n-3 polyunsaturated fatty acids as modulators of thermogenesis in Ames dwarf mice

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Abstract Lipids, commonly split into saturated and mono- and polyunsaturated fatty acids, are key constituents of all biological membranes, and their exact proportions in different tissues were previously shown to be related to lifespan in mammals. As a mechanism, it was put forward that long-chain and highly unsaturated n-3 fatty acids may act as “pacemakers” in membranes while the n-6 fatty acid class may act as a counterbalance. Previously, long-lived Ames dwarf mice (Prop1^{df/df}) were found to have lower n-3 fatty acids and higher n-6 throughout their tissues. We exposed 32 adult (8 months old) Ames dwarf mice to three isocaloric diets differing in their fatty acid composition (saturated vs. rich in n-3 and n-6) for 2 months while measuring their body masses, subcutaneous body temperatures and finally membrane fatty acid profiles. Prominently, we found that individuals from all three groups quickly increased their body masses by ca. 20% and had 0.45 °C higher subcutaneous body temperatures than at baseline ($F_{1,12,16} = 22.27$; $p < 0.001$). Conceivably, experimental diets also largely reflected lipid composition found in the tissues with over 50% n-3 fatty acids in heart phospholipids from animals from the n-3-enriched feeding group. Our study indicates that fatty acid-

enriched diets well affected body mass, subcutaneous body temperature and membrane fatty acid composition in Ames dwarf mice with no visible adverse effects on their health. Experimental feeding increased subcutaneous body fat and insulation, most likely explaining the higher subcutaneous temperatures.

Keywords Ames dwarf mouse · Thermogenesis · Membrane phospholipids · Fatty acid supplementation · Dietary intervention · Membrane pacemaker hypothesis · Subcutaneous body temperature · n-3 · n-6

Introduction

For much of the past decades, fats have suffered from bad press. Fat or adipose tissue was seen as superfluous and ugly, indicative of an indolent or intemperate lifestyle (Pond and Mattacks 1998). Scientists have been slow to undermine this attitude. Lipids, being as fundamental to life as proteins, were poorly researched despite their important functions in the body. Indeed, lipids consisting of saturated and mono- and polyunsaturated fatty acids are key constituents of all biological

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membranes (Valencak and Azzu 2014). Membranes themselves are dynamic structures that generally are built from bilayers of amphipathic molecules held together by non-covalent bonds (reviewed in Naudí et al. 2013). Apart from this function, they are important precursors of tissue hormones such as eicosanoids, and they have direct effects on eyesight as well as susceptibility for certain diseases (Cordain et al. 2005; Simopoulos 1999; Valencak et al. 2015). Finally, lipids play an eminent role in adaptation to cold and positively influence hypometabolic processes such as torpor and hibernation (Arnold et al. 2015; Munro and Thomas 2004).

Polyunsaturated fatty acids are essential dietary components that mammals cannot synthesise *de novo* but rather have to be taken up with the diet: Linoleic (C18:2 n-6) and alpha-linolenic (C18:3 n-3) acid have to be ingested in order to be either metabolised immediately or possibly elongated and inserted with double bonds enzymatically in mammalian metabolism (Simopoulos 1999). Membrane fatty acid composition has a marked effect on membrane fluidity, which is kept relatively constant respective to an animal's body temperature and reflects an animal's thermal environment ("homeoviscous adaptation"; Cossins 1977; Hazel 1995). Generally, membranes rich in unsaturated fatty acids have increased membrane fluidity (Hochachka and Somero 2002).

Several correlations between membrane fatty acid composition and both metabolism and ageing have been put forward, being summarised under the concept of fatty acids being "membrane pacemakers" (Else and Hulbert 2003; Hulbert 2005). This hypothesis suggests that fatty acid molecules, e.g. those from the n-3 polyunsaturated fatty acid class, may boost trigger metabolism by fulfilling a "pacemaking" function while affecting the activity of membrane-bound proteins (Hulbert et al. 2007). Specifically, the "membrane pacemaker hypothesis" predicts that highly polyunsaturated membranes have distinct physical properties that cause the proteins in the membranes to have a high molecular activity and further a higher rate of metabolism in the cell, the tissues and, consequently, the whole animal (Hulbert and Else 2005). Yet, intraspecific studies have failed to confirm that variation in membrane lipid composition was associated with basal metabolic rate (Brzęk et al. 2007; Haggerty et al. 2008). Similarly, there is growing evidence, which is incompatible with the metabolic pacemaker hypothesis of metabolism, "the

