Effects of polyunsaturated fatty acids (PUFAs) on hibernation and torpor:
A review and hypothesis.

Thomas Ruf and Walter Arnold

Research Institute of Wildlife Ecology,
University of Veterinary Medicine, Savoyenstr.1, A-1160 Vienna, Austria.

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Corresponding author:
Thomas Ruf
Research Institute of Wildlife Ecology,
University of Veterinary Medicine
Savoyenstr.1
A-1160 Vienna, Austria
e-mail: thomas.ruf@fiwi.at
phone: +43 1 489 0915 150
fax: +43 1 489 0915 550
ABSTRACT

Polyunsaturated fatty acids (PUFAs) can have strong effects on hibernation and daily torpor in mammals. High dietary PUFA contents were found to increase proneness for torpor, decrease body temperatures, prolong torpor bout duration, and attenuate hibernation mass loss. The mechanism by which PUFAs enhance torpor and hibernation is unknown however. Based on a review of the literature, and on re-examining own data on alpine marmots, we propose that effects on hibernation are not due to PUFAs in general, but to shifts in the ratio of n-6 PUFAs to n-3 PUFAs in membrane phospholipids. Specifically, high ratios of n-6 to n-3 PUFAs increase the activity of the Ca^{2+} Mg^{2+} pump in the sarcoplasmatic reticulum of the heart (SERCA) and counteract Q_{10} effects on SERCA activity at low tissue temperatures. Therefore, high n-6 to n-3 PUFA ratios in cardiac myocyte membranes appear to protect the hibernating heart from arrhythmia, which in hypothermic non-hibernators is caused by massive increases in cytosolic Ca^{2+}. The resulting reduced risk of cardiac arrest during hypothermia may explain why increased dietary uptake of n-6 PUFAs, but not of n-3 PUFAs, can strongly enhance the propensity for hibernation, and allows heterotherms to reach lower body temperatures, with associated increased energy savings. Therefore, at least for herbivorous hibernators, such as marmots, linoleic acid (C18:2 n-6), the dietary source of all n-6 PUFAs, appears to represent a crucial and limited resource in natural environments.

Keywords: Polyunsaturated fatty acids; Hibernation; Torpor; Hypothermia; SERCA; Heart
INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential dietary components that mammals can not synthesize \textit{de novo}. PUFAs are well known for their effects on certain aspects of human health, in particular on cardiovascular function (46,58). In animals, specifically in ectotherms and mammalian heterotherms, an important function of PUFAs, and of monounsaturated fatty acids (MUFAs), appears to be the maintenance of cell membrane function at varying tissue temperatures. "Homoeoviscous adaptation", that is, the adjustment of the proportion of unsaturated fatty acids in membrane phospholipids to changes in tissue temperature is thought to regulate membrane fluidity and the functioning of trans-membrane proteins (2, 67, 73). The importance of PUFAs for mammalian heterotherms is demonstrated by their effects on hibernation and daily torpor. As reviewed by Geiser (28), Florant (19) and more recently by Munro and Thomas (55) and Dark (14) both experimental trials and field studies show positive effects of increased PUFA content in the diet, or in white adipose tissue (WAT) stores, on the propensity of animals to enter torpor, and, at least in several studies, on torpor bout duration, minimum body temperatures tolerated, and energy savings (e.g., 7, 21, 22, 29, 30, 72). Some, albeit limited, evidence indicates that MUFAs may also exert positive effects on torpor (31, 26). These results on effects of unsaturated fatty acids are not unequivocal, however, which may be related to the composition of the specific diets used in experimental studies (55, and see below). Also, to date, the specific trans-membrane proteins and pathways by which unsaturated fatty acids exert their effects on hibernation are entirely unknown.
Here, we propose such a mechanism, based on reviewing the relevant literature, and on re-analysing own data on hibernation in free-living alpine marmots (*Marmota marmota*).

In short, we hypothesize that (i) in hibernators, the primary target of PUFAs is the Ca\(^{2+}\) Mg\(^{2+}\) pump in the sarcoplasmatic reticulum (SR) of the heart (SERCA 2a\(^{1}\)). This transmembrane ATPase is the key enzyme which ensures proper Ca\(^{2+}\) handling of myocytes and hence functioning of the heart, in particular in deep hibernation. (ii) We review evidence that SERCA activity is not regulated by total membrane PUFA content, but by the ratio of n-6 to n-3 PUFAs, (which have their first double bond at the 3rd and 6th carbon of the fatty acid chain, respectively; Fig. 1). High ratios of n-6 to n-3 PUFAs in heart SR membranes strongly up regulate SERCA activity and thus help to ensure Ca\(^{2+}\) re-uptake into the SR and hence contraction of myocytes even during severe hypothermia, a condition which is indeed fatal, due to ventricular dysfunction, for non-hibernators. High n-6 to n-3 PUFA ratios in cardiac myocytes allow hibernators and daily heterotherms to decrease minimal body temperatures, increase torpor bout duration, and maximize energy savings. Below, we summarize the available evidence for each aspect of this hypothesis.

