Membrane Phospholipid Fatty Acid Composition Regulates Cardiac SERCA Activity in a Hibernator, the Syrian Hamster (*Mesocricetus auratus*)

Sylvain Giroud¹ *, Carla Frare¹ *, Arjen Strijkstra²,³,⁴, Ate Boerema³,⁵, Walter Arnold¹, Thomas Ruf¹

¹ Research Institute of Wildlife Ecology, Department of Integrative Biology and Evolution, University of Veterinary Medicine, Vienna, Austria, ² University Medical Center Groningen, Departments of Chronobiology & Molecular Neurobiology, University of Groningen, Groningen, The Netherlands, ³ Department of Chronobiology, University of Groningen, Groningen, The Netherlands, ⁴ Wildlife Management, University of Applied Sciences Van Hall Larenstein, Leeuwarden, The Netherlands, ⁵ University Medical Center Groningen, Nuclear Medicine and Molecular Imaging, University of Groningen, Groningen, The Netherlands

Abstract

Polyunsaturated fatty acids (PUFA) have strong effects on hibernation and daily torpor. Increased dietary uptake of PUFA of the n-6 class, particularly of Linoleic acid (LA, C18:2 n-6) lengthens torpor bout duration and enables animals to reach lower body temperatures (Tb) and metabolic rates. As previously hypothesized, this well-known influence of PUFA may be mediated via effects of the membrane fatty acid composition on sarcoplasmic reticulum (SR) Ca²⁺ ATPase 2a (SERCA) in the heart of hibernators. We tested the hypotheses that high proportions of n-6 PUFA in general, or specifically high proportions of LA (C18:2 n-6) in SR phospholipids (PL) should be associated with increased cardiac SERCA activity, and should allow animals to reach lower minimum Tb in torpor. We measured activity of SERCA from hearts of hibernating and non-hibernating Syrian hamsters (*Mesocricetus auratus*) in vitro at 35°C. Further, we determined the PL fatty acid composition of the SR membrane of these hearts. We found that SERCA activity strongly increased as the proportion of LA in SR PL increased but was negatively affected by the content of Docosahexaenoic acid (DHA; C22:6 n-3). SR PL from hibernating hamsters were characterized by high proportions of LA and low proportions of DHA. As a result, SERCA activity was significantly higher during entrance into torpor and in torpor compared to inter-bout arousal. Also, animals with increased SERCA activity reached lower Tb during torpor. Interestingly, a subgroup of hamsters which never entered torpor but remained euthermic throughout winter displayed a phenotype similar to animals in summer. This was characterized by lower proportions of LA and increased proportions of DHA in SR membranes, which is apparently incompatible with torpor. We conclude that the PUFA composition of SR membranes affects cardiac function via modulating SERCA activity, and hence determines the minimum Tb tolerated by hibernators.

Introduction

Torpor and hibernation are states with reduced metabolic rate and hence body temperatures (Tb), a strategy employed by many birds and mammals to survive harsh environmental conditions [1]. However, vital organs must remain functional despite depressed oxygen consumption and single-digit Tb. This is most important for the heart that has to maintain circulation by regular contractions to guarantee sufficient perfusion of the organism.

At Tb below 20°C, non-hibernators experience severe arrhythmias and ventricular fibrillation that leads to cardiac arrest [2]. This heart dysfunction is due to a massive increase in cytosolic calcium accompanied by calcium waves and by calcium overload [3,4]. In contrast to non-hibernators, hibernating mammals remain in sinus rhythm even if Tb approaches 0°C [2]. This outstanding ability of the hibernator’s heart is due to the maintenance of sufficiently fast calcium removal into the sarcoplasmic reticulum (SR) after contraction, despite low Tb (see [5] and [4] for reviews). Indeed, an increased rate of calcium reuptake and larger calcium stores were reported in the SR of hibernating Richardson’s ground squirrels [6]. Moreover, the mRNA and protein levels of SR-calcium ATPase (SERCA 2a in the heart, subsequently called SERCA for simplicity), the pump removing calcium into the SR, were found to be increased by 3-fold in myocytes of hibernating woodchucks compared to those of animals in the non-hibernating season [7]. These results support the role of SERCA as the key enzyme that ensures proper calcium handling in cardiomyocytes and hence functioning of the heart at low Tb.

Interestingly, the activity of this transmembrane pump seems to be affected by the fatty acid composition of the surrounding phospholipids (PL). Specifically, there are reports of positive effects of certain polyunsaturated fatty-acids (PUFA), namely those of the...
n-6 class, on SERCA activity in non-hibernating mice. Small increases in the n-6/n-3 PUFA ratio were associated with large increases in SERCA activity [8]. It was recently hypothesized that such an enhancing effect of n-6 PUFA on SERCA activity and therefore on calcium handling could explain the maintenance of proper cardiac function at low Tm [9]. This hypothesis could also explain data from in-vivo studies in hibernators. Both experimental trials and field studies showed positive effects of increased PUFA content in the diet, or in white adipose reserves, on torpor bout duration, tolerance of low Tm, and energy savings [10–15]. Although not explicitly discriminated in these papers, the major PUFA added to the diet in these studies was Linoleic acid (LA, C18:2) of the n-6 family. Conversely, several studies reported negative effects of n-3 PUFA on SERCA activity [8,16,17] and it seems that n-3 fatty acids have adverse effects on hibernation [18].