membrane pacemaker hypothesis of ageing" (Hulbert et al. 2007). This hypothesis postulates that those species having membranes with high contents of polyunsaturated fatty acids, especially n-3 polyunsaturated fatty acids, have shorter lives because of their higher risk of lipid peroxidation and thus possibly reinforced production of oxidative stress (Hulbert et al. 2007; Pamplona and Barja 2007). In support of an existing relationship between membrane lipidomic profiles and lifespan, we observed previously that homozygous long-lived and heterozygous non-mutant sibling Ames dwarf mice differed significantly not in the total amounts of polyunsaturated fatty acids but in the proportions of n-3 and n-6 fatty acids (Valencak and Ruf 2013). Similarly, long-lived naked mole rats (*Heterocephalus glaber*) were reported to differ from laboratory mice in the amounts of n-3 and n-6 polyunsaturated fatty acids (Hulbert et al. 2006b). In addition, our previous feeding experiment in inbred mice pointed to a good match between dietary and tissue lipid profiles in laboratory mice, yet no lifespan differences were observed (Valencak and Ruf 2011).

In summary, there is no clear picture on the causal relationship between membrane composition and lifespan but there are very robust observations that long-lived creatures possess membranes rich in n-6 fatty acids and to the same extent, low in n-3 fatty acids (Hulbert et al. 2006b; Valencak and Ruf 2007; Valencak and Ruf 2013). While evidence against oxidative stress being the cause for this effect has been accumulating in the past few years (Speakman and Selman 2011), new concepts focusing on the original role of lipid molecules as important metabolic pacemakers are sought after. We here present an experiment to test the hypothesis.

We hypothesised that n-3 and n-6 polyunsaturated fatty acids differentially modulate proton leak by affecting membrane pumps as proposed originally (Else and Hulbert 2003; Hulbert 2005). This effect may be particularly relevant to membranes from those mammalian model systems having a consistently lower body temperature while living a long life such as the Ames dwarf mouse (Brown-Borg et al. 1996; Hunter et al. 1999). Mutation of the *Prop1* gene leads to an altered growth hormone pathway in the Ames dwarf mice and causes a lower body temperature, which may reflect in a membrane fatty composition richer in n-6 and poorer in n-3 fatty acids (Valencak and Ruf 2013).

To further test the membrane pacemaker hypothesis in an experimental approach, we exposed long-lived Ames dwarf mice to three isocaloric diets differing in lipid fatty acid composition while monitoring the subcutaneous body temperatures of homozygous Ames dwarf mice and analysing their membrane fatty acid composition. As membrane lipidomic composition is largely responsive to dietary intervention in mice (Valencak and Ruf 2011), we hypothesised that by manipulating membrane fatty acid composition to one (n-3) or another (n-6) direction, we could induce body temperature changes in the animals which may be caused by altered activity of the membrane pumps. If that case was observed, we would conclude that membrane fatty acids, via their role as “pacemaking” molecules for important membrane proteins, may exert an important influence on thermoregulation in mice even in the extraordinary model of the Ames dwarf mouse having a consistently lower body temperature than outbred or inbred mouse strains generally have (Hunter et al. 1999).

Materials and methods

Animals, maintenance, measurements and experimental diets

Prior to the study, we purchased Ames dwarf mice from Charles River Laboratories, Bad Sulzfeld, Germany (Stock #001618 JAX), which were born at The Jackson Laboratories (JAX) in Bar Harbour (ME, USA) and then transported to Austria by Charles River. The 32 homozygous animals (Prop1 df/df) were used in this study whereas we started a breeding colony from pairing heterozygous females and males for later studies. Homozygous dwarf mice of both sexes were kept in groups of 2 to 4 individuals in polycarbonate cages (Eurostandard Type II Long, Tecniplast, Italy, size 365 × 207 × 140 mm, floor space 530 cm³) throughout the study. As animals were already delivered in harmonic groups from Charles River, we did not observe any agonistic or aggressive behaviour between the animals but rather observed sociable and huddling groups of Ames dwarf mice (Fig. 1).

All mice were housed at 22 ± 2 °C on a 16-h:8-h L:D photoperiod in a clean room being entered only by four trained persons every day. Major murine pathogens were ruled out after screening co-housed sentinel animals (heterozygous Ames dwarf mice) regularly

Fig. 1 Homozygous Ames dwarf mice (Prop1 df/df)

(AnLab, Czech Republic). Humidity in the animal room was set at 50% and was continuously monitored. Food (see below for details) and water were available to the animals ad libitum, and food intake was measured by weighing food amounts missing from the hoppers and by dividing the food intake by the number of animals in the respective cage. Cages were cleaned once per week when the animals were carefully transferred into clean cages with new bedding and enrichment material (Lignocel FS 14, Rettenmaier, Austria). At the onset of the experiment, all 32 homozygous Ames dwarf mice were subcutaneously implanted a temperature-sensitive “LifeChip” (Destron Fearing Bio-Thermo LifeChip, size 12 × 2 mm) and were randomly allocated to one of the three feeding groups. The passive “LifeChip” was implanted subcutaneously in the dorsolateral region (below the region with brown adipose tissue, above the hips) by using a sterile single-use injection device and allowing (when connected with the pocket reader) the read-out of an ID number as well as the subcutaneous body temperature (Fig. 2). No anaesthesia was needed