**RESULTS AND DISCUSSION**

**SERCA and the hibernating heart**

The singular importance of Ca\(^{2+}\) handling in cardiac myocytes, and several of the special adaptations of hibernators to maintain Ca\(^{2+}\) homoeostasis at low body temperature have been extensively reviewed before (3,81). Thus, we will focus on the most important findings, and some additional results: Non-hibernators exposed to severe hypothermia
typically die from heart failure, i.e. loss of contractibility of cardiac myocytes and ventricular fibrillation (e.g. 9, 11, 50, 79). This is caused by a massive increase in cytosolic Ca\(^{2+}\) accompanied by Ca\(^{2+}\) waves, which cause arrhythmia. Hibernators in the winter acclimated state when they display torpor, however, have the capability to maintain cyclic release of Ca\(^{2+}\) from the SR, which initiates cardiac contraction, and rapid removal of Ca\(^{2+}\) into the SR. This has been related to several seasonal adjustments. Hibernators down regulate the entry of Ca\(^{2+}\) into myocytes through ion channels, and simultaneously up regulate the removal of Ca\(^{2+}\) into intracellular storage compartments by SERCA (Fig. 2). In hibernating ground squirrels and hamsters, the volume of the longitudinal SR in myocytes, which is responsible for Ca\(^{2+}\) storage, is 2-3 times larger than in summer-acclimated individuals (5, 50, 62, 71). Further, in hibernating woodchucks, the mRNA levels of SERCA in myocytes and SERCA protein levels were increased threefold compared to animals in the non-hibernating season. Simultaneously, hibernating woodchucks showed a ~50 % reduction in both mRNA and protein levels of phospholamban, a potent inhibitor of SERCA (82). An up regulation of the gene expression of SERCA, and reduced levels of phospholamban expression were also found in hearts of hibernating ground squirrels (8). Together, these adjustments lead to much faster rates of re-uptake of Ca\(^{2+}\) into the SR which explains why myocytes in hibernators at low body temperature have significantly faster relaxation rates than in hypothermic non-hibernators (80). Similar adjustments apparently also occur in daily heterotherms. Dibb et al. (15) found that in Djungarian hamsters, increased SR Ca\(^{2+}\) content and enhanced uptake by SERCA can be induced by short photoperiod alone, and probably represents a prerequisite for daily torpor with body temperatures of down to 12 °C in this species (32).
Apart from its protective effect against ventricular dysfunction via maintaining proper Ca\(^{2+}\) handling, increased SERCA density and activity may additionally serve to preserve cardiac function by local heat production (8,3). ATP hydrolysis by SERCAs (namely the SERCA 1 isoform) can lead to substantial amounts of chemical energy released as heat (53) and this avenue of thermogenesis is also used by the 'heater organ' of billfishes which can create large thermal gradients between this organ (and the adjacent eye and brain) and ambient water temperature (54). Clearly, this mechanism would counteract temperature-dependent (Arrhenius) effects on SERCA activity in torpid mammals.

In summary then, there seems to be a general consensus that enhanced SERCA-driven Ca\(^{2+}\) re-uptake into the SR is an important pathway by which hibernators maintain functionality of the heart, and hence survival at low body temperature. However, an important factor in this context that has, to our knowledge, not been considered until now is the effect of PUFAs in the surrounding SR membrane on SERCA activity.

High ratios of n-6 to n-3 fatty acids in membrane phospholipids significantly increase SERCA activity.

It has long been known that Ca\(^{2+}\) ATPase activity can be affected by the physico-chemical properties of the surrounding membrane. For instance, the activity of SERCA reconstituted in microsomes can be strongly inhibited by replacing natural by synthetic phospholipids, such as dipalmitoyllecithin (57, and literature cited there). This inhibition
of SERCA has been related to increased membrane microviscosity impeding the conformational change required for the translocation of Ca\(^{2+}\) (57), but it seems unclear whether the bulk membrane property of viscosity is a major regulative factor of Ca\(^{2+}\) transport in natural SR membranes. Another determinant of SERCA activity is the thickness of the lipid bilayer, which is determined by the length of phospholipid fatty acid chains. In experiments by Lee (47, 48) the activity of SERCA was maximal when the protein was reconstituted in phosphatidyl cholines containing (monounsaturated) fatty acids with a chain length of C18 to C20, but significantly reduced at shorter or longer chain lengths.

