFAs. Thus, if this finding reflects a causal relationship, muscle phospholipid n–3/n–6 ratios may play an important role in determining MLSP. However, our data do not support the hypothesis that longevity of mammals is linked to membrane unsaturation (i.e. PUFA content or UI) or to DHA alone, as suggested by previous studies that were based on eight mammalian species (Pamplona *et al*., 1998, 1999a; Portero-Otin *et al*., 2001;

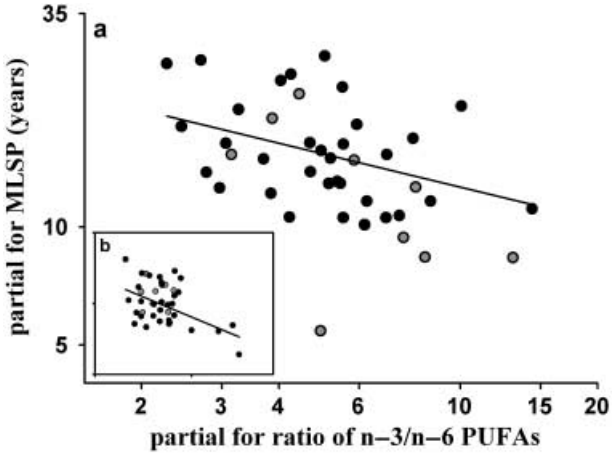

Fig. 3 The relation between the n–3/n–6 polyunsaturated fatty acids (PUFA) ratio in muscle phospholipids and maximum lifespan (MLSP) in mammals. Partial regression plot after adjusting for the influence of body weight ($F_{1,39}$ = 173.9; P < 0.0005, panel a). The inset graph (panel b) shows a partial residual plot computed from a phylogenetically corrected regression. Note that the phylogenetic regression showed the same principal relation as the conventional regression. Grey dots represent data obtained from the literature, while black dots represent values from this study.

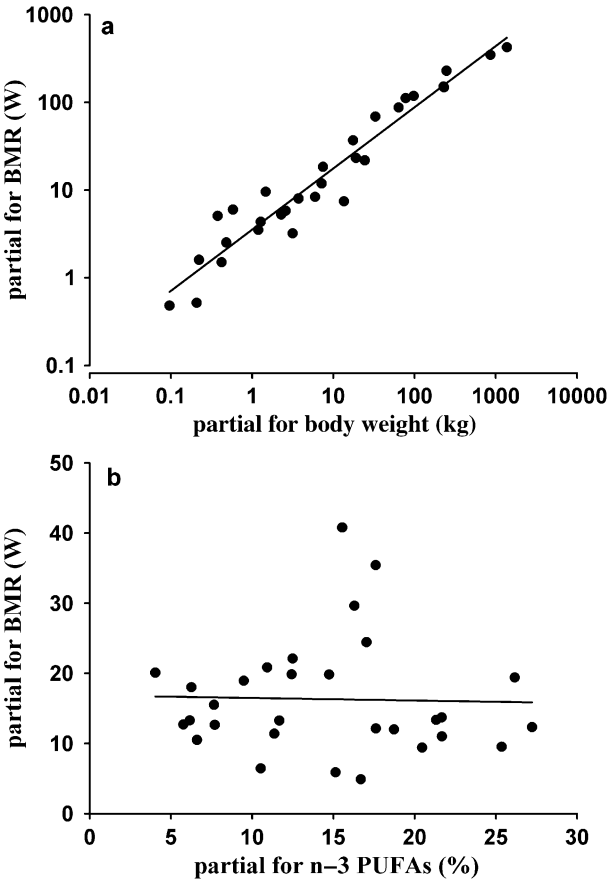

Fig. 4 Correlates of basal metabolic rate (BMR) in mammals. (a) Partial regression plot of the relation between body weight and BMR. (b) Partial regression of the relation between n–3 PUFAs in muscle phospholipids and BMR. While BMR was strongly related to body weight, the total fraction of n–3 PUFAs in muscle phospholipids did not explain any residual variation in BMR.

Hulbert, 2005). As outlined in the introduction, we would argue that those analyses, which did not correct for the known influence of body weight on both membrane unsaturation (or DHA) and MLSP, may merely reflect spurious correlations between these variables. Also, a tendency for an effect of DHA content on MLSP in a sample that was body weight adjusted (Speakman, 2005b) was not confirmed by our present comparison.