Fig. 2 Ames dwarf mouse during the read-out process of its subcutaneous body temperature

for the implantation of the “LifeChips”, and no adverse behavioural or physiological effects were observed following the procedure. All mice were 8 months old (at the onset of our study) to make sure they were fully adult (due to the retarded growth in Ames dwarf mice). The allocation into the feeding groups was done without dissolving the existing harmonic groups or forming new groups. As we knew ahead from Hunter et al. (1999) that irrespective of the feeding group, the heterozygote (non-dwarf) Ames mice would have a higher body temperature, we refrained from a control group consisting of heterozygote animals. During the 10-day entry phase, the implantation site of the transponder was monitored as well as individual body weight and subcutaneous body temperature while all mice received rodent maintenance chow (Ssniff No. 1234, Ssniff, Germany). We then carefully exposed them to the respective fatty acid-enriched diets by adding larger amounts of the new diet every day while removing the standard rodent chow over the course of 1 week. All mice are readily and widely picked up the experimental diets. After full habituation (1 week) to the experimental enriched with saturated, n-6 polyunsaturated or n-3 polyunsaturated diets (composition detailed in Table 1), we collected food intake data by weighing the mass of pellets missing from the hoppers every day and dividing them by the number of animals in the cage. Experimental animals were fed on the three different diets for 50 days. All experimental diets were isocaloric at an energy content of 17.9 MJ/kg (Atwater) and contained 15.1% crude fat, 17.6% crude protein 53.4 N-free extracts and 16% sugar and were produced for the purpose of our study (Ssniff, Soest, Germany). To minimise autoxidative effects in the fatty acid-enriched diets, the pellets were stored at -18°C . Once per week, fresh food pellets from the three qualities were thawed overnight in the animal room (to adjust to temperature and humidity), were weighed and were placed in the hoppers while the remaining uneaten pellets were discarded. We did not observe large between-individual differences in body masses during the experiment, so we felt confident that any possible variance caused by dividing the observed food intake by the number of animals was minor in comparison with the potential negative side effects of single housing of Ames dwarf mice. All measurements (body masses, subcutaneous body temperature and food intake) took place daily during 9:00 and 10:00 am to minimise variation due to the circadian rhythm of body temperature in mice (Hunter et al. 1999).

Table 1 Fatty acid composition (%) of the three experimental diets (Ssniff Special Diets) and individual sample size in the three groups

	Saturated fatty acid diet (SFD)	n-6 enriched (n6)	n-3 enriched (n3)	Baseline diet (V118x Ssniff, Germany, from Valencak and Ruf 2013)
Sample size	11	11	10	7
homozygous individuals				
C14:0	0.41	0.01	0.71	0.19
C16:0	4	1.08	2.35	11.38
C18:0	2.11	0.39	0.55	3.51
C20:0	0.03	0.07	0.04	0.43
C16:1	0.44	0	0.84	0.58
C18:1	5.28	1.88	2.7	23.81
C20:1	0.02	0.07	0.3	0.17
C18:2	1.64	10.82	2.79	53.39
C18:3	0.18	0.18	0.33	5.94
C20:4	0.02	0	0.07	0
C20:5	0	0	1.87	0
C22:5	0	0	0.24	0
C22:6	0	0	1.21	0
Beef tallow (%) (premier jus)	10	0	0	NA
Palm oil (%)	3	0	0	NA
Safflower oil (%)	0	13	0	NA
Eicosapentaenoic oil (%)	0	0	10	NA
Soybean oil (%)	2	2	5	NA

Lipid profiling

After 50 days on the experimental diets, all animals were sacrificed by cervical dislocation and tissues were rapidly removed, flash frozen in liquid nitrogen and stored in Eppendorf tubes at -80°C until lipid extraction and analysis (<2 months). Total hindleg *musculus vastus* and heart sampling, lipid extraction, analysis have been detailed elsewhere but were alike for both tissues (Valencak and Ruf 2011, 2013). Both sexes were used in our study as we are not aware of any sex-specific differences in membrane lipid composition (Valencak and Ruf 2013). Briefly, lipids were extracted using

chloroform and methanol (2:1 v/v), separated on silica gel thin-layer chromatography plates (Kieselgel 60, F254, 0.5 mm, Merck), then made visible under ultra-violet light with the phospholipid fraction isolated. Phospholipid extracts were transesterified by heating (100 °C) for 30 min in acidic atmosphere (H_2SO_4 in methanol), extracted into hexane and analysed by gas liquid chromatography (GLC) using a Shimadzu GC 2010 with Autosampler and FID (Kyoto, Japan). Fatty acid (FA) methyl esters of a set of 13 FA were identified by comparing retention times with those of FA methyl standards (Sigma-Aldrich, St. Louis, USA) as outlined elsewhere in Valencak et al. (2015) and by using GC solution Analysis software (version 2.42.00, Shimadzu, Kyoto, Japan). Concentrating on the characterisation of the phospholipid class, we used no internal standard for FA GC as the distribution of the lipid classes in our small sample was unknown.