Convincing evidence for a role of fatty acid composition in regulation SERCA activity in natural membranes comes from experiments that have used dietary manipulations to change PUFA composition in lipid bilayers (69, 70, 74). Swanson et al. (69) found that the addition of menhaden oil, corn oil, or olive oil to the diet of mice caused significant changes in the fatty acid composition of SR membranes in the heart that were accompanied by very large effects on SERCA activity and SR Ca\(^{2+}\) uptake. SERCA activity was not, however, associated with total PUFA content or the degree of unsaturation of membranes (i.e. the number of double bonds in each fatty acid). Instead, SERCA activity increased by a factor > 5 as the ratio of n-6 to n-3 PUFAs increased (Fig. 3). Significant increases in SERCA activity and Ca\(^{2+}\) uptake in response to increased n-6 to n-3 PUFA ratios following dietary manipulation were also found in rat myocyte SR (70), as well as in a slightly different model system, i.e., skeletal muscle SR membranes in rainbow trout (74). The data on SERCA activity in this ectothermic species, measured over a large range of temperatures (0-30 °C), are particularly
important to understand the role of membrane composition for Ca\(^{2+}\) homoeostasis at low
tissue temperature. Increasing the proportion of n-6 PUFAs in the SR fully compensated
for the effects a 10-15 °C decrease in temperature on SERCA activity (Fig. 4). Thus,
shifts towards a high n-6 to n-3 ratio in membrane phospholipid composition should
substantially contribute to the maintenance of proper Ca\(^{2+}\) handling of cells during
voluntary hypothermia in heterotherms (Fig. 2).

In the above studies, experimental diets caused simultaneous shifts in both the n-6 and
n-3 content of membrane fatty acids, which seems quite typical (12, 64). Therefore, it is
not possible to clearly distinguish between enhancing effects of n-6, and inhibitory
effects of n-3 PUFAs, or both, on SR Ca\(^{2+}\) uptake. Also, the actual biochemical
mechanism by which PUFAs affect SERCA activity is unknown. A simple explanation
would be that high proportions of n-3 PUFAs lead to an increase in membrane
thickness, which suppresses SERCA activity in vivo (47). This is plausible, at first
glance, because the most common (41,64) fatty acid with a SERCA activity impairing
chain length of >C20 belongs to the n-3 class (C22:6 n-3 docosahexaenoic acid (DHA)).

However, it seems that positive effects on SERCA activity in the experiments outlined
above, and the beneficial effects of PUFAs on hibernation (see below) are specifically
due to a replacement of n-3 by n-6 PUFAs. If membrane thickness alone was the crucial
factor, n-6 PUFAs in hibernators could be substituted with other optimal-length fatty
acids such as stearic acid, which is not supported by the current evidence. Therefore,
we consider it more likely that n-6 and n-3 PUFAs (and, perhaps, the n-9 MUFA oleic
acid) exert their specific effects on SERCA by other mechanisms. One of these
potential mechanisms (reviews in 51, 68) is that unsaturates, due to their large packing
free volume (i.e., the volume outside the van der Waals radii of atoms), ease the
insertion of proteins into membranes, and hence may increase protein density.

Another (not mutually exclusive) possibility is that the physical properties of unsaturates
in the surrounding membrane bilayer, particularly the intrinsic bilayer curvature, affect
the conformation and specific activity of trans-membrane enzymes. If this is indeed the
key mechanism, it would seem plausible that specific proteins, such as SERCA, are
activated by specific fatty acids, such as n-6 PUFAs. As mentioned before, there is
some indication that MUFAs, namely oleic acid (C18:1 n-9), can mimic the positive
effects of n-6 PUFAs on hibernation (31, 26). If these effects of oleic acid should also be
related to enhanced SERCA activity in the heart, this could mean that the crucial factor
for effects on SERCA may be a similarity in the structure of n-6 and n-9 fatty acids, i.e.,
the location of the first bend in the carbon chain at position 6, or further (at C9) towards
the hydrophilic end of the membrane bilayer. Ca$^{2+}$ transport into the SR involves rapid
conformational changes of the transmembrane domains of SERCA (Fig 2; 52).
Therefore, it is easy to envision that the physical properties of the fatty acids surrounding
the moving domains, such as disturbed packing of the lipids at a specific depth of the
bilayer caused by these bended unsaturates, may affect the activity of this pump.
Recently, biochemical modelling (in that case, of the effects of DHA on rhodopsin activity
in photoreceptor cells) has demonstrated that certain fatty acids may even penetrate the
core of membrane proteins and displace interactions between protein domains, thereby
facilitating the formation of active states (34). Currently, these types of direct interactions
within the membrane bilayer seem a likely explanation for the observed effects of n-6
PUFAs on SERCA activity.
However, it is also possible that PUFA effects in this context are not due to their impact on physical membrane properties, but to the well known role of both dietary and membrane-derived PUFAs in modulating the expression of various genes (45, 65). Conceivably then, PUFAs may alter the expression of SERCA, phospholamban, or other proteins involved in heart SR Ca\textsuperscript{2+} uptake, but possible effects of PUFAs on gene expression and direct effects on enzyme activity are not mutually exclusive.