Rather than statistically adjusting for confounding body size effects, some previous studies used the approach of comparing animals with similar size but different longevity. In particular, Pamplona *et al*. (1996, 1999b) found significantly higher degrees of membrane unsaturation in rats, compared with pigeons with much higher longevity but equal body weight. Arguably, however, these comparisons still represent correlations, albeit with a sample size of two species only. In view of the residual variability in MLSP (cf. Fig. 2a), it is easy to envision that comparisons of a small number of species of similar weight may suggest relations that disappear in more extensive

Table 3 The relation between muscle phospholipid composition and basal metabolic rate (BMR) in mammals. Shown are regression statistics for separate models using each fatty acid class and body weight as predictors of BMR. Bootstrap results are estimated slopes and 95% confidence intervals of repeated regressions using random picks of individual specimen from each species. Values in bold face indicate significant effects ($P \leq 0.05$)

	Conventional regression				Phylogenetic regression				Bootstrap results		
	Slope	SE	*t*	*P*	Slope	SE	*t*	*P*	Lower CI	Slope	Upper CI
DHA	−0.104	0.105	−0.985	0.333	−0.085	0.075	−1.135	0.266	−0.297	−0.101	0.088
Linoleic acid	0.027	0.195	0.138	0.890	0.370	0.203	1.824	0.079	−0.548	0.005	0.360
Linolenic acid	0.057	0.103	0.559	0.581	0.127	0.086	1.478	0.151	−0.157	0.005	0.285
UI	−0.147	0.650	−0.227	0.822	−0.539	0.409	−1.317	0.199	−1.315	−0.158	0.998
PI	−0.412	0.423	−0.974	0.339	−0.582	0.262	−2.221	**0.035**	−1.176	−0.408	0.331
PUFA	−0.128	0.579	−0.222	0.826	−0.476	0.434	−1.097	0.282	−1.149	−0.116	0.826
n–3 PUFA	−0.059	0.185	−0.320	0.752	−0.295	0.218	−1.351	0.188	−0.297	−0.063	0.212
n–6 PUFA	−0.361	0.266	−1.355	0.186	−0.169	0.312	−0.559	0.580	−0.929	−0.331	0.283
n–3/n–6	0.141	0.195	0.722	0.476	−0.205	0.263	−0.779	0.442	−0.243	0.120	0.518
MUFA	0.113	0.167	0.680	0.502	0.184	0.139	1.322	0.197	−0.190	0.106	0.406
UFA	1.711	2.130	0.803	0.429	0.327	1.564	0.209	0.836	−1.868	1.633	6.145
SFA	−0.712	1.035	−0.688	0.497	0.503	0.882	0.570	0.574	−2.789	−0.670	1.245

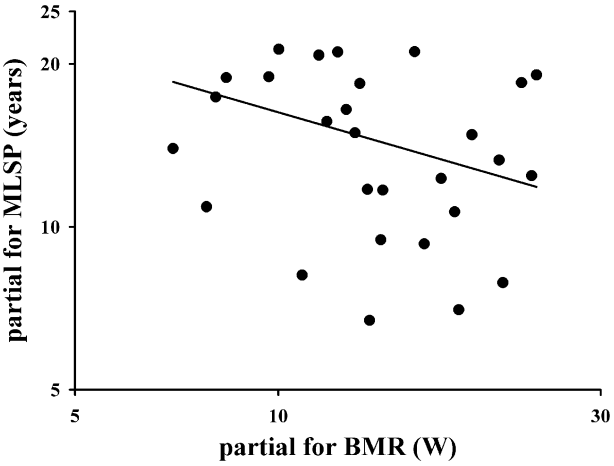

Fig. 5 The relation between basal metabolic rate (BMR) and maximum lifespan (MLSP) ($F_{1,27}$ = 2.474; P = 0.127, n.s.). The figure shows a partial regression plot after adjusting for body weight effects. Note that this analysis was restricted to subset of 30 mammalian species for which published data on BMR were available.

comparative analyses. Further evidence for a link between membrane unsaturation (particularly, DHA content) and longevity comes from a recent study on three mouse strains that differed in both phospholipid profiles and MLSP (Hulbert *et al*., 2006). Clearly, this approach not only eliminates body size differences but also minimizes genetic differences, and hence the probability of spurious correlations. Therefore, it may well be that there is indeed some association of high membrane unsaturation with decreased longevity, that remained undetected in our comparative study which is certainly affected by additional confounding differences between species. Even so, our analysis still shows that the magnitude of this association, if it exists at all, is much smaller than suggested by previous across-species correlations that have ignored body weight effects.