To our knowledge, two studies [19,20] have tested so far the effect of a diet specifically enriched in a n-3 fatty acid (ω-Linolenic acid, LNA) on hibernation, but only one [19] assessed the fatty acid composition in the tissues. The authors found that a n-3 PUFA enriched diet, leading to a high content in adipose tissues, strongly reduced the propensity to hibernate in yellow banded-marmots [19]. Taken together, these data suggest antagonistic effects of n-6 and n-3 PUFAs on SERCA activity, cardiac function and hence hibernation.

A role for membrane composition in regulating hibernation was further supported by the finding that free-living alpine marmots show a rapid increase of n-6 PUFA in PL just prior to hibernation, with highest levels reached in the heart [21]. Seasonal changes in the n-6 and n-3 PUFA contents of heart PL have also been reported in hibernators fed a constant diet. Specifically, higher levels of LA (C18:2 n-6) and Arachidonic acid (AA, C20:4 n-6) and lower proportions of Docosahexaenoic acid (DHA, C22:6 n-3) and Docosapentaenoic acid (DPA, C22:5 n-3) were reported in hibernators fed a constant diet. Specifically, higher levels of LA (C18:2 n-6) and Arachidonic acid (AA, C20:4 n-6) and lower proportions of Docosahexaenoic acid (DHA, C22:6 n-3) and Docosapentaenoic acid (DPA, C22:5 n-3) were reported in hibernating squirrels compared with summer active animals [22]. More recently, Geiser et al. [23] demonstrated that exposure to short photoperiod triggers a significant increase in n-6 PUFA and a concomitant reduction in n-3 PUFA (notably C22:6 n-3) in muscle PL of Peromyscus maniculatus, a species that exhibits daily torpor mainly under short days. Together, these findings point to an up-regulation of n-6 PUFA content and down-regulation of n-3 PUFA content in cardiac PL prior to hibernation or torpor that is independent of dietary intake. It seems that these changes in cardiac PL composition may be a prerequisite for animals to enter hibernation, possibly to keep the heart functioning at low Tm.

In the present study, we therefore determined SERCA activity in hearts from hibernating Syrian hamsters (Mesocricetus auratus), and investigated its relation to the fatty acid composition of SR PL. Specifically, we tested the hypotheses that high proportions of n-6 PUFA in general, or specifically in LA (C18:2 n-6), relative to n-3 PUFA in SR phospholipids should be associated with increased cardiac SERCA activity, and should allow animals to reach lower minimum Tm in torpor.

**Materials and Methods**

**Ethics Statement**

The experiment was approved by the Animal Experimental Ethics Committee of the University of Groningen under license numbers DEC-2954 and DEC-4746a.

**Animals**

Syrian hamsters (Mesocricetus auratus, 18 males, 29 females), five months old, were obtained from the breeding colony of the Zoological Laboratory (University of Groningen, Haren, The Netherlands) in two different years (2003 and 2008). Each year, animals were transferred to a climate-controlled room and were kept in standard laboratory cages under a summer photoperiodic regime (LD 14:10) at an ambient temperature of 21 ± 1°C. Some hamsters were sacrificed under these conditions (summer, n = 7). After 9 weeks of acclimatization, the light-dark cycle was changed to a short photoperiod (LD 8:16) with a constant ambient temperature kept at 21°C, in order to induce a winter phenotype. After another 3–7 weeks, ambient temperature was changed to 5 ± 1°C with continuous dim red light (<0.5 Lux), to trigger hibernation [24]. At all stages, the animals had ad libitum access to lab chows (Arie Blok Tiervoeding, Woerden, The Netherlands, www.arieblok.nl) and water. Hamsters were maintained under these constant conditions until sacrificed at different states within the hibernating cycle. All animals were killed by decapitation, 10 minutes after intra-peritoneal injection of pentobarbital (60 mg/Kg). Tm was measured immediately after decapitation in the animal’s mouth using a Voltcraft K102 (Conrad, the Netherlands) thermometer, equipped with a polytetrafluoroethylene insulated 1 mm bare tip copper constantan (Type T) thermocouple. Prior to use, the thermometer was calibrated in a water bath between 0°C and 45°C, by using a mercury thermometer (accuracy ±0.25°C). Hearts were quickly removed and stored at −80°C until subsequent determination of SERCA activity and SR PL composition at the Research Institute of Wildlife Ecology.

**Time-points in the Hibernating Cycle**

Hibernation cycles were defined as a succession of torpid and euthemic states, lasting up to 7 days. Locomotor activity of hamsters was continuously monitored with passive infrared motion detectors. Animals were sacrificed during midwinter hibernation cycles of maximal length, i.e. between the 5th and 10th bout of torpor [24], at three different time-points: during entrance into torpor while Tm was still decreasing (“cooling”, n = 9), during deep torpor (“torpid”, i.e. at least 24 h after cessation of locomotor activity, n = 18) and during inter-bout arousal (“IBA”, n = 13).

**Isolation of Cardiac SR**

Cardiac homogenates were prepared from approximately half of each hamster heart (~300 mg). Hearts were washed and minced in ice-cold isotonic saline (0.9% NaCl). A protease inhibitor cocktail (Sigma P8340) was added to the suspensions. Tissues were then homogenized in 2 ml of a buffer containing 30 mM histidine (pH 6.9), 300 mM sucrose, 600 mM KCl, 0.5 mM DTT, 10 mM EDTA, 50 mM Na₂HPO₄ and 1 mM PMSF, by 10 strokes with a motor-driven Teflon/glass homogenizer (tube volume, 5 ml, Wheathon, USA). Homogenates were then centrifuged at 24.600 g for 15 min to remove mitochondria, cell debris and most of the membranes, including sarcolemma, but not SR. Homogenates therefore contained SERCA protein embedded in membrane vesicles, and SERCA represents the major fraction (~80%) of the total membrane protein of the longitudinal SR [26]. Supernatants with 10% glycerol were stored in
in 200 µl- aliquots at –80°C. All steps were performed at 4°C within 1 h to minimize enzyme denaturation.