The evidence from hibernation studies

To date, there has been no systematic experimental study on the effects of various ratios of n-6/n-3 uptake on hibernation or torpor patterns. However, positive effects of PUFA-enriched diets on the propensity for torpor, minimum body temperature, metabolic rate and torpor bout duration were all caused by diets with increased linoleic acid content, i.e., the major PUFA of the n-6 class (55). These positive effects of high dietary linoleic acid content, in particular on torpor bout duration and torpor propensity, were observed in several genera of daily heterotherms (Peromyscus, Phodopus, Sminthopsis) and hibernators (Acrobates, Cynomys, Tamias, Spermophilus, Marmota) (55). There is also some evidence indicating that high amounts of dietary oleic acid (C18:1 n-9) can partly (31) or even fully (26) compensate for low n-6 intake, and that this MUFA also leads to increased torpor bout duration and decreased body temperatures during hibernation.
To our knowledge, there is only one study by Hill and Florant (38) which has tested the effects of a diet specifically enriched in an n-3 fatty acid, namely α-linolenic acid (C18:3 n-3) on hibernation. Interestingly, Hill and Florant (38) found that the n-3 PUFA enriched diet had a very strong negative effect on hibernation propensity in yellow bellied-marmots: Only two animals on this n-3 rich diet showed normal hibernation, while one animal terminated the hibernation season very early, and another 5 out of 8 animals stayed euthermic throughout winter, and continued to feed, which was completely unexpected for this species. Some evidence for an adverse effect of n-3 fatty acids on hibernation propensity was also reported by Frank and Storey (25). While the proneness for hibernation in ground squirrels fed PUFA-rich diets was generally reduced in that study, the propensity for hibernation was clearly lowest (with no animal entering hibernation) in the single group on a diet that contained not only linoleic acid, but also α-linolenic acid (C18:3 n-3).

Differential effects of n-6 and n-3 PUFAS on hibernation are also evident in our own studies on free-living alpine marmots. In a previous study, we found that high PUFA content of WAT triglyceride depots in fall was associated with lower minimum body temperatures, and with a significantly lower mass loss over the subsequent hibernation season (7). A re-analysis of these effects, using an extended data set, shows that hibernation mass loss in marmots actually was not attenuated by PUFAs as such, but by high levels of n-6 PUFAs in WAT triglycerides only (Fig. 5). There was a significant correlation between n-6 PUFA proportion and mass loss, such that high n-6 PUFA was associated with a reduction in mass loss. Importantly for n-3 PUFAs,
there was no effect of FA proportion on mass loss. Given that PUFA contents in WAT triglyceride stores are only a correlate for the phospholipid composition of heart or other tissue membranes (75), it is surprising to still find a strong association between mass loss and WAT n-6 PUFA content.

The main mechanism by which high n-6 to n-3 PUFA ratios lead to attenuated mass loss in marmots apparently involves decreased gradients between body and ambient temperatures during hibernation, and consequently decreased energy expenditure during the winter season. These temperature gradients were significantly correlated with both WAT linoleic acid content and hibernation mass loss. The large variation of WAT n-6 PUFA contents shown in Fig. 5 is probably related to individual differences in dietary fatty acid uptake. We found significant differences in the linoleic acid content of dietary plants between study areas and even between marmot territories within study sites (7). Also, it is well known from both laboratory and field studies that diets rapidly affect the composition of WAT lipid stores (20, 31, 19, 24). Thus, n-6 PUFAs may in fact represent a limited dietary resource for these hibernators, even if they should select diets rich in linoleic acid, as has been suggested for several heterotherms (23, 19, 28).