The second, possibly confounding factor in comparative studies, i.e. phylogenetic correlations, had very little effect on our regression models of longevity vs. phospholipid profiles. We were not surprised, however, to find that our results were virtually unaffected by phylogenetic corrections, because our particular sample consisted of a wide range of mammalian species with little within-taxon clustering. In further comparative studies in this context, possibly including more closely related groups of species, phylogenetic relations may well have strong effects on the statistical analysis.

From a physiological perspective, the lack of an association of MLSP with either DHA, total PUFA content, or UI in our analysis was somewhat surprising since undoubtedly, PUFAs, especially those from the n–3 class are more peroxidation prone than their inert, saturated counterparts, and their propensity for oxidative damage potentially may be detrimental for tissues (Hulbert, 2005). Also, as reviewed by Hulbert (2005), species with highly peroxidizable membranes do not possess correspondingly effective antioxidant defences, which suggests that those defence systems are not limiting for the aging process. However, even using the relative peroxidizability (Holman, 1954; Pamplona *et al*., 1998) as a predictor variable we found no evidence for a relation of the overall susceptibility of membranes to lipid peroxidation on MLSP. Taken together, these results indicate that the significant effect of the ratio of n–3 to n–6 PUFAs on MLSP observed here is likely due to differences in their chemical structure other than peroxidizability, and possibly related to membrane effects caused by the position of their first double bond.

There is further independent evidence for an important role of n–3/n–6 PUFA ratios in determining longevity. For instance, while it is generally difficult to substantially alter phospholipid fatty acid composition in muscles through dietary manipulation (e.g. Hulbert & Else, 1999, 2000; Turner *et al*., 2005a), one dietary manipulation that does affect fatty acid composition is caloric restriction. Interestingly, caloric restriction leads to a decrease in the percentage of n–3- and an increase of n-6 PUFAs (Lambert *et al*., 2004; Hulbert *et al*., 2005), and it is well established that a reduction in caloric intake, without malnutrition, also significantly extends MLSP in a wide range of organisms (Masoro, 1993; Sohal & Weindruch, 1996). Further, it has been noted that the well-known lifespan difference between mammals and birds of similar size is accompanied by a lower n–3/n–6 PUFA ratio in birds (Hulbert, 2003). These observations clearly point to a link between longevity and n–3/n–6 PUFA ratios in membranes. In view of our present results it seems questionable, however, whether the increased longevity in birds, or in calorically restricted animals, can be attributed to higher lipid peroxidation resistance, as has been suggested previously (Hulbert, 2005).

One could argue that some of the discrepancies between the present and some previous studies (apart from our adjustment for body weight) could be due to the fact that we analysed fatty acid composition of skeletal muscle rather than liver or heart phospholipids, as done, for instance, by Pamplona *et al*. (1996, 1998, 1999a,b). However, we know from an intraspecific study in hares (Valencak *et al*., 2003), and from other published data (Hulbert *et al*., 2002), that fatty acid contents in phospholipids from muscle and heart are highly correlated. In addition, some of the previous studies relating membrane composition to MLSP also were partly based on skeletal muscle phospholipids (e.g. Hulbert, 2003, 2005). Thus, it is unlikely that the lack of an effect of overall membrane unsaturation or peroxidizability on MLSP in our data set was due to a specific characteristic of muscle tissue.

There are, of course further, possibly confounding factors that may affect tissue phospholipid fatty acid composition, such as diet differences, stress, or levels of exercise in captive animals. However, the reported effect sizes of experimental manipulations of diet or exercise, for instance, are very small compared with the between-species range of differences in fatty acid composition studied here (Andersson *et al*., 2000; Scollan *et al*., 2001). Also, as demonstrated by our bootstrap analysis, within-species variability (that may have been partly caused by those confounding factors) is unlikely to mask or substantially affect any of the across-species relations investigated.

Basal metabolic rate and fatty acids

Our results clearly show that after adjusting for the impact of body weight there was no effect of PUFA content or any other aspect of membrane phospholipid composition on BMR within the 30 species compared. To our knowledge, this is the first time the relationship between membrane fatty acids and BMR has been addressed with a statistical approach that avoids spurious correlations with body weight. It clearly shows that mammals with a high degree of membrane unsaturation for their size do not have higher than expected BMRs, and vice versa. As for the relation between longevity and phospholipid profiles, this result was almost unaffected by phylogenetic correction of regressions (Table 2). The only notable discrepancy with conventional models was some indication for association between BMR and the peroxidizability index (PI) in phylogenetic regressions (Table 3). Contrary to previous reports of a positive relationship between membrane unsaturation and BMR (e.g. Hulbert & Else, 2004), this regression model indicated a decrease of BMR as PI increased. However, as mentioned before, in view of a relatively high false discovery probability due to the multiplicity of *P* values computed, we do not consider this result convincing evidence for a genuine relation between these variables.