**SERCA Activity Measurements**

ATPase activities were measured by a standard coupled enzyme assay, in which the rate of ATP hydrolysis was calculated from spectrophotometric recording (Perkin-Elmer 550, Germany) of NADH oxidation at 340 nm (ε = 6.22 mM−1 cm−1). The assay was performed according to the method previously described by Simonides et al. [27], using a sample volume optimized for the Syrian hamster’s heart. SERCA activity was determined by adding 100 nM thapsigargin (TG), a specific inhibitor of SERCA [28], during the assay. Only SERCA protein embedded in the SR membrane vesicles remains functional. The difference between ATPase activities recorded before and after TG addition was attributed to SERCA. The standard reaction mixture contained 50 mM imidazole (pH 6.9), 100 mM KCl, 10 mM MgCl₂, 10 mM NaN₃, 0.5 mM DTT, 10 mM PEP, 5 mM ATP, 10 µM CaCl₂, 5.3 unit.ml⁻¹ pyruvate kinase, 17.5 unit.ml⁻¹ lactate dehydrogenase, 300 µM NADH and 2 µM calcium ionophore (A23187) in a final volume of 1 ml. The reaction was started by addition of the sample (0.1–0.2 mg.ml⁻¹ final volume). Assays were performed at a temperature of 35°C, which corresponds to euthermic T_b (Fig. 1). Protein concentrations were determined with the Bradford method [29]. Data presented are means from 4 replicate measurements of SERCA activities, and are expressed as µmol ATP hydrolyzed per mg total protein and minute.

**Lipid Analyses**

Lipids were extracted from the isolated SR membranes following the procedure of Folch et al. [30]. The phospholipids (PL) were then isolated using thin-layer chromatography [31], and further saponified and converted into fatty acid methyl esters (FAME) as reported previously [32]. FAME were identified by gas-liquid chromatography using a Perkin-Elmer FID AutoSystem XL autosampler chromatograph equipped with a 30 m × 0.25 mm × 0.25 µm HP INNOWax capillary column, using the following parameters: injector 240°C, column 130–180°C at 4°C/min, 180–200°C at 3°C/min, 200–240°C at 15°C/min, 240°C for 8 min. The relative fatty acid composition was quantified using external fatty acid methyl ester standards (Supelco) run after every 20 samples and Turbochrom 6.3 software (Perkin Elmer). Concentrations of single fatty acids were calculated as mass % of total identified peaks of 13 fatty acids of chain length 14 to 22.

**Statistics**

Data analyses were carried out using R 2.15.1 [33]. Residuals from statistical models were tested for normality (using Shapiro-Wilk tests), and if necessary, response variables were log-transformed. General linear models with Tukey-like post-hoc multiple comparison tests (R package ‘multcomp’ [34]) were used to assess differences in SERCA activity and SR PL composition between summer active animals, non-hibernating winter animals, and hibernating animals during IBA, cooling and torpor. For regression analyses we applied ranged major axis (RMA) models (R package ‘lmodel2’ [33]) to account for measurement errors in both the dependent and predictor variable. Finally, we carried out a path analysis (R package ‘lavaan’ [35]) to determine the most likely cause and effect relationship between the proportion of a given fatty acid among SR PL, SERCA activity and T_b of the animals. All reported values are means ± standard deviation.

**Figure 1. Body temperatures according to the animal physiological states.** Body temperatures of summer hamsters (“summer”, n = 7), non-hibernating winter hamsters (“winter”, n = 7), and hibernating hamsters during inter-bout arousal (“IBA”, n = 13), entrance into a hibernating bout (“cooling”, n = 9), and deep torpor (“torpid”, n = 18). Groups differing significantly (p<0.001, Tukey’s post-hoc comparisons) are denoted by different superscripts.

doi:10.1371/journal.pone.0063111.g001
Results

Body Temperature

$T_b$ of hamsters was close to ambient during deep torpor, on average $1.7 \pm 0.6^\circ C$ above, and significantly lower than in animals sacrificed during entrance into hibernation. $T_b$ of summer hamsters was similar to the $T_b$ of non-hibernating hamsters during winter and to the $T_b$ of hamsters during IBA (Fig. 1).

Cardiac SERCA Activity

A general linear model revealed significant differences in SERCA activities in hearts from summer, non-hibernating winter, IBA, cooling and torpid hamsters ($F = 9.42, p<0.001$). SERCA activities, regardless of the original $T_b$ of the hamsters, were all measured in vitro at 35°C. This resulted in the cancellation of Arrhenius effects in order to reveal the impacts of fatty acids on SERCA activity. SERCA from cooling hamsters had higher activity, similar to SERCA from torpid individuals, whereas SERCA from hamsters during IBA as well as from non-hibernating winter and summer animals had significantly lower activity (Fig. 2). Relating $T_b$ of torpid animals, measured immediately before obtaining the tissue for SERCA preparation, to SERCA activity determined in vitro at 35°C, we found that minimum $T_b$ reached in deep torpor decreased as SERCA activity increased (Fig. 3).