Despite the evidence for an active selection of diets rich in n-6 PUFAs, their beneficial effects on hibernation could simply have evolved as an adaptation to seasonal changes in the fatty acid composition of the diet, at least in herbivores (or in their predators). In spring, plant material is typically dominated by green parts rich in $\alpha$-linolenic acid (C18:3 n-3), while linoleic acid (C18:2 n-6) should become increasingly abundant in flowers and seeds in summer. However, analyses of natural diets of marmots and prairie dogs
indicate that the supply with n-6 and n-3 PUFAS may actually be constant (37) or fluctuate unsystematically between seasons (49). Also, as illustrated in Fig. 6, our data on alpine marmots demonstrate that seasonal changes in the fatty acid composition of tissue membranes, namely in the heart, do not simply mirror changes in diet composition. There are three aspects of these time courses, obtained from free-ranging marmots, that should be noted. First, the ratio of n-6 to n-3 fatty acids in heart membranes was approximately 10-fold higher than in stomach content total lipids. Secondly, n-6 content in the heart decreased from early spring to summer in heart phospholipids, while it increased in the diet. Third, and most importantly, the time courses in both stomach and heart samples within the fall sampling period suggest that marmots may actively select n-6 rich diets, and increasingly incorporate n-6 fatty acids into heart membranes just prior to the onset of the hibernation season. We have evidence that this remodelling of membranes also involves differential depletion of fatty acids from WAT depots, and is reversed immediately after emergence from hibernation in spring (Arnold et al., unpublished observations).

Also, even in hibernators fed standard laboratory diets, seasonal changes in n-6 and n-3 PUFA content in heart phospholipids have been observed. In the hearts of hibernating ground squirrels Aloia and Pengelley (1) found an increase in C18:2 n-6 and C20:4 n-6, and a pronounced reduction in C22:6 n-3 and C22:5 n-3 PUFAs in hibernating squirrels compared to summer active animals. Especially the reduction in 22:6 n-3 (DHA) led to a decrease in the overall degree of unsaturation of membranes. This effect was unexpected, because the major impact of PUFAs on hibernation was thought to be due to increased membrane fluidity (1), which is particularly enhanced by DHA (68). Further,
Geiser et al. recently (33) demonstrated that short photoperiod alone is sufficient to cause significant changes in the muscle lipid composition of *Peromyscus maniculatus*, a species that exhibits daily torpor predominantly under short days. A major shift induced by short photoperiod, in animals kept on a constant diet, was a significant increase in n-6 PUFAs and a concomitant decrease in n-3 PUFAs, namely DHA.

Together, these findings point to an active –diet independent– up regulation, not of membrane PUFA content or unsaturation as such, but of the n-6 to n-3 fatty acids ratio in heart membranes (which are dominated by SR) in preparation for hibernation and daily torpor. This supports the view that high ratios of these two fatty acid classes serve to maintain functionality of the heart at low body temperature.

**Implications and trade-offs**

If high n-6/n-3 PUFA ratios in the SR enhance proper Ca^{2+} handling in the hypothermic heart, the implications for torpor and hibernation are straightforward: Very low n-6 to n-3 PUFA ratios in heart SR as caused by a limitation of dietary uptake of linoleic acid, or an increased uptake of α-linolenic acid, should primarily affect the propensity of animals for voluntary hypothermia (as in the experiment by Hill and Florant (38), see above), because torpor without adequate SERCA function will be associated with a high risk of cardiac arrhythmia and sudden death. Even without dietary manipulations, cardiac arrhythmia at intermediate body temperatures during arousals seems to occur quite regularly in hibernators (16). Further, within physiological limits that allow voluntary hypothermia at all, individuals with high n-6 to n-3 ratios in heart SR will be able to
decrease body temperature to lower minima. This prediction is in accordance with one of
the most consistent outcomes of dietary manipulations in both hibernators and daily
heterotherms (55, 14).