One could be tempted to think that our statistical analysis is overly conservative and may tend to overlook biologically important effects of PUFAs. Arguably, however, this is not the case. First, correlations of biological traits with body weight are the rule rather than the exception, and the parsimony principle dictates the null hypothesis of their independence from each other (cf. Speakman, 2005b). Second, the residual variation in our present data was still large enough to detect certain relations, such as the effect of n–3/n–6 PUFA ratios on MLSP, if they are strong. Furthermore, it is well known that residual variation in weight-adjusted BMR does not simply reflect measurement error, but is also due to biologically important factors, such as phylogeny, geographical distribution, or nutritional characteristics (Lovegrove, 2000; White & Seymour, 2004). Since membrane unsaturation, according to our analysis, did not explain any of this variance, its role as a determinant of BMR, if it exists at all, is clearly of limited importance.

The lack of notable effects of membrane composition on BMR in our study is difficult to reconcile with several studies demonstrating a role for PUFAs, namely DHA, in promoting membrane-associated processes that require energy, such as the sodium pump activity under *in vitro* conditions (Wu *et al*., 2001, 2004; Turner *et al*., 2003, 2005a,b). Similarly, PUFAs are thought to increase proton permeability, which should result in increased metabolic rate (Brookes *et al*., 1998). Therefore, we propose that in order to regulate BMR to a species-specific, optimal level, mammals may adjust the molecular activity or density of membrane proteins, such as ion pumps, independently from their degree of membrane unsaturation. Hence, membrane PUFA content may explain a certain fraction of BMR (that is correlated with body weight), but biologically significant differences between species of similar weight are caused by PUFA independent mechanisms. In this context it is interesting that Turner *et al*. (2005a) have recently shown that mammals in fact show considerable differences in the concentration of membrane sodium pumps (Na^+-K^+-ATPase), one of the major contributors to BMR (Hulbert & Else, 2004, 2005; Turner *et al*., 2005a,b). Adjustments of sodium pump density and/or other metabolically active molecules could explain both the influence of PUFAs on the activity of membrane proteins *in vitro* and the lack of a body-weight-independent effect of unsaturation on total BMR.

Longevity vs. basal metabolic rate

Longevity was not associated with BMR in our study. This finding is in agreement with a recent comparison of 249 mammalian species that also showed no relation between MLSP and BMR when the influence of body weight was accounted for (Speakman, 2005b). Results of other cross-species comparisons remain controversial: while some studies suggest a negative relation between BMR and longevity, at least among certain mammalian orders (White & Seymour, 2004), others show that longer lifespan in similar sized species is associated with a higher BMR (Austad & Fischer, 1991). Similarly, voluntary exercise and its associated increase in metabolic rate does not shorten the lifespan of rats or humans (Van Voorhies, 2001), and there is no correlation between lifespan and individual metabolic rate in either mice or flies (Speakman *et al*., 2000; Hulbert *et al*., 2004). Furthermore, caloric restriction (with its positive effect on lifespan) may be associated with either decreased or increased metabolic rate in mice (Masoro *et al*., 1982; Demetrius, 2004), and mice strains with genetically knocked-out insulin signalling in their white adipose tissue (FIRKO) live significantly longer despite their increased mass-specific energy turnover (Blüher *et al*., 2003). Finally, it has been noted that although birds and mammals have essentially similar metabolic rates, birds are much longer lived than similar-sized mammals (e.g. Speakman, 2005a). These observations indicate that there is no physiological constraint that immediately links high metabolic rate, as such, to low lifespan in mammals of comparable size. This confirms our conclusion that the negative impact of membrane n–3/n–6 PUFA ratios on MLSP in mammals is almost certainly not due to potential effects of membrane fatty acid composition on BMR.