Phospholipid Composition of Heart Sarcoplasmic Reticulum Membranes

In cardiac SR membrane PL, LA (C18:2 n-6), AA (C20:4 n-6) and DHA (C22:6 n-3) were the three major PUFA comprising 96.95% ($\pm 0.74\%$) of the total PUFA content. Proportions of LA were significantly lower (Table 1, $F = 10.32, p=0.002$), and proportions of DHA significantly higher (Table 1, $F = 8.81, p=0.005$), in hamsters in the non-hibernating state compared to animals in the hibernating state. This suggests that values from IBA hamsters rather resembled those of cooling and torpid groups (Table 1), despite a $T_b$ similar to summer and non-hibernating winter animals (Fig. 1). Post-hoc tests between all five groups did not contradict these results, although not all single comparisons reached statistical significance. For instance, LA levels of non-hibernating winter hamsters were statistically equivalent to those of summer and IBA animals, which in turn did not significantly differ from those of cooling and torpid animals (Table 1). Regarding DHA, non-hibernating winter hamsters had significantly higher levels compared with any of the hibernating states (cooling, torpid, IBA), but DHA levels of summer animals did not significantly differ from those of all the other physiological states (Table 1). As to be expected, the LA/DHA ratio was significantly higher in hibernating hamsters versus non-hibernating animals (Table 1; $F = 12.00, p=0.001$), as was the total n-6/n-3 PUFA ratio (Table 1; $F = 7.43, p=0.009$), despite the lack of a corresponding difference in the second n-6 PUFA AA (Table 1; $F = 0.001, p=0.98$). From the remaining n-3 PUFA only ALA (C18:3 n-3) differed significantly between the five compared groups due to, for some reason, a slightly higher concentration in cooling, and possibly also torpid animals (Table 1; comparison of hibernating state vs. non-hibernating state, $F = 3.87, p=0.055$). However, concentrations of ALA in SR PL were in all groups very low (<0.5%, Table 1). The n-6/n-3 PUFA ratio in non-hibernating winter hamsters did not significantly differ from that

![Figure 2. Levels of cardiac Sarcoplasmic Reticulum Calcium ATPase 2a (SERCA) activity.](https://www.plosone.org/article/fichiers/pone.0063111.s002.jpg)
of the summer and cooling animal groups, which had ratios similar to torpid and IBA hamsters (Table 1).

We also found significant differences among several proportions of saturated fatty acids, such as Palmitic acid (C16:0), Stearic acid (C18:0) between the five groups (Table 1). These effects were, however, due to differences between summer and winter animals but unrelated to thermoregulatory states (Table 1). The same result was found for monounsaturated fatty acids.

Cardiac SERCA Activity and Phospholipid Composition

SERCA activity was positively associated with the proportion of LA among SR PL (Fig. 4a), but negatively with the proportion of DHA (Fig. 4b), and thus positively with the ratio of LA/DHA (Fig. 4c). These relations were also significant for torpid animals only (LA: intercept = -1.39, slope = 0.03, adjusted $R^2 = 0.33$, $p = 0.01$; DHA: intercept = 0.65, slope = -0.13, adjusted $R^2 = 0.12$, $p < 0.05$; LA/DHA: intercept = -1.21, slope = 0.24, adjusted $R^2 = 0.37$, $p = 0.01$), i.e., for the state when the presumed influence of PL-composition on SERCA activity is most critical.

No significant relation was found between SERCA activity and the n-6/n-3 PUFA ratio (intercept = -2.92, slope = 0.64, $p = 0.15$). SERCA activity increased, however, with the proportion of DPA (C22:5 n-3), the precursor of DHA, both among all hamsters and in torpid animals only (intercept = -1.22, slope = 0.47, adjusted $R^2 = 0.10$, $p = 0.01$), but the proportion of DPA among SR PL was only <2%. Also, no significant association existed between AA and SERCA activity.

Causal Relations

Path analysis indicated that the proportion of LA among SR PL, and, to a lesser degree that of DHA, were associated with SERCA activities, which in turn determined the minimum $T_b$ reached (Table 2). Results of this causal analysis were significant both in the pooled sample from all states discriminated but also for torpid animals only. As a consequence, minimum $T_b$ in torpid animals decreased as the LA/DHA ratio increased (Fig. 5).

Discussion

Up-regulated SERCA Activity in Membranes Rich in LA

Our results suggest that a high proportion of LA in the SR membrane, rather than a high total n-6 PUFA content, up-regulates SERCA activity and determines the minimum $T_b$, a hibernator can tolerate during torpor. This conclusion is mainly based on the significant correlation between SERCA activity and levels of LA (Fig. 4a). SERCA activity was therefore not affected by the n-6/n-3 PUFA ratio, as previously suggested [9]. Indeed, the effect in the Syrian hamster was specific to LA, as we found no association between AA and SERCA activity. $T_b$ of torpid hamsters measured in this study can be considered as the minimum $T_b$ reached during the respective torpor bout. Syrian hamsters reach minimum $T_b$ under ambient temperatures like in this experiment within 24 hours, thus long before samples were taken from torpid animals [36,37]. Therefore, the observed negative association between $T_b$ of torpid animals and SERCA activity would suggest that incorporation of LA into the sarcoplasmic membrane may compensate Arrhenius effects on SERCA activity and thus enable tolerance of lower $T_b$. Lastly, our path analysis supports such a sequence of causal relations.

