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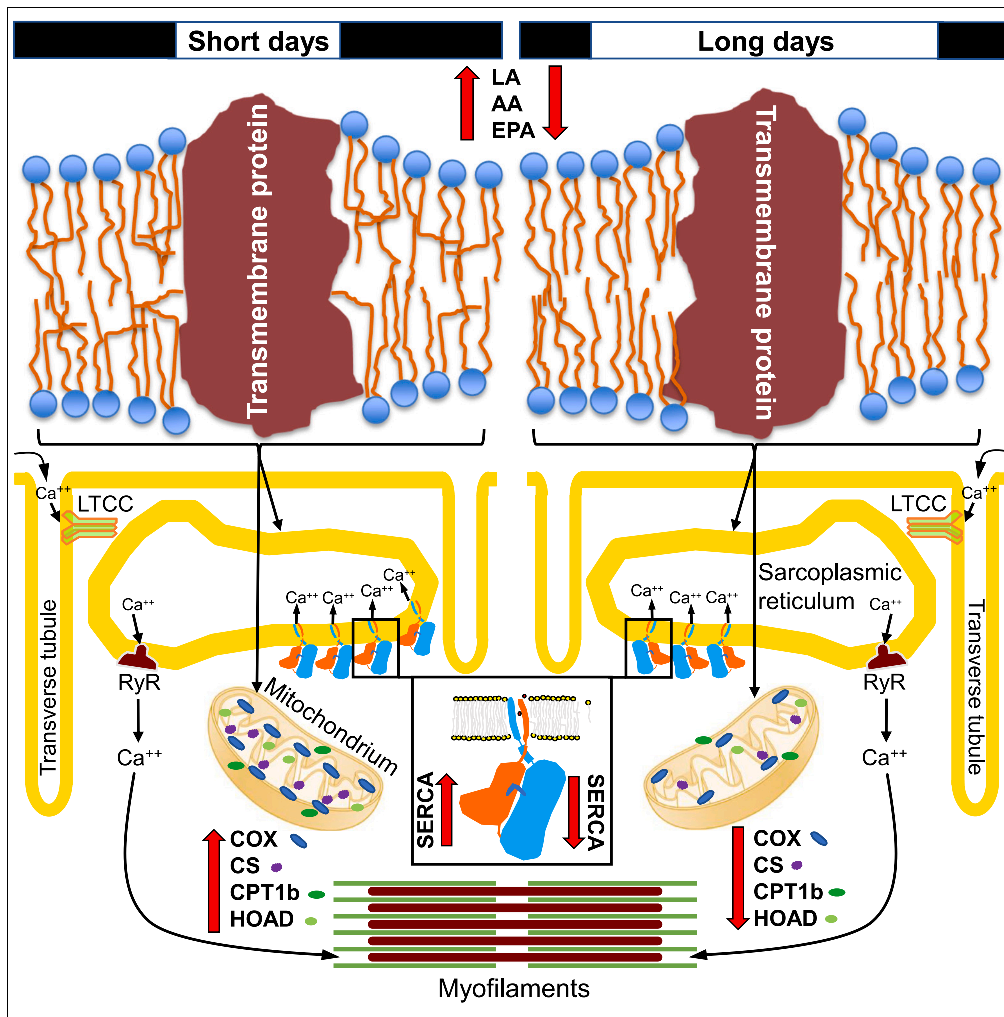

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Highlights

During winter, more polyunsaturated fatty acids (PUFAs) were found in phospholipids

Key metabolic enzymes were more expressed during winter

Their activity was boosted by some of the PUFA incorporated in phospholipids

Seasonal changes were independent of intake of PUFA but governed by photoperiod

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Summer fades, deer change: Photoperiodic control of cellular seasonal acclimatization of skeletal muscle

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SUMMARY

We found major seasonal changes of polyunsaturated fatty acids (PUFAs) in muscular phospholipids (PL) in a large non-hibernating mammal, the red deer (*Cervus elaphus*). Dietary supply of essential linoleic acid (LA) and α-linolenic acid (ALA) had no, or only weak influence, respectively. We further found correlations of PL PUFA concentrations with the activity of key metabolic enzymes, independent of higher winter expression. Activity of the sarcoplasmic reticulum (SR) Ca^{++}-ATPase increased with SR PL concentrations of n-6 PUFA, and of cytochrome *c* oxidase and citrate synthase, indicators of ATP-production, with concentrations of eicosapentaenoic acid in mitochondrial PL. All detected cyclic molecular changes were controlled by photoperiod and are likely of general relevance for mammals living in seasonal environments, including humans. During winter, these changes at the molecular level presumably compensate for Arrhenius effects in the colder peripheral body parts and thus enable a thrifty life at lower body temperature.