Conclusions

Our study demonstrates a strong relation between the ratio of n–3 to n–6 membrane fatty acids and maximum longevity in mammals, which is independent of body weight and phylogeny. Our results do not support previous hypotheses suggesting a simple relationship between membrane unsaturation, peroxidizability, BMR, and lifespan. Thus, our analysis underlines the importance of avoiding 'common pitfalls of comparative studies' (Speakman, 2005b), such as failure to statistically eliminate correlations of the trait under consideration with body weight. Further research is needed to clarify the mechanisms by which

ratios of membrane n–3 to n–6 PUFAs affect longevity in mammals, and probably in other vertebrates.

Experimental procedures

We examined muscle phospholipid fatty acid composition of mammals from six taxonomic orders (see Supplementary Table S1). Muscle samples consisted of tissues from zoo animals in Vienna and Salzburg, Austria as well as from the zoo Munich, Germany ($n_{Species}$ = 13). Zoo animals were killed for population management reasons, or died subsequent to trauma. Additionally, we sampled muscle material from fresh road kills ($n_{Species}$ = 6), from animals shot during hunting ($n_{Species}$ = 10), and finally, from breeding-colony animals ($n_{Species}$ = 4). In each case, we took 0.5 g of hind leg muscle, *Musculus vastus* (*M. vastus*), from the fresh carcass. In small mammals, when we were unable to gain this amount of muscle material from the *M. vastus* alone, we used muscle tissues from the entire hind legs. We felt justified to pool tissues in these cases because muscle (and fibre type) specific differences in total phospholipid fatty acid profiles are very small compared to the range of between-species difference investigated here (Kriketos *et al*., 1995; Valencak *et al*., 2003), or not detectable at all (Blackard *et al*., 1997; Nikolaidis *et al*., 2006).

All animals investigated were healthy individuals of both sexes. To avoid any effect of maturational changes in phospholipid composition, we excluded juvenile specimens from our analysis, and only used adult individuals. We tested for, but found no effect of gender on any individual fatty acid or any of the fatty acid classes determined (ANCOVA with adjustment for body weight, $0.10 < P < 0.84$). Thus, we pooled data obtained from males and females. We only used fresh tissues without any discoloration or gunshot wounds (hunting samples) and collected warm road kills as described in Valencak *et al*. (2003). Immediately after sampling, muscles tissues were placed in plastic bags and stored at –18 °C.

Lipids were extracted from muscle samples (0.5 g each) and lipid classes were separated on silica gel thin layer chromatography plates. All solvents contained butylhydroxytoluol in order to avoid oxidative modification of PUFAs. Phospholipid extracts were transesterified under nitrogen, extracted into hexane, and analysed by GLC (PerkinElmer Autosystem XL with autosampler and FID; Norwalk, CA, USA) using a capillary column (HP INNOWax, 30 m × 0.25 mm; Hewlett Packard, Palo Alto, CA, USA). Fatty acid methyl esters were identified by comparing retention times with those of standards (Sigma-Aldrich, St. Louis, MO, USA), and peaks were integrated using the Turbochrom 4.1 Software (PerkinElmer). Details about chemical analyses and substances used are given elsewhere (Valencak *et al*., 2003). For 33 species, we determined the proportions of the following phospholipid acids: C14:0, C15:0, C16:0, C17:0, C18:0 (saturated fatty acids, SFA), C16:1n–7, C18:1n–9 (MUFA), C18:2n–6, C18:3n–3, C20:4n–6, C20:5n–3, C22:5n–3, and C22:6n–3 (PUFA). Exact proportions of single fatty acids are not given here but can be obtained from the authors. Sample sizes within species ranged from one to 244 individuals (given in Supplementary Table S1). We felt justified to include single specimens because fatty acid concentrations showed only little to moderate within-species variation [coefficients of variation for all fatty acid (classes) investigated ranged from 2.3% to 36.67%, overall mean: 15.68%]. Nonetheless, we computed additional bootstrap statistics (see below) to test for the possible effects of including species for which no replicates were available.

Our fatty acid data set was enlarged with values obtained from the literature ($n_{Species}$ = 9, see Supplementary Table S1). We included only studies in which the same set of fatty acids was given as determined in our laboratory (except for three cases in which C14:0, C15:0 or C17:0 had not been determined due to their negligible amount [typically < 1%]). We computed mean proportions for each species when more than one reference was available (*Bos taurus*, *Sus domestica*, *Oryctolagus cuniculus*).

Data on body weight were obtained, if possible, from the actual specimen used for tissue collection (n = 25), or taken from the literature (n = 9). Basal metabolic rates for 30 from our set of 42 mammal species could be compiled from different sources (Supplementary Table S1), and units were transformed into Watt if necessary.