Previous studies are in line with this major result of our study. Hibernating Richardson’s ground squirrels, for instance, show increased rates of calcium reuptake and larger SR calcium stores.
Table 1. Fatty acid proportions (% of total fatty acids) of cardiac sarcoplasmic reticulum phospholipids (means ± standard deviation), ratio of certain fatty acid proportions, and unsaturation index ("UI") of summer hamsters ("Summer", n = 7), non-hibernating winter hamsters ("Winter", n = 7), and hibernating hamsters during entrance into a torpor bout ("Cooling", n = 9), during deep torpor ("Torpid", n = 18) and during inter-bout arousal ("IBA", n = 13).

| Fatty Acid | Non-hibernating state | Hibernating state | ANOVA |
|------------|------------------------|-------------------|-------|
|            | Summer                 | Winter            | Cooling | Torpid | IBA     |
|            |                        |                   |         |        |         |
| C14:0      | 0.05±0.01ab            | 0.06±0.02a        | 0.05±0.01ab | 0.04±0.01b | 0.05±0.01a | 7.30 | <0.001 |
| C15:0      | 0.14±0.04ab            | 0.11±0.05b        | 0.06±0.01b | 0.06±0.01b | 0.07±0.01b | 15.08 | <0.001 |
| C16:0      | 20.49±1.09bc           | 18.60±2.51emm     | 16.54±1.51m | 16.70±1.28m | 17.64±1.28m | 3.98 | <0.001 |
| C16:1(n-7) | 0.25±0.04ab            | 0.27±0.08ab       | 0.35±0.07abc | 0.36±0.08abc | 0.39±0.08b | 5.67 | <0.001 |
| C17:0      | 0.88±0.22a             | 0.65±0.23b        | 0.50±0.04b | 0.51±0.07b | 0.57±0.08b | 12.44 | <0.001 |
| C18:0      | 19.92±0.94b            | 23.03±0.98b       | 23.31±0.78b | 23.27±2.75b | 22.72±0.97b | 5.04 | 0.002 |
| C18:1(n-9) | 9.88±1.06a             | 8.38±0.83b        | 8.45±1.05b | 8.41±0.85b | 8.58±1.03b | 3.45 | 0.015 |
| C18:2(n-6) | 21.61±2.20ab           | 20.47±2.41ab      | 24.18±1.07ab | 23.77±2.91b | 22.53±2.35ab | 3.58 | 0.012 |
| C18:3(n-3) | 0.19±0.08abc           | 0.19±0.16         | 0.37±0.04abc | 0.28±0.15abc | 0.19±0.15abc | 3.60 | 0.012 |
| C20:4(n-6) | 15.69±1.61             | 15.82±1.90        | 15.09±1.24 | 15.56±2.27 | 16.43±2.09 | 0.70 | 0.598 |
| C20:5(n-3) | 0.10±0.03              | 0.10±0.04         | 0.14±0.03 | 0.12±0.03 | 0.11±0.03 | 2.36 | 0.066 |
| C22:5(n-3) | 1.07±0.19              | 1.00±0.41         | 1.26±0.13 | 1.22±0.21 | 1.13±0.30 | 1.59 | 0.191 |
| C22:6(n-3) | 9.73±1.29ab            | 11.25±1.23ab      | 9.51±1.10b | 9.40±0.69b | 9.50±1.32b | 4.11 | 0.006 |
| MUFA       | 48.39±1.62             | 48.82±3.14        | 50.56±1.84 | 50.32±3.24 | 49.90±1.97 | 1.16 | 0.342 |
| SFA        | 10.13±1.06b            | 8.65±0.87ab       | 8.80±1.11ab | 8.77±0.99b | 8.97±0.88ab | 2.81 | 0.035 |
| Σn=6       | 37.30±2.04ab           | 36.29±3.26        | 40.46±2.04 | 40.58±3.69 | 41.05±1.79 | 0.72 | 0.582 |
| Σn=3       | 11.09±1.46             | 12.53±1.30        | 11.29±1.17 | 11.00±0.64 | 10.94±1.55 | 2.48 | 0.056 |
| n=6/n=3    | 3.43±0.60abc           | 2.93±0.51a        | 3.51±0.33abc | 3.58±0.29abc | 3.64±0.64abc | 2.97 | 0.029 |
| C18:2/C22:6| 2.26±0.40abc           | 1.85±0.39abc      | 2.58±0.37abc | 2.55±0.48abc | 2.43±0.51abc | 4.09 | 0.006 |
| UI         | 1.81±0.05              | 1.86±0.09         | 1.83±0.10 | 1.82±0.12 | 1.84±0.09 | 0.32 | 0.865 |

The unsaturation index was calculated as followed: UI = Σ([% each fatty acid] × no. double bonds/fatty acid]). Groups differing significantly with p<0.05 (Tukey’s post-hoc comparisons) are denoted by different superscripts.