Statistical analysis

Statistical analyses were conducted in R (3.5.3; R Development Core Team 2019). We compared body masses, food intakes, subcutaneous body temperatures and individual fatty acid contents between the three feeding groups. Due to the fact that data were obtained from the same animals repeatedly, we adjusted for repeated measurements by computing linear mixed-effects models using individual intercepts as the random factor (library nlme; Pinheiro et al. 2019). Two-way interactions between the experimental group and time were computed in the same models. Prior to parametric testing, data were tested for normality and for inhomogeneity of variances. Figures were produced in Prism 7 (GraphPad Software) with means and standard errors of the mean provided.

All experiments described here were evaluated and approved first by the local ethics committee at the University of Veterinary Medicine and the Austrian Federal Ministry of Education, Science and Research (code BMWF-68.205/0057-II/3b/2012), and fully comply with the current laws in Austria, where the experiments were performed.

Results

Time courses of body masses during the experiment

At baseline, ahead exposure to the three diets, body masses of individual mice did not differ from each other

and amounted to $13.3 \text{ g} \pm 0.55$, $13.7 \text{ g} \pm 0.6$ and $13.6 \text{ g} \pm 0.47$ in the SFA, the n-6 and the n-3 groups respectively ($F_{2,1215} = 0.27$; $p = 0.77$; Fig. 3). During the course of our 50-day feeding study, body masses increased in all individuals by ca. 20% ($F_{1,1215} = 734.07$, $p < 0.0001$, Fig. 2). Although this increase was observed in all three feeding groups, it was most pronounced in individuals from the n-3 group (2-way interaction experimental group *time $F_{2,1215} = 25.75$; $p < 0.0001$, Fig. 3). Mean body masses over the 50-day experiment (following baseline) amounted to 15.6 ± 1.11 , 16.2 ± 1.17 and $16.8 \pm 0.94 \text{ g}$ for SFA, n-6 and n-3 Ames dwarf mice respectively.

Energy intake

Gross energy intake (GEI) was similar in all three groups independent of fatty acid composition ($F_{1,23} = 0.0098$; $p = 0.922$, Fig. 4). In kilojoules per day, individual food intake amounted to $22.4 \text{ g} \pm 1.4 \text{ kJ/day}$, $25.6 \text{ kJ/day} \pm 0.7$ and $22.83 \text{ kJ/day} \pm 0.54$ in the SFA, n-6 and n-3 groups respectively. We observed no significant difference in food intake over the course of the experiment ($F_{1,23} = 0.0098$; $p = 0.92$, time courses not shown). Due to the necessary group housing, however, energy intake shown in Fig. 4 may be considered a rough estimate for each individual only. The n-6 group seemed to have a higher intake, but this tendency was driven by two individuals having a higher intake. The energy intake of ca. 23 kJ/day was ca. half of 46.5 kJ/day which was measured in heterozygote Ames dwarf mice on the same diets (Lenzhofer, unpublished data).

Fig. 3 Time courses of body masses (g) in homozygous Ames dwarf mice exposed to rodent chow in the beginning and on three diets differing in n-3 and n-6 content for 3 months

Fig. 4 Gross energy intake (GEI) in kJ in d⁻¹ in homozygous Ames dwarf exposed to three isocaloric diets differing in fatty acid composition

Subcutaneous body temperature

Time courses of subcutaneous body temperatures of Ames dwarf mice at baseline and during experimental feeding are given in Fig. 5. While we observed no significant body temperature differences between the three experimental groups ($F_{2,30} = 1.81$; $p = 0.18$), there was an average increase following the experimental feeding on the body temperatures of ca. 0.45 °C ($F_{1,1216} = 22.27$; $p < 0.001$, Fig. 5). With relation to the respective time courses and as visible in Fig. 5, we observed a tendency towards a significant two-way interaction between the dietary composition of the group and time with individuals from the SFA and n-6 groups having a 0.3 °C (= 0.86%) lower subcutaneous temperature than the n-3 group from ca. day 7 onwards despite individual variation and potential imprecision caused by the subcutaneous transponder ($F_{2,1216} = 2.58$; $p = 0.07$; Fig. 5). Mean observed subcutaneous body temperatures in the three groups amounted to 34.69 °C ± 0.06, 34.78 °C ± 0.07 and 34.99 °C ± 0.08 in the SFA, the n-6 and the n-3 groups respectively. Visible in Fig. 5 is that body temperatures in the n-3 group showed an increase

Fig. 5 Time courses of subcutaneous body temperatures (°C) in Ames dwarf mice at baseline and during exposure to three diets differing in fatty acid composition

within the first 10 days of feeding and then plateaued again (Fig. 5).