Data on MLSP were mainly gained from Carey & Judge (2000), $n_{Species}$ = 39, but completed with Grzimek (1988), $n_{Species}$ = 2 (*Rattus norvegicus*, *Cavia aperea*), and MacDonald & Barret (1993), $n_{Species}$ = 1 (*Cervus nippon*). For each species we only considered the highest longevity record which was available (Carey & Judge, 2000).

Fatty acid percentages were combined into classes of SFA, MUFA, PUFA, UFA (unsaturated fatty acids) according to their degree of unsaturation, and, additionally, PUFAs were attributed to the n–3 and n–6 family. For all animals an unsaturation index (UI) was computed (Couture & Hulbert, 1995) as well as the ratio between n–3 and n–6 PUFAs. The UI represents the average number of double bonds per 100 fatty acid molecules (Couture & Hulbert, 1995: UI = (%MUFA × 1) + (%Dienoic × 2) + (%Trienoic × 3) + (%Tetraenoic × 4) + (%Pentaenoic × 5) + (%Hexaenoic × 6)). Further, a peroxidisability index (P1) was computed as follows and as given in Pamplona *et al*. (1998): PI = (%MUFA × 0.025) + (%Dienoic × 1) + (%Trienoic × 2) + (%Tetraenoic × 4) + (%Pentaenoic × 6) + (%Hexaenoic × 8).

Prior to computing regression models, longevity, body weight, and BMR data as well as fatty acid classes (n–3, n–6 PUFAs, UFAs, MUFAs, SFAs, UI) and single fatty acids (C18:3, C20:5, C22:5, C22:6) were log transformed to linearise these traits. To assess the impact of body weight, BMR and lifespan on fatty acid proportions, we used multiple linear regression models and entered either longevity or BMR as the response variable. Body weight was always entered as a covariate. We used this multiple regression approach, rather than comparing residuals from individual regressions against body weight (e.g. Speakman, 2005b), because it avoids bias and allows to estimate P values based on the correct degrees of freedom (with all variables entered into a single model; Freckleton, 2002). Note that this statistical procedure statistically eliminates possibly confounding effects of body mass on both MLSP *and* on phospholipid fatty acid

composition. We computed separate analyses for each fatty acid class because percentages of fatty acids inevitably showed strong multicolinearity. While this leads to the problem of multiple *P* values, we followed the arguments by Nakagawa (2004) and did not employ further Bonferroni-like corrections. All analyses were carried out with the statistical package R (R-Project, Version 2.3.1; R Development Core Team, 2005). To illustrate the partial influence of variable correlations in multiple regression models, we used partial regression plots, also called added variable plots [function av. plot in library 'car', Fox (1997)], as described in Sall (1990).

To test for the influence of including data with very different levels of uncertainty for each species (i.e., replicates of fatty acid composition ranging from $n = 1$ to $n = 244$) we employed a bootstrap test with 10 000 runs per predictor variable investigated. For each run, we randomally picked data from only one individual per species (sampled with replacement, cf. Efron & Tibshirani, 1993) and recomputed regression models. Note that this procedure uses both random omission of species during each run and incorporation of all information available on within-species variation for each trait to assess possible bias, particularly that caused by including species represented by single specimens. We provide 95% confidence intervals of the coefficient estimates from this bootstrap procedure for comparison with conventional statistics based on means from each species.

To adjust for phylogenetic relationships, we used phylogenetic generalized least square (GLS) models (Garland & Ives, 2000). This approach is functionally identical to independent contrasts regressions (Garland & Ives, 2000). The distance matrix on which these models were based, was constructed from the phylogenetic relationships between the 42 mammalian species investigated shown in Fig. 1. The topology in this dendrogram was constructed combining information on evolutionary relationships given in two recent sources (Arnason *et al*., 2002; Murphy *et al*., 2001). Due to a large number of polytomies, we used arbitrary branch lengths (Pagel, 1992) to generate the distance matrix. It has been demonstrated that this use of arbitrary branch lengths has little effect on hypothesis testing in phylogenetic regressions (Garland & Diaz-Uriarte, 1999). Multiple linear GLS models with longevity and BMR as the dependent variable, and log transformed body weight as well as the content of muscle fatty acids as predictor variables were computed with the R-library PHYLOGR (Diaz-Uriarte & Garland, 2000).

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Supplementary Material

The following supplementary material is available for this article:

Table S1 Original data and sources.

This material is available as part of the online article from:
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