doi:10.1371/journal.pone.0063111.t001

Figure 4. Cardiac Sarcoplasmic Reticulum Calcium ATPase 2a (SERCA) activity and fatty acid composition. SERCA activity as a function of the proportions (% of total fatty acids) of (a) Linoleic acid (LA 18:2 n-6), (b) Docosahexanoic acid (DHA 22:6 n-3), and (c) the ratio of LA/DHA in the sarcoplasmic reticulum membrane. Black dots indicate data from torpid animals, black triangles from cooling animals, grey dots from animals during inter-bout arousals, and open dots from summer and non-hibernating winter animals (Difference of slopes between groups: (a) F = 1.78, p = 0.15; (b) F = 0.89, p = 0.48; (c) F = 2.07, p = 0.10; regression statistics for all 54 animals from linear ranged major axis analysis: (a) intercept = −1.70, slope = 0.05, adjusted R² = 0.27, p<0.01; (b) intercept = 0.50, slope = −0.12, adjusted R² = 0.04, p = 0.04; (c) intercept = −1.29, slope = 0.03, adjusted R² = 0.18, p = 0.02).

doi:10.1371/journal.pone.0063111.g004
compared to active animals [6,38]. However, these earlier studies failed to demonstrate significantly higher SERCA activity during hibernation, although this pump removes 70–92% of the total calcium present in the cytosol after excitation and contraction of cardiac myocytes [39]. Furthermore, an enhanced cytosolic clearance of calcium has been observed in hibernating ground squirrels versus non-hibernating rats, and this difference was attributed to differences in SERCA activity [3].

SERCA protein levels have been reported to be increased by 3-fold in myocytes of hibernating woodchucks compared to those of animals in the non-hibernating season [7]. In our study, total SERCA activity may have also been affected by changes in the amount of SERCA protein, i.e. between seasons. However, since our analysis shows effects of membrane fatty acid composition on SERCA activity per mg protein (e.g. Fig. 4), we would conclude that fatty acids affect the specific activity of the SERCA pump rather than the expression of SERCA protein. This relies on previous findings showing that SERCA accounts for the largest fraction of the total SR membrane protein [26]. Still further studies on this subject should validate this conclusion by additionally determining levels of either mRNA or protein levels of both SERCA and its inhibitor, phospholamban.

### Adverse Effects of Docosahexanoic Acid on SERCA Activity

In contrast to LA, SERCA activity was negatively associated with the DHA content of the SR membrane, although the relation was weak. Interestingly, the non-hibernating hamsters were characterized by high DHA but low LA content in their SR PL.

### Figure 5. Body temperature as a function of the ratio of the proportions (% of total fatty acids) of Linoleic acid and Docosahexanoic acid (LA 18:2 n-6/DHA 22:6 n-3) in the sarcoplasmic reticulum membrane of torpid hamsters. Linear ranged major axis analysis: intercept = 10.29, slope = −1.61, p<0.01, R² = 0.40.
doi:10.1371/journal.pone.0063111.g005

### Table 2. Path analyses between Linoleic acid (LA) or Docosahexanoic acid (DHA), log-transformed cardiac Sarcoplasmic Reticulum Calcium ATPase (SERCA2a) activity and body temperature (Tb) in all hamsters (summer, winter, cooling, torpid and inter-bout arousal) and for torpid animals only.

| Variables          | All hamsters | Torpid hamsters |
|--------------------|--------------|-----------------|
|                    | Estimate     | Z-value | p-value | Estimate    | Z-value | p-value |
| Tb ~ SERCA2a       | −64.22±10.62 | −6.05 | <0.001  | −4.44±1.02 | −4.34 | <0.001 |
| SERCA2a ~ LA 18:2n-6 | 0.03±0.01   | 4.62  | <0.001  | 0.02±0.01  | 3.27   | 0.001  |
| SERCA2a ~ DHA 22:6n-3 | −0.03±0.01 | −1.88 | 0.06    | −0.06±0.03 | −1.93 | 0.05   |

The model detects potential causal effects between LA or DHA and SERCA activity (SERCA2a ~ LA 18:2n-6, SERCA2a ~ DHA 22:6n-3), and between SERCA activity and Tb (Tb ~ SERCA2a) of the hamsters. The “Estimates” correspond to the coefficients of the different regressions calculated by the model, and inform on the relative strength of the respective dyadic relations.
doi:10.1371/journal.pone.0063111.t002
Apparently, this phenotype is incompatible with the use of torpor. Hill and Florant [19] reported similar inhibitory effects of an ALA (the precursor of DHA) enriched diet on hibernation propensity in yellow bellied marmots. Among eight marmots tested, only three entered hibernation, whereas the other five individuals stayed euthermic throughout winter and continued to feed. Further, recent experiments conducted on garden dormice (Eliomys quercus) revealed that animals fed a diet supplemented with fish oil, containing high amounts of DHA, significantly delayed the onset of hibernation compared to individuals fed a diet supplemented with corn oil high in LA (Giroud, Arnold and Ruf; unpublished data).

The apparent incompatibility of high DHA contents in PL with hibernation could be due to the known strong inhibitory effects of n-3 fatty acids on SERCA activity [8,16]. Such inhibition can be cardio-protective when operating at high Tb, and is apparently mediated by DHA [40,41]. Low Tb on the other hand, apparently requires removal of DHA and increased LA content in SR membranes leading to enhanced calcium handling in order to prevent cardiac arrest caused by Arrhenius effects [17].