INTRODUCTION

Changes of body temperature (T_b) cause changes in the fatty acid (FA) composition of cellular membranes, a phenomenon well known for ectotherms.[1] Similar changes occur in so-called 'heterothermous' endothermic organisms, where T_b in peripheral body parts can drop to single-digit values.[2] Indirect evidence for such changes was reported decades ago: the melting point of bone marrow fat of mammals declines with distance from the body core,[3] and more unsaturated FA with lower melting point are indeed found in the body's periphery.[4,5] Furthermore, there seem to be seasonal differences with respect to the FA composition of peripheral body parts,[6,7] possibly caused by changes in cell temperature that in ectotherms can lead within hours to measurable adjustments of the FA composition of phospholipids (PL).[8] In hibernating mammals T_b can decline close to the freezing point, and these species apparently prepare for a life at low T_b by integrating polyunsaturated FA (PUFA) into PL.[9,10]

Integrating FA with lower melting points into PL was traditionally viewed as a measure to maintain membrane fluidity at low T_b.[11,12] However, work that is more recent emphasizes that such integration boosts the activity of membrane-bound enzymes (reviewed in[13]). For instance, the activity of the sarcoplasmic reticulum Ca^{++}-ATPase (SERCA) is increased in membranes rich in linoleic acid (LA, n-6 C18:2), a mechanism presumably pivotal during hibernation by ensuring proper Ca^{++} handling in cardiac myocytes at T_b close to the freezing point.[13–16] Incorporation of n-6 PUFA into PL is a well-known phenomenon of cold acclimatization,[17] and is the predominant process of remodeling the acyl composition of heart PL in Alpine marmots prior to hibernation.[18] Moreover, an increase of LA in muscle tissue can be induced by experimental exposure to short photoperiod.[19] Further, a comparison of 36 species of mammals revealed a positive relation between the LA concentration in muscle PL and maximum running speed.[20] This result suggested a role of the same mechanism in skeletal muscle even when operating at high T_b, *i.e.*, improved contractibility of muscle cells due to higher activity of SERCA in a PL environment rich in LA, although a different SERCA isoform is present in fast-twitch fibers.[21] We therefore suspected similar changes in a non-hibernating heterotherm, the red deer, to maintain sufficient SERCA activity in skeletal muscle cells despite a lower T_b in the body's periphery during winter.[22]

In contrast to LA, high concentrations of long-chain n-3 PUFA, notably docosahexaenoic acid (DHA, n-3 C22:6), seem to hinder life at low T_b (reviewed in[16,23]), as DHA incorporated in PL seem to decrease SERCA activity.[14,15,24,25] On the other hand, DHA and its precursor eicosapentaenoic acid (EPA, n-3 C20:5) are known to stimulate oxidative metabolism. Key enzymes of the Krebs cycle and β-oxidation have been reported to be more active in tissues containing high concentrations of EPA and/or DHA in PL.[26–29] In line with these results, the thermogenic capacity of hibernating Alpine marmots increases during the course of hibernation along with an increase of DHA in PL,[18] and physical

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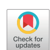

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exercise in humans increases the concentration of DHA in skeletal muscle PL, thus decreasing the ratio of n-6 to n-3 FA.[30,31] Altogether, higher concentrations of n-3 PUFA in PL are expected to be a feature of the summer phenotype with high metabolic rate to accumulate fat reserves for the next winter period. In line with this view, incorporation of n-3 PUFA into PL can indeed be induced by exposure to long photoperiod.[19]