Fatty acid composition

Expectedly, heart and muscle phospholipid lipidomic profiles largely reflected the fatty acid profiles from the diet (Tables 2 and 3).

We were particularly interested in the relationship between heart phospholipid n-3 polyunsaturated fatty acids and subcutaneous body temperatures in the three experimental groups, but there was no significant correlation ($t = 0.9$, $r^2 = 0.229$; $df = 25$, $p = 0.36$). We observed similar results in muscle phospholipids ($r^2 = 0.397$, $df = 8$, $p = 0.257$).

Discussion

Effect of experimental diets on body mass, body temperature and thermal insulation

The results of our study show that Ames dwarf mice were very responsive to weight gain when fed diets enriched with fats of different lipidomic profiles (Fig. 3). As their body mass increased, their subcutaneous body temperature was elevated, clearly above baseline levels (Fig. 5). However, this effect was observed similarly in all feeding groups and fatty acid compositions (Fig. 5, $p > 0.05$) potentially indicating that dietary lipids were not responsible for the observed effects on body temperature. Rather, all Ames dwarf mice increased body fat in their fat depots but specifically subcutaneously as we observed when collecting tissues at the end of our study (Lenzhofer et al., personal observation) resulting in a 20% total body mass gain during our study (Fig. 3). Supported by the extra energy from the three experimental diets, Ames dwarf mice built an isolating subcutaneous fat layer observed in all experimental animals after the experiment. In view of their lower body temperature and their high heat loss over the body surface (compared to non-dwarf animals), it may have helped them conserve their body temperature by reducing heat dissipation to the environment while resulting in elevated subcutaneous body temperatures measured in our study (Fig. 5). This effect may be particularly economic in long-lived Ames dwarf mice due to their lower core body temperature as shown in Hunter et al. (1999) and therefore increased demand for

Table 2 Compiled heart polar lipid fatty acid composition (phospholipids) in percentage from homozygote Ames dwarf mice exposed to three diets rich in saturated (SFA), in n-6 and n-3 fatty acids (this study) and already published data from Valencak andRuf (2013). Means $\pm$ SEM. Sample sizes are given for each group in the table. Please note that four samples could not have been successfully interpreted due to a software problem of the gas chromatograph and were excluded from the study

	SFA	n-6	n-3	Rodent chow
$N_{\text{individual mice}}$	9	8	11	6
C14:0	0.012 $\pm$ 0.003	0.005 $\pm$ 0.0003	0.013 $\pm$ 0.001	0.08 $\pm$ 0.006
C15:0	0.054 $\pm$ 0.002	0.036 $\pm$ 0.002	0.05 $\pm$ 0.001	0.05 $\pm$ 0.003
C16:0	12.5 $\pm$ 0.13	11.53 $\pm$ 0.5	10.103 $\pm$ 0.073	13.45 $\pm$ 0.13
C17:0	0.35 $\pm$ 0.009	0.3 $\pm$ 0.006	0.275 $\pm$ 0.006	0.35 $\pm$ 0.02
C18:0	21.4 $\pm$ 0.24	22.41 $\pm$ 0.36	19.9 $\pm$ 0.272	20.6 $\pm$ 0.25
C16:1 n-7	0.3 $\pm$ 0.01	0.13 $\pm$ 0.02	0.185 $\pm$ 0.006	0.24 $\pm$ 0.005
C18:1 n-9	9.17 $\pm$ 0.237	4.94 $\pm$ 0.16	5.12 $\pm$ 0.08	7.32 $\pm$ 0.7
C18:2 n-6	12.27 $\pm$ 0.67	19.77 $\pm$ 0.8	9.04 $\pm$ 0.46	19.9 $\pm$ 0.4
C18:3 n-3	0.04 $\pm$ 0.004	0.475 $\pm$ 0.42	0.782 $\pm$ 0.024	0.08 $\pm$ 0.03
C20:4 n-6	12.66 $\pm$ 0.2	14.89 $\pm$ 0.43	4.46 $\pm$ 0.381	12.4 $\pm$ 0.6
C20:5 n-3	0.07 $\pm$ 0.009	0.031 $\pm$ 0.009	0.1 $\pm$ 0.035	0.12 $\pm$ 0.01
C22:5 n-3	3.23 $\pm$ 0.27	1.685 $\pm$ 0.21	3.312 $\pm$ 0.15	3.5 $\pm$ 0.33
C22:6 n-3	27.93 $\pm$ 0.96	23.822 $\pm$ 1.13	45.77 $\pm$ 1.211	21.9 $\pm$ 1.2
Σ SFA	34.31 $\pm$ 0.381	34.26 $\pm$ 0.88	30.341 $\pm$ 0.353	34.6 $\pm$ 0.2
Σ MUFA	9.486 $\pm$ 0.249	5.08 $\pm$ 0.18	5.3 $\pm$ 0.09	7.6 $\pm$ 0.7
Σ PUFA	56.21 $\pm$ 2.1	60.7 $\pm$ 3.02	64.4 $\pm$ 2.3	57.9 $\pm$ 0.9
Σ n-3	31.28 $\pm$ 1.24	26.01 $\pm$ 1.8	50.87 $\pm$ 1.43	25.6 $\pm$ 1.3
Σ n-6	24.94 $\pm$ 0.9	34.7 $\pm$ 1.3	26.01 $\pm$ 1.8	32.3 $\pm$ 0.7