Potential Mechanisms

Although the actual biochemical mechanisms by which PUFA could influence SERCA activity are unknown, the most likely explanation would be that physical properties of certain unsaturated fatty acids affect the conformational change and hence the specific activity of the trans-membrane SERCA pump. These interactions are now well understood and can be described in terms of protein-induced perturbations to the membrane shape that feed-back on trans-membrane proteins (review in [42]). They may also involve specific chemical interactions. For example, biochemical modeling has recently demonstrated that certain fatty acids (DHA in the study) may displace interactions between protein domains and therefore facilitate the formation of active states [43]. One of the first membrane proteins that was shown to be affected by the properties of the surrounding lipids was in fact SERCA (review in [44]). Specifically, SERCA 1a activity in skeletal muscles was regulated by membrane contents of both cholesterol and phosphatidylethanolamine [44]. Our data suggest that, in the context of hibernation, the beneficial effects on cardiac SERCA are due to a replacement of DHA with LA in SR PL. Therefore, a previously suspected influence of the n-6/n-3 PUFA ratio [9] may only be correlational, caused by a potential inhibitory effect of DHA and a possible facilitating effect of LA on SERCA.

If a low content of DHA in SR PL is detrimental at high Tb, but a high LA content necessary for adequate calcium handling during deep torpor, one would expect according membrane adjustments during rewarming and entrance into torpor. We did not find such differences between the SR membranes of IBA and cooling or torpid hamsters. This could be due to the fact that in our study IBA hamsters were sampled after artificially induced rewarming. This may have limited the time for the animals to complete changes in membrane composition, and explain the lack of significant alterations. Also, SERCA activity of IBA hamsters was similar to summer and non-hibernating winter animals (Fig. 2), despite a membrane composition that was intermediate between non-hibernating animals and cooling or torpid individuals (Table 1). This suggests additional influences on SERCA activity apart from those of membrane composition. A prime candidate molecule for such an influence is the SERCA inhibitor phospholamban (PLB) [45]. Generally, PLB gene expression has been found to be low during hibernation [7,46], presumably because inhibition of SERCA is superfluous at low Tb. However, even low levels of active PLB could be responsible for the unexpected low SERCA activity of our IBA hamsters. If so, PLB should be deactivated prior to entrance into the next torpor bout in order to guarantee maximal SERCA activity when Arrhenius effects become severe due to low Tb. SERCA inhibition by PLB is relieved by phosphorylation via cAMP-dependent protein kinase, which in turn is activated through epinephrine release after sympathetic stimulation [47]. Interestingly, a sympathetic burst is typical prior to entry into torpor as described in some hibernating species [48,49], and more recently in hibernating dormice [50]. Furthermore, it has been demonstrated experimentally that a functional sympathetic nervous system is a prerequisite for the use of torpor. Djungarian hamsters did not enter torpor after blockade of sympathetic action by administering 6-hydroxydopamine [51].

The immediate result of sympathetic stimulation is a transient increase of metabolic and heart rate, hence the opposite of what one would expect in preparation for torpor, but a phenomenon that has long been known to occur [52]. However, these inevitable side effects of sympathetic stimulation are short term, presumably in contrast to the effect on PLB. On one hand, Arrhenius effects could preserve phosphorylation and thus inactivation of PLB at low Tb. On the other hand, repeated beta-adrenergic stimulation occurs during torpor when non-shivering thermogenesis is initiated in order to defend an already low Tb against a further decrease [48,49], or for rewarming at the end of a torpor bout. In the latter case, efficient suppression of PLB may well be pivotal because heart rate is increasing tremendously although Tb is still very low [53–55].

Conclusion and Perspectives

In conclusion, our findings suggest that replacing DHA with LA in SR PL up-regulates SERCA activity, thus possibly enabling hibernators to tolerate low Tb without jeopardizing heart function. In contrast, a high content of DHA in SR PL could be responsible for dampening SERCA activity to ensure proper calcium handling at high Tb [40,41], but appears to be incompatible with the use of torpor. We are well aware that these findings correspond to associations between SR composition, SERCA activity and Tb, which ask for more experimental follow-up studies. However, the fact that these associations can be detected even in animals fed identical rodent chow leads us to think that effects of differences in SR PL may be even stronger in free-living hibernators exposed to a higher variability in food resources, or even with limited access to essential PUFA [21]. Furthermore, the role of PLB during hibernation and its regulation by β-adrenergic stimulation also needs experimental clarification by measuring levels of phosphorylated and dephosphorylated PLB during IBA, cooling, deep torpor, and rewarming.

Acknowledgments

The authors thank W. Gregor for help with the measurement of SERCA activity, M. H. Le and M. Hämmerle for biochemical analyses of fatty acid composition.

Author Contributions

Provided the tissues: AS AB. Conceived and designed the experiments: WA TR. Performed the experiments: SG CF. Analyzed the data: SG CF TR. Wrote the paper: SG.
References

1. Geiser F, Ruf T (1995) Hibernation versus daily torpor in mammals and birds: physiological variables and classification of torpor patterns. Physiol Zool 68: 935–966.

2. Johansson BW (1996) The hibernator heart—nature’s model of resistance to ventricular fibrillation. Cardiovasc Res 31: 826–832.

3. Liu B, Belke DD, Wang LC (1997) Ca2+ uptake by cardiac sarcoplasmic reticulum at low temperature in rat and ground squirrel. Am J Physiol 272: R1112–1127.

4. Wang SQ, Lakatta EG, Cheng H, Zhou ZQ (2002) Adaptive mechanisms of intracellular calcium homeostasis in mammalian hibernators. J Exp Biol 205: 2957–2967.

5. Andrews MT (2007) Advances in molecular biology of hibernation in mammals. Bioessays 29: 431–440.

6. Belke DD, Milner RE, Wang LC (1991) Seasonal variations in the rate and capacity of cardiac SR calcium accumulation in a hibernating species. Physiol Zool 64: 354–361.