Animals lack the respective enzymes for *de novo* biosynthesis of LA and α-linolenic acid (ALA, n-3 C18:3). These precursors of all longer-chain PUFA must be obtained from the diet and are often referred to as 'essential fatty acids' (EFA). There is ample evidence that dietary intake of PUFA influences the PL-FA composition of membranes in mammals and birds e.g.,[27–29,32,33] However, the effect of diet on the acyl composition of membranes is limited.[34] It seems that there is a homeostatically regulated balance of n-6 to n-3 PUFA with organ specific set points.[35] As T_b ubiquitously follows a daily rhythm, typically superimposed on seasonal variation and with potentially profound amplitude in peripheral tissues, particularly when food supply is limited,[22,36–40] remodeling of the PL-FA composition may occur accordingly.[13] Nevertheless, the necessary EFA must be obtained via the diet, although temporary storage in white adipose tissue and later mobilization for incorporation into PL has been reported.[18]

In this study, we investigated seasonal acclimatization at the outlined molecular level in a strongly seasonal large herbivore, the red deer (*Cervus elaphus*). We aimed to manipulate the acyl composition of PL by feeding different amounts of LA and ALA. We determined the FA composition of sarcoplasmic reticulum (SR) and mitochondrial PL during winter and summer to differentiate the contribution of EFA supply and season on the acyl composition of PL. Furthermore, we measured the influence of PUFA concentrations in SR PL on the activity of SERCA, and of PUFA concentrations in mitochondrial PL on the activities of key mitochondrial enzymes involved in the ATP generating metabolism (cytochrome *c* oxidase, COX; citrate synthase, CS), and of β–oxidation (3-hydroxyacyl coenzyme A dehydrogenase, HOAD; carnitine palmitoyltransferase I, CPT1).

Acclimatization to seasonally changing temperatures should take place well in advance of the approaching energetic challenge to be prepared in good time. Such anticipating acclimatization is typically accomplished by photoperiodic control of the according reaction.[41–43] We therefore tested whether changes in the acyl composition of PL are subject to photoperiodic control by experimentally administering melatonin. We expected that experimentally increased levels of circulating melatonin during summer induce a molecular winter phenotype.

RESULTS

Determinants of concentrations of polyunsaturated fatty acids in phospholipids

Daily intake of LA and ALA with pelleted food by the experimental animals was highly variable. This was the intended result of experimental manipulation of the type of oil supplementation (sunflower seed oil *vs.* linseed oil), and the feeding regime (*ad lib. vs.* restricted, Table 1). Further, the well-known reduction of voluntary food intake during winter of red deer[39,44,45] also contributed to reduced daily LA and ALA intake. Since we were interested whether PUFA concentrations in PL varied seasonally, *i.e.*, differed between samples taken in February *vs.* August, on top of potential effects of diet manipulations, we omitted samples taken after melatonin treatment (Table 1) from downstream analyses.

When regressing concentrations of n-6 PUFA in SR PL against daily intake of LA (Figure 1A, a-b), and concentrations of n-3 PUFA in mitochondrial PL against daily intake of ALA (Figure 1A, c-f), we found only one significant correlation, higher concentrations of ALA in mitochondrial PL with higher intake of ALA ($F_{(1,85)} = 16.2$, $p = 0.0001$). This effect was more pronounced in winter (interaction of ALA intake with season, $F_{(1,85)} = 10.1$, $p = 0.0021$, Figures 1A, c).

Independent of daily intake, significantly higher winter concentrations were found in SR PL for LA ($F_{(1,91)} = 12.0$, $p = 0.0008$, Figures 1A, a), arachidonic acid (AA, n-6 C20:4; $F_{(1,86)} = 5.5$, $p = 0.0219$, Figures 1A, b), and in mitochondrial PL for DHA ($F_{(1,65)} = 10.5$, $p = 0.0019$, Figures 1A, f).