endogenous thermogenesis. Our chosen method for the quantification of body temperature, the passive, temperature-sensitive “LifeChip” (Destron Fearing, USA), allows for non-surgical implantation and stress-free read-out of the subcutaneous body temperatures, was ideal to reflect the small, yet significant increase in subcutaneous temperatures during the course of our feeding trial. While the transponders used in Hunter et al. (1999) assessed core body temperatures of 34 °C, our “LifeChip” similarly revealed 34.36 °C at baseline on average. Being located below the skin in the dorso-lateral area (to avoid the area of the main interscapular brown adipose tissue depot), our daily “LifeChip” measurements read showed increasing subcutaneous temperatures of up to 34.86 °C during the course of the experiment. However, subcutaneous and body core temperature measurements cannot easily be compared. While body core temperatures as assessed in Hunter et al. (1999) reflect the accurate and constantly regulated read-out of the Ames dwarf mice body temperature, subcutaneous temperature may need to be interpreted in a different way as they are responsive to a change in

insulation, e.g. caused by a thicker subcutaneous fat layer. Thus, we may have observed no changes in the core body temperatures in our Ames dwarf mice on the experimental diets if we would have measured them. A manifest increase of core body temperature in the Ames dwarf mice would have led to known and described behavioural and physiological changes in the individuals similarly to what is observed in lactating female MF1 mice whose core body temperature is increased by ca. 2 °C (Gamo et al. 2016): a thinning of fur, a down-regulation of the expression of uncoupling protein 1 (UCP-1) to shut down non-shivering thermogenesis, a regressive food and energy intake etc. A temporarily elevated body temperature could also affect comforting behaviour, body mass and finally locomotor activity of individual mice, but we did not observe any of these effects (Lenzhofer et al., personal observation). Rather, we observed continuous weight gain (Fig. 3) and consistently high food intakes (Fig. 4). Food ingestion is associated with a rise in body temperature in various rodent species (Refinetti 2010), but the endogenous circadian rhythm of body temperature persists even in

Table 3 Compiled muscle polar lipid fatty acid composition (phospholipids) in percentage from homozygote Ames dwarf mice exposed to three diets rich in saturated (SFA), in n-6 and n-3 fatty acids (this study) and already published data from Valencak and Ruf (2013). Means $\pm$ SEM. Please note that muscle polar lipid

profiles given here are only based on a subsample of data that were rescued from a software breakdown of the gas chromatography computer (see sample sizes given below). Due to the overall small variation in muscle phospholipid lipid profiles, we however felt it would be justified to present the diminished dataset here