Another effect of n-6 PUFA enriched diets is increased torpor bout duration, which can
be profound (e.g. 21, 29, 30, 72). Since torpor bout duration seems to be linked to
energy turnover in the hibernating state (27, 29, 31), minimizing the lowest tolerated
body temperature, and hence metabolism, by establishing high n-6/n-3 ratios in heart
phospholipids would also explain their effect on the length of torpor bouts. Alternatively,
torpor bout duration may be directly affected by the impact of high n-6 to n-3 ratios on
heart SERCA activity. The translation and transcription of SERCA, as of all proteins, is
sharply and acutely down regulated during entrance into torpor and only resumed during
arousals. (10, 35, 77,78). Degradation of proteins, on the other hand, is also decreased
several fold at low body temperatures, but may not be entirely blocked (overview in 78).
During torpor entrance, protein damage may be even increased due to the stress of
severe changes in metabolism (78). In view of the evidence outlined above we would
expect that high SR n-6 to n-3 PUFA ratios in the heart will partially compensate for a
decrease in SERCA activity resulting from proteolysis over time. Thus, high n-6 to n-3
ratios could prolong the period hibernators can remain torpid until the need to restore
SERCA activity via protein synthesis forces them to re-warm to euthermic temperatures.
As SERCA has a very long half life of 14.5 days in normothermic young rats, and of 18.4
days in aged rats (18) this explanation is not entirely unrealistic, given that maximum
torpor bout duration in hibernators ranges from several days to weeks (32). However, at
present, such a role of restoration of SERCA in dictating periodic arousals is speculative.
Despite the beneficial effects of n-6 PUFAs on various aspects of hibernation, the results of feeding n-6 PUFA enriched diets are not unequivocal. In fact, some studies show adverse effects on hibernation propensity and patterns (overview in 55, see also (43, 56)). These controversial observations may have several explanations. First, given our evidence for strong regulatory mechanisms that control phospholipid composition in cardiac tissue of marmots (Fig. 6), dietary manipulations may have only a limited influence on actual SR membrane composition and SERCA activity, and will also depend on the actual concentrations ingested and absorbed, and on the composition of WAT lipid depots. Second, diets enriched in n-6 PUFAs may be particularly ineffective in certain species, namely seed eaters, in which linoleic acid is probably not a limited resource at all. Third, there is accumulating evidence that the risks associated with the torpid state, such as impaired cardiac, but also neuronal, or immune function (13, 61) create a trade-off that explains why certain mammals, especially those that continue to feed over winter, abandon hibernation if energy supply is very high (42, 56). Thus, linoleic acid-enriched diets may have adverse effects on torpor and hibernation propensity in certain species simply due to their high caloric value. Even if isocaloric diets are used, food with added PUFA-rich items (e.g. peanuts) may be perceived by the animals as energetically more valuable (56), and increasing the number and type of food items can have strong adverse effects on torpor propensity (63). Clearly, further experiments with isocaloric diets with an identical number and type of food items, but different n-6 to n-3 PUFA ratios are required to clarify these questions.
An undisputed adverse effect of high PUFA content on membranes is their high susceptibility to peroxidation by radical oxygen species (ROS) that are generated during mitochondrial respiration (40, 46). Lipid peroxidation leads to deleterious products such as reactive aldehydes that cause damage to membranes as well as enzymes, and inhibit DNA and protein synthesis (overview in 40). Therefore, it has been argued that optimal levels of PUFA intake in hibernators result from a trade-off between their beneficial effects on cellular function during hypothermia, and peroxidation-related cellular damage (e.g. 24, 55). This trade-off is thought to be the underlying reason why some hibernators reduce torpor when provided with large amounts of PUFAs (56), or, when given the choice, do not simply maximize linoleic acid intake, but appear to ingest an "optimal", intermediate amount (24).

Previously, it has been suggested that oxidative damage should be most pronounced in tissues with high proportions of n-3 PUFAs, in particular DHA (C22:6 n-3), because they are much more susceptible to peroxidation than n-6 PUFAs (39, 40). However, research on cardiovascular function in humans and animal models has demonstrated that probably the most damaging and reactive product of lipid peroxidation is the aldehyde 4-hydroxy-2-nonenal (HNE) (e.g. 44, 46, 59). In contrast to other ROS species, HNE is relatively long-lived and acts not only in the immediate proximity of membranes but can diffuse from the site of its origin and damage even distant targets (17, 46). Importantly, HNE originates not from n-3-PUFAs but is formed by superoxide reaction with n-6 PUFAs. HNE is produced from hydroperoxides of linoleic acid and arachidonic acid, and the nine carbons of HNE represent the last nine carbons of these n-6 PUFAs (17, 66, 4). Not surprisingly then, increases in the ratio of n-6 to n-3 PUFAs in heart membranes,
which occur, for instance, as a result of ageing, lead to elevated levels of HNE formation. In fact, this process may be one of the major causes of impaired cardiac function in the elderly (46). Together, these results indicate that not all PUFAs have equal effects in increasing the risk of oxidative damage, but that high amounts membrane n-6 PUFAs, as a source of HNE production, may be much more detrimental than n-3 PUFAs.

In the current context these findings are important because they could explain why hibernators or daily heterotherms do not maintain maximal n-6 PUFA levels at all times, but apparently restrict high n-6 to n-3 ratios in the heart and other tissues to the fall and winter season only (Fig. 6, (2, 25), see also (36)). Contrary to previous arguments that were based on the high susceptibility of n-3 PUFAs to oxidative degradation by free radicals alone (e.g. 40), these results imply that replacing n-6 by n-3 PUFAs, whenever possible (i.e., in hibernators during the summer euthermic season) may even protect against oxidative damage. This protective effect is also thought to be one of the reasons for the well known beneficial influence of n-3 enriched diets on cardiac function in humans (46). Thus, seasonal remodelling of cardiac membranes in hibernators such as the alpine marmot appears to be an adaptive seasonal strategy that serves two purposes: In winter, an increase in the ratio of n-6 to n-3 PUFAs counteracts the risks of hypothermia for cardiac function, while decreased n-6 to n-3 levels in the summer-active state lower cellular damage caused by otherwise enormously increased HNE production due to an almost doubled euthermic basal metabolic rate (60).
Perspectives and Significance

In summary, our analysis supports the view that proper Ca\(^{2+}\) handling of cardiac myocytes is the crucial adaptation that separates hibernators and daily heterotherms from homoeothermic mammals (3, 81). Further, we propose that the key to understanding the impact of PUFAs on hibernation is to recognize the opposing effects of n-6 and n-3 PUFAs on SERCA activity in cardiac membranes, and the risk of low n-6/n-3 ratios for cardiac arrest during hypothermia.

One might be tempted to think that the effects of these essential fatty acids are quite specific and limited to the particular problem of maintaining cellular function at low body temperatures. Arguably however, this is not the case. For instance, we recently found that n-6 to n-3 PUFA ratios in skeletal muscle phospholipids are closely associated with (body-size corrected) differences in maximum running speed in mammals, an effect which may be also mediated via their effects on SERCA activity (64). Further, n-6 to n-3 PUFA ratios appear to be the only aspect of membrane fatty acid composition that is clearly associated with maximum life span across mammals (76). Thus, it seems that the ratio of n-6 to n-3 PUFAs in biological membranes may have important general physiological functions, and can create crucial trade-offs, that go far beyond their effect on seasonal adaptation and hibernation alone.
FOOTNOTES

1 There are several isoforms of Ca^{2+} ATPase or SERCA (EC 3.6.3.8).

SERCA 1 is expressed in white muscle and brown adipose tissue. Red muscle expresses both SERCA 1 and SERCA 2a. SERCA 2b and SERCA 3 are expressed in blood platelets and lymphoid tissues (53). Note that, unless stated otherwise, our use of the label SERCA refers to the muscle specific isoform SERCA 2a.

2 Munro and Thomas (55) list two studies using high n-3 PUFA diets. However, Table 1 in Munro and Thomas (55) contains a type error. 18:3 content in the medium PUFA diet of Thorp et al. (72) was 3.1, rather than 31 %. Thus, there was in fact only one study with enriched 18:3 content (50.9 %; (38)).

3 Among mammals, there is the exception of ruminants and humans, which take up trans fatty acids produced by bacterial endosymbionts and during food processing.

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Figure Legends

Fig. 1. Chemical structures of the two precursors of all polyunsaturated fatty acids, linoleic acid, and α-linolenic acid. Polyunsaturated fatty acids contain two or more double bonds, which usually occur at every third carbon atom. In n-6 (ω6) PUFAs, the first double bond is located at the 6th carbon, counting from the methyl terminus (the ω carbon) of the molecule, and in n-3 PUFAs the first double bond is located at the 3rd carbon. In mammals, all double bonds in membrane PUFAs are in the cis configuration, which causes a bend in the fatty acyl chain. While saturated fatty acyl chains are straight, packed close together, and form rigid arrays in membranes, the "kinks" of PUFA hydrocarbon chains interfere with this highly ordered packing structure, because the cis configuration does not allow rotation around the C=C linkage. Thus, insertion of PUFAs produces flexible, fluid membranes which may affect the function of transmembrane proteins (48, 51, 68).

Fig. 2. Model of Ca^{2+} handling in cardiomyocytes of hibernators (modified from Andrews (3) and Bers (6)). In the hibernating state, Ca^{2+} entry via L-type calcium channels (LTCC) is decreased. This is mainly due to lowered expression of calcium-calmodulin protein kinase II, leading to reduced phosphorylation of LTCC, and hence decreased probability of open channels (3). Calcium entering the cell causes the ryanodine receptors (RyR) to release sarcoplasmic reticulum (SR) calcium stores into the cytosol which binds to the contractile apparatus. In the hibernation season, Ca^{2+} reuptake into the SR is enhanced by decreased expression of phospholamban, increased expression
of Ca\textsuperscript{2+} ATPase (SERCA 2a), and by increased SERCA activity caused by high n-6 to n-3 PUFA ratios in phospholipids of the surrounding SR membrane. The enlarged cartoon of SERCA shows the principal domains of the Ca\textsuperscript{2+} pump (52) and its position in the SR membrane. The transport of two Ca\textsuperscript{2+} ions per cycle into the SR involves strong movements of the transmembrane domains, which may be affected by the fatty acid composition of the bilayer (52).

**Fig. 3.** The relationship between SERCA activity and the phospholipid n-6/n-3 PUFA ratio in cardiac sarcoplasmatic reticulum of CD-1 white mice fed four diets differing in n-3 and n-6 PUFA content. Data are presented as the relative activity of ATP hydrolysis with a group fed corn oil arbitrarily set at 100 %, and all other groups compared to this value for ATPase activity. The n-6/n-3 PUFA ratio represents the $\sum$ (n-6 fatty acids) / $\sum$ (n-3 fatty acids) of SR total phospholipids. Each point represents the mean ± SEM, n = 3 (3 pools of 3 hearts per replicate). Data were taken from Fig. 3 in (69).
Fig. 4. SERCA activity in the SR as a function of temperature in rainbow trout skeletal muscle (73); (shown are data on the Serca 1 isoform, which, in addition to SERCA 2a, is also expressed in red muscles). Fish were fed different diets prior to the measurements of SERCA activity. Numbers within the legend give $\sum$ (n-3 fatty acid) / $\sum$(n-6 fatty acids) in the diet. Numbers on the right of the graph give the n-3/n-6 ratio in the phosphatidylcholine fraction of SR membranes (which represented the largest fraction of phospholipids; >50%). Note that the diet with a considerably increased n-3 to n-6 PUFA ratio (11.4) led to a substantially reduced n-6 PUFA content in the SR phospholipids (n-3/n-6 ratio 8.7), and to a significant decrease in SERCA activity. Data were taken from Fig. 6 and Tables 2 and 6 in (74).

Fig. 5. Hibernation mass loss as a function total lipid n-6 PUFA content (gray symbols) and n-3 PUFA content (white symbols) in WAT depots of free-living alpine marmots (N=75) sampled in fall, just prior to hibernation onset. Linoleic acid (C18:2 n-6) and $\alpha$-linolenic acid (C18:3 n-3) represented >98 % of n-6 and n-3 PUFAs, respectively, in WAT. Mass loss significantly decreased as WAT n-6 PUFA content increased (slope=-1.68, t= -3.55, P<0.001) but was independent of WAT n-3 PUFAs (slope= -0.12, t= -0.72, P=0.47). The slopes of the two regression lines were significantly different (t=2.98, P=0.003). Animals were captured and weighed prior to hibernation (September) and shortly after emergence from hibernacula (April). Note that alpine marmots do not feed during the hibernation season. Thus, body fat reserves are the sole source for PUFAs during winter. For methods of WAT sampling and fatty acid analysis see (7) and (75).
Fig. 6. Seasonal changes in the ratio of n-6 to n-3 PUFAs in the diet (as determined from stomach contents) and in heart phospholipids of free-living alpine marmots (n=8-14 per sampling period; means±SEM). The inset graphs show the data points sampled in fall on an actual time scale. This is to illustrate that n-6 to n-3 ratios increased slightly in stomach contents (slope=+0.005; t=2.14; P=0.06) and rapidly in the heart (slope=+0.22; t=6.97; P<0.0001) within the fall, pre-hibernation sampling period (September). Tissues and stomach contents were obtained from marmots culled for management reasons. For details on the study areas, tissue sampling, and measurement of fatty acids see (7) and (75).
Linoleic acid (18:2 n-6)

α-linolenic acid (18:3 n-3)
increased n-6/n-3 ratio

LTCC $[\text{Ca}^{2+}]$↓

RyR $\text{Ca}^{2+}$←

SR $\text{Ca}^{2+}$←

PLB $\downarrow$

SERCA $\uparrow$

$\text{Ca}^{2+}$
Proportions of C18:2 n-6 and of C18:3 n-3 in prehibernation WAT