Photoperiodic control of changes of fatty acid concentrations in phospholipids

To corroborate photoperiodic control of seasonal differences in the concentrations of PUFA in PL (Figure 1A), we disabled the physiological perception of daylength by administering melatonin experimentally. After implantation in early summer of the last study year (Table 1), the constant diffusion of melatonin out of the subcutaneous depots induced three times higher circulating melatonin levels in the experimental animals compared to peak winter values reported for Iberian red deer (*Cervus elaphus hispanicus*),[46] despite long summer days. Results show for n-6 PUFA that this treatment induced a change toward the winter phenotype of higher concentrations in the SR PL already in August, but statistically significant only in LA (C18:2, Figure 1B). The value of the LA derivate AA was in samples from August intermediate between the lower summer and higher winter level without melatonin treatment (C20:4, Figure 1B). A similar picture appeared regarding EPA concentrations in the SR (C20:5, Figure 1B). Conversely, we found no clear seasonal, or treatment induced differences for ALA, docosapentaenoic acid (DPA), and DHA (C18:3, C22:5, C22:6, Figure 1B).

In mitochondrial PL, no seasonal or melatonin treatment effects were found in ALA and DPA (C18:3, C22:5, Figure 1C). EPA concentrations also did not differ between summer and winter samples but were consistently lower in melatonin treated animals (C20:5, Figure 1C), in contrast to the pattern found in SR PL (C20:5 Figure 1B). The LA concentration was higher in mitochondrial PL in winter than in summer, as for SR PL. There was no increase of LA in mitochondrial PL in summer samples after melatonin treatment, unlike in SR PL (C18:2, cf. Figures 1B and 1C). Winter concentrations of AA were higher in mitochondrial PL than summer concentrations, similar to SR PL, but the melatonin treatment did not increase the summer concentrations and even led to a decrease in winter samples (C20:4, Figure 1C). Lastly, DHA concentration in mitochondrial PL were higher in winter compared to summer controls and intermediate in summer samples after the melatonin treatment (Figure 1C, C22:6).

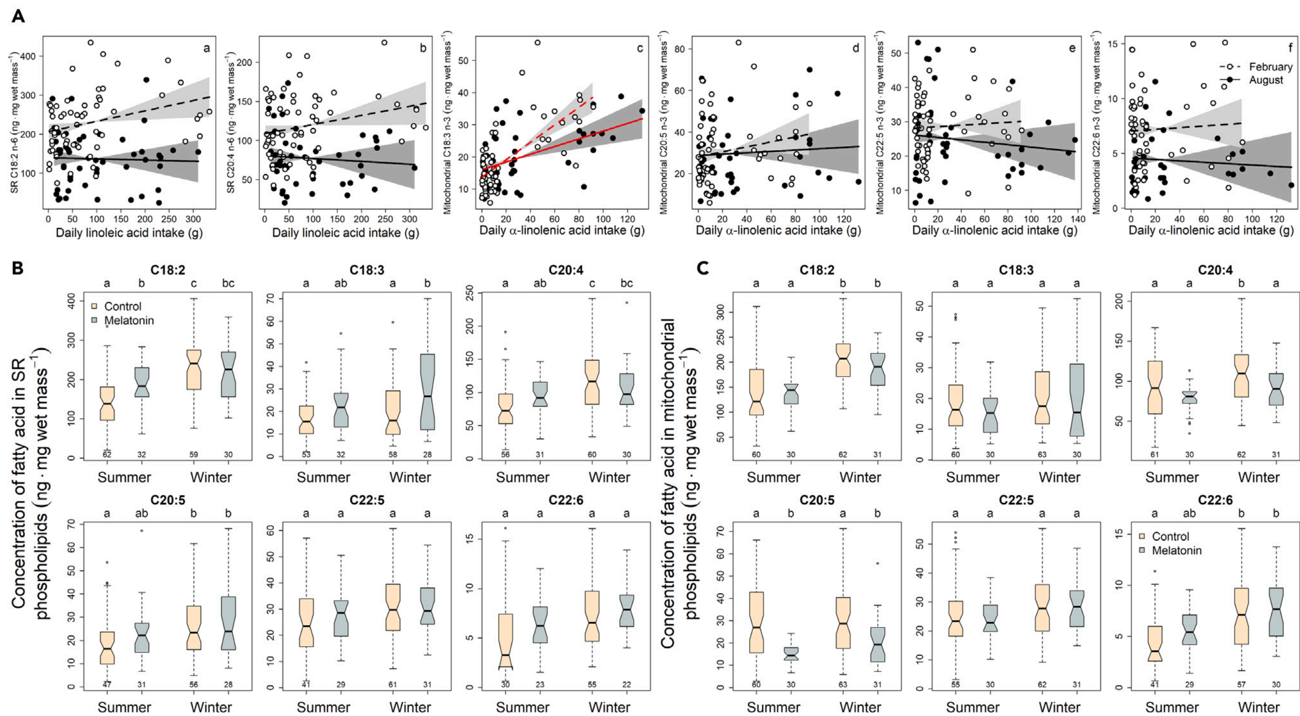

Figure 1. The influence of dietary intake of LA and ALA and of photoperiod on PUFA concentrations in phospholipids

(A) Relations of n-6 PUFA concentration in SR phospholipids with daily intake of LA (a-b), and of n-3 PUFA concentrations in mitochondrial phospholipids with daily intake of ALA (c-f). Intake data shown are means of daily intakes during 13 days of unchanged feeding regime and oil supplementation prior to a biopsy. Open symbols and dashed regression lines indicate samples from February, closed symbols and sold regression lines indicate August samples. Red regression lines indicate significant slopes with $p < 0.05$, shaded areas 95% confidence intervals of regression lines. For statistical details, see text.

(B) Boxplots of concentrations of PUFA identified in SR phospholipids in biopsy material sampled in February (Winter), and August (Summer). Bisque: Samples taken before implanting melatonin depots. Azure: Samples taken from the same individuals after implanting melatonin depots. Different letters above each graph indicate differences between groups with $p < 0.05$ (Tukey-like post-hoc multiple comparison), numbers above abscissas are sample sizes.

(C) Boxplots of concentrations of PUFA identified in mitochondrial phospholipids in biopsy material sampled in February (Winter), and August (Summer). Bisque: Samples taken before implanting melatonin depots. Azure: Samples taken after implanting melatonin depots. Different letters above each graph indicate differences between groups with $p < 0.05$ (Tukey-like post-hoc multiple comparison), numbers above abscissas are sample sizes.

Expression of key metabolic enzymes

All enzyme genes tested had higher expression levels during winter compared to summer (Figure 2A). The melatonin experiment suggested photoperiodic control of these seasonal differences. Except for COX, relative gene expression values in summer samples after the application of melatonin were intermediate between the high winter and low summer levels without melatonin treatment; for HOAD the summer values after melatonin treatment were already as high as in winter samples (Figure 2A).

All gene expression levels correlated positively with the activities of the respective enzyme, but statistically significant only for CS and HOAD (Figure 2B, SERCA: $F_{(1,164)} = 3.4$, $p = 0.0670$; COX: $F_{(1,139)} = 1.0$, $p = 0.3195$; CS: $F_{(1,105)} = 14.4$, $p = 0.0002$; CPT1: $F_{(1,60)} = 2.5$, $p = 0.1173$; HOAD: $F_{(1,79)} = 4.1$, $p = 0.0475$).

Influences of fatty acid composition of phospholipids on enzyme activities

For analyses of associations between the concentrations of specific PUFA in PL and enzyme activities, we used all samples, *i.e.*, those taken before and after melatonin treatment, to test whether such relations existed in principle. In each statistical model, predictors included were: the logarithmized PL concentration of the respective PUFA, the logarithmized expression level of the respective gene to adjust for differences in gene expression, and the logarithmized RNA concentration in a sample to adjust for its influence on relative mRNA values.

Sarcoplasmic reticulum Ca^{++}-ATPase

Independent of the level of relative SERCA1 gene expression, the total activity of all SERCA isoforms correlated positively with the concentrations of the two n-6 PUFA LA and AA in SR PL (effect of C18:2, $F_{(1,155)} = 17.9$, $p < 0.0001$; of C20:4, $F_{(1,149)} = 8.9$, $p = 0.0033$, Figure 3A), whereas statistically significant relations were absent for all n-3 PUFA (Figure 3A). However, all n-3 PUFA also correlated positively with SERCA activity adjusted for relative gene expression, in the case of DHA with borderline statistical significance