	SFA	n-6	n-3	Rodent chow
$N_{\text{individual mice}}$	2	5	3	6
C14:0	0.497 $\pm$ 0.013	0.492 $\pm$ 0.081	0.815 $\pm$ 0.211	0.16 $\pm$ 0.005
C15:0	0.473 $\pm$ 0.02	0.415 $\pm$ 0.06	0.543 $\pm$ 0.099	0.1 $\pm$ 0.005
C16:0	21.799 $\pm$ 0.83	22.235 $\pm$ 1.09	22.986 $\pm$ 0.97	21.3 $\pm$ 0.3
C17:0	0.646 $\pm$ 0.05	0.50 $\pm$ 0.07	0.65 $\pm$ 1.3	0.39 $\pm$ 0.01
C18:0	15.483 $\pm$ 1.1	13.032 $\pm$ 1.06	13.044 $\pm$ 0.54	16.1 $\pm$ 0.2
C16:1 n-7	1.366 $\pm$ 0.28	1.309 $\pm$ 0.16	1.310 $\pm$ 0.16	0.79 $\pm$ 0.007
C18:1 n-9	7.074 $\pm$ 1.001	5.932 $\pm$ 0.79	5.301 $\pm$ 1.11	7.8 $\pm$ 0.2
C18:2 n-6	16.503 $\pm$ 0.49	13.37 $\pm$ 2.83	10.32 $\pm$ 1.55	19.5 $\pm$ 0.7
C18:3 n-3	0.875 $\pm$ 0.08	0.617 $\pm$ 0.13	1.622 $\pm$ 0.8	0.5 $\pm$ 0.03
C20:4 n-6	14.982 $\pm$ 0.17	14.196 $\pm$ 1.4	5.56 $\pm$ 0.64	10.6 $\pm$ 0.4
C20:5 n-3	0.859 $\pm$ 0.09	0.684 $\pm$ 0.16	2.41 $\pm$ 0.53	0.2 $\pm$ 0.01
C22:5 n-3	4.83 $\pm$ 1.8	2.25 $\pm$ 0.25	4.56 $\pm$ 0.64	4.5 $\pm$ 0.4
C22:6 n-3	14.62 $\pm$ 0.73	20.99 $\pm$ 2.02	30.19 $\pm$ 2.8	18.02 $\pm$ 0.8
Σ SFA	38.26 $\pm$ 0.22	36.17 $\pm$ 1.3	37.37 $\pm$ 0.66	38.03 $\pm$ 0.2
Σ MUFA	8.44 $\pm$ 1.31	7.24 $\pm$ 0.8	6.61 $\pm$ 0.97	8.6 $\pm$ 0.3
Σ PUFA	52.66 $\pm$ 1.56	52.21 $\pm$ 2.5	54.65 $\pm$ 0.85	53.4 $\pm$ 0.3
Σ n-3	21.18 $\pm$ 1.24	24.55 $\pm$ 1.6	38.77 $\pm$ 2.2	30.1 $\pm$ 0.8
Σ n-6	31.5 $\pm$ 0.32	27.57 $\pm$ 3.1	15.88 $\pm$ 1.8	23.3 $\pm$ 0.5

the absence of daily oscillations in food consumption (Refinetti 2010). According to our behavioural observations, Ames dwarf mice mostly huddle together and sleep during daytime, interrupted by regular bouts to pick up food and drink water or some general activity in the cage with their conspecifics (Lenzhofer et al., personal observation). The extra subcutaneous fat layer did not impair any activity in the cage, nor did they become sluggish as observed in obese laboratory mice (Valencak TG, personal observation). We rather suggest that the addition of fat to the diet, irrespective of the lipidomic signature of the fat (n-3, n-6 or saturated), allowed the mice to increasingly allocate the available energy away from shivering and thermoregulation, but towards an increased general activity in the cage (personal observation, Lenzhofer et al.) and that this might have led to the higher subcutaneous body temperatures in addition to the gain of insulation through the subcutaneous body fat. The newly accumulated white adipose tissue layer has to be considered a soft subcutaneous adipose tissue layer which, due to its location outside the body cavity,

may also largely hold back potential negative consequences of excess lipids on metabolism. Similarly, Hill et al. (2016) have reported that Ames dwarf mice readily became obese on the “green” high fat diet containing 60 kcal of fat (D12492, New Brunswick, NJ, USA), but they were still more insulin sensitive than control mice and appeared relatively unaffected by visceral and subcutaneous adiposity (Hill et al. 2016). Future studies will have to explain why Ames dwarf mice and why and how altered IGF signalling affects the deposition of adipose tissue subcutaneously and if there may be less lipotoxicity arising from subcutaneous depots than from intra-abdominally located, perirenal white adipose tissue.

Membrane composition in Ames dwarf mice on fat-enriched diets

Expectedly, membrane lipidomic profiles of mice given fat-enriched diets reflected the fatty acid composition of the diets: heart phospholipids in Ames dwarf mice from

the n-3 group contained almost 51% n-3 fatty acids (Table 2). These data confirm our previous feeding experiment in C57BL6 mice where we observed ca. 47% n-3 fatty acids in heart phospholipids from a similarly n-3-enriched feeding group (Valencak and Ruf 2011). The observed accordance of very high n-3 contents in two very different mouse models (inbred C57BL6 and Ames dwarf mice) due to experimental feeding suggests that mutation at the *Prop1* locus with the consequently altered growth hormone signalling and endocrine pathways does not “immunise” Ames dwarf mice against accumulation of dietary n-3 fatty acids in membrane phospholipids. Our new finding raises several questions. What are the long-term metabolic consequences of a doubled n-3 fatty acid content in the membranes (Fig. 5)? Is the extraordinary long lifespan of Ames dwarf mice shortened if membrane composition is shifted towards n-3 fatty acids as would be proposed by the membrane pacemaker hypothesis of ageing (Hulbert et al. 2007)? Do Ames dwarf mice with higher tissue n-3 lipid contents produce more detrimental reactive oxygen species (ROS) or other free radicals as would be suggested by the membrane pacemaker hypothesis of ageing (Hulbert et al. 2007; Pamplona and Barja 2011)? Given the importance of altered insulin growth factor 1 (IGF-1) signalling in the Ames dwarf mice for their metabolic homeostasis (reviewed recently in Aguiar-Oliveira and Bartke (2019) and Bartke and Quainoo (2018)), do altered lipidomic profiles due to experimental feeding affect the metabolic pathways involved in nutrient sensing and insulin signalling? While these questions will have to be addressed in future studies, we conclude from our present study that, despite the significantly lower metabolic rate observed in homozygous Ames dwarf mice (Westbrook et al. 2009), the speed at which body weight gain took place in our experimental animals on diets enriched with fat was not different from C57BL6 mice (Valencak and Ruf 2011). The observed incorporation of essential n-3 and n-6 fatty acids into the tissues up to several folds the levels over control animals (Tables 2 and 3, Fig. 5, Valencak and Ruf 2011) rather occurred within ca. half of the time than the reported feeding period of about 19 months as performed in the C57BL6 mice (Valencak and Ruf 2011). Thus, Ames dwarf mice apparently do not differ from non-mutant mice in their susceptibility to match their tissue lipidomic profiles with their diet. Previously, we observed that long-lived Ames dwarf mice naturally had significantly lower n-3 fatty acids in their muscle

and heart phospholipids than normal-sized and shorter-lived heterozygous control animals (Valencak and Ruf 2013). We stated that the lower body temperature and metabolism along with the lower mitochondrial metabolism may be the reason for the significantly lower n-3 fatty acid contents in all examined membranes except for brain tissue (Valencak and Ruf 2013). Similarly, Hulbert et al. (2006a) observed lower membrane n-3 contents in different mouse strains with longer lifespans. In addition, Hulbert et al. (2006b) reported very low membrane n-3 contents in heart tissue of 4% from naked mole rats as opposed to 28% found in mice. In conclusion, long-lived rodents which were studied so far all possess membranes low in n-3, well in line with the predictions from the “membrane pacemaker hypothesis of ageing” (Pamplona and Barja 2011).

Our new data reveal almost 51% n-3 fatty acids in heart phospholipids in otherwise healthy and intact Ames dwarf mice seem to contradict an incompatibility between high tissue n-3 fatty acids and an exacerbated metabolism with side effects although we did not measure oxidative stress in our study. In mammals, membrane lipid profiles are considered a highly regulated, species-specific trait with few intraspecific variations (Valencak and Ruf 2007; Valencak and Ruf 2011; Valencak and Ruf 2013). Yet, we can infer from dietary supplementation studies that the regulation of the membrane lipidomic profiles is dysregulated up when excess essential dietary fatty acids enter the metabolic system and that altered endocrine signalling as well as the lower metabolic rate of Ames dwarf mice is by no means a barrier to the broad incorporation of n-3 fatty acids (Tables 2 and 3). Rather, we were surprised to observe no side effects of an altered tissue and membrane lipidomic signature as all Ames dwarf mice seemed at good health with the extra bonus of improved body insulation. We cannot rule out that an increase in membrane n-3 fatty acids up to 50% in heart phospholipids may have subtle and long-term health and metabolic effects which were not detected in our study due to the time frame chosen. Future studies will also have to elucidate the effects of a high membrane n-3 percentage on the thermoregulatory costs of Ames dwarf mice exposed to cold ambient temperatures. It was reported that in hibernators, n-3 fatty acids are related to the occurrence, duration and extent of deep torpor (reviewed in Ruf and Arnold 2008). In line with the benefit of having an insulating subcutaneous adipose tissue layer, the tissue accumulation of certain lipid

molecules due to the diet may bring about increased resilience against energetic stressors such as lowered ambient temperatures. This effect is well-described in cold-exposed fish and was previously attributed to “homeoviscous adaptation” (Cossins 1977; Hazel 1995). Endotherms with an endogenously lower body temperature such as long-lived Ames dwarf mice may particularly benefit from an increased tissue membrane fluidity and viscosity. This suggests a need to partly revise the “membrane pacemaker hypothesis of metabolism and ageing” in its current form. To date, it is unlikely that membrane lipid n-3 content is the only limiting factor for determining cellular activity causal for metabolism and lifespan but the repeatedly confirmed, robust correlation between membrane lipidomic n-3 and n-6 content and lifespan deserves future experimental studies.

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