7. Yatani A, Kim SJ, Ushio H, Koizumi C, Visuthi V, Kiron V, et al. (1997) Effect of dietary fatty acids on hibernation by golden-bellied marmots (Marmota flaviventris). Physiol Behav 68: 431–437.

8. Swanson JE, Lokes BR, Kinsella JE (1989) Ca2+-Mg2+-ATPase of mouse cardiac sarcoplasmic reticulum is affected by membrane n-6 and n-3 polyunsaturated fatty acid content. J Nutr 119: 364–372.

9. Ruf T, Arnold BW (2000) Effects of polyunsaturated fatty acids on hibernation and torpor: a review and hypothesis. Am J Physiol Regul Integr Comp Physiol 294: R1044–1052.

10. Brus U, Frey F, Puchta S, Tataruch F, Ruf T, et al. (2000) Essential fatty acids: their impact on free-living Alpine marmots (Marmota marmota). In: Life in the Cold, edited by Heldmaier G and Klingenspor M Berlin, Germany, Springer 2000: p215–222.

11. Florant GL, Hester L, Ameenuddin S, Rintoul DA (1993) The effect of a low calcium diet on the rate of calcium accumulation in mammalian cardiac sarcoplasmic reticulum. Am J Physiol Heart Circ Physiol 266: H2219–J. Membr Biol 131: 35–42.

12. Swanson JE, Lokes BR, Kinsella JE (1989) Ca2+-Mg2+-ATPase of mouse cardiac sarcoplasmic reticulum is affected by membrane n-6 and n-3 polyunsaturated fatty acid content. J Nutr 119: 364–372.

13. Frank CL (1992) The influence of dietary fatty acids on hibernation by golden-bellied marmots (Marmota flaviventris). Physiol Behav 68: 431–437.

14. Johansson BW (1996) The hibernator heart—nature’s model of resistance to ventricular fibrillation. Cardiovasc Res 31: 826–832.

15. Hill VL, Florant GL, Wang LC (1992) Seasonal variation in calcium uptake by cardiac sarcoplasmic reticulum in a hibernator, the Richardson's ground squirrel, Spermophilus citellus. Journal of Thermal Biology 17: 53–56.

16. Berto DM (2000) Calcium fluxes involved in control of cardiac myocyte contraction. Circ Res 87: 775–781.

17. Kranias EG (2000) Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. Pharmacol Ther 98: 355–377.

18. Phillips R, Ursell T, Wiggins P, Sems P (2009) Emerging roles for lipids in shaping membrane-protein function. Naturwissenschaften 96: 379–393.

19. Braulke LJ, O'Brien R (1960) Circulatory changes in the Thirteen-lined ground squirrel during the hibernating cycle. In: Lyman CP & Dawe AR (eds) Symposium Biological Papers of the University of Alaska, number 27 Fairbanks, Alaska, USA: Institute of Arctic Biology, University of Alaska: 71–80.

20. Arnold W, Ruf T, Frey-Roos F, Brus U (2011) Diet-independent remodeling of cellular membranes precedes seasonally changing body temperature in a hibernator. PLoS One 6: e18041.

21. Axela RC, Pengelly ET (1979) Lipid composition of cellular membranes of hibernating mammals. Chem Zool 11.

22. Geiser F, McAllan BM, Kenagy GJ, Bergert SE, Pitman MC (2006) A role for direct interactions in the shaping membrane-protein function. Nature 459: 379–385.

23. Brittsan AG, Kranias EG (2000) Phospholamban and cardiac contractile function. J Mol Cell Cardiol 32: 2131–2139.

24. Branch KM, Dhruv ND, Hanse EA, Andrews MT (2005) Digital transcriptome analysis indicates adaptive mechanisms in the heart of a hibernating mammal. Physiol Genomics 23: 227–234.

25. Lindemann JP, Jones LR, Hatchaway DR, Henry BG, Watanabe AM (1983) beta-Adrenergic stimulation of phospholamban phosphorylation and Ca2+-ATPase activity in guinea pig ventricles. J Biol Chem 258: 464–471.

26. Oertel R, Heldmaier G (2000) Regulation of body temperature and energy requirements of hibernating Alpine marmots (Marmota marmota). Am J Physiol Regul Integr Comp Physiol 278: R509–R504.

27. Heldmaier G, Oertel R, Ewert R (2004) Natural hypometabolism during hibernation and daily torpor in mammals. Respir Physiol Neurobiol 141: 317–329.

28. Lyman C, O'Brien R (1960) Circulatory changes in the Thirteen-lined ground squirrel during the hibernating cycle. In: Lyman CP & Davis AR (eds) Mammalian Hibernation, Proceedings of the first international symposium on natural mammalian hibernation, May 13–15, 1959 Bulletin of the Museum of Comparative Zoology at Harvard College 124: 353–372.

29. Hampton M, Nelson BT, Andrews MT (2010) Circulation and metabolic rates in a natural hibernator: an integrative physiological model. Am J Physiol Regul Integr Comp Physiol 299: R1478–1488.

30. Milsom WK, Zimmer MB, Harris MR (1999) Regulation of cardiac rhythm in hibernating mammals. Comp Biochem Physiol A Mol Integr Physiol 124: 303–309.

31. Strumwasser F (1959) Thermoregulatory, brain and behavioral mechanisms during entrance into hibernation in the squirrel, Citellus beeseyi. Am J Physiol 196: 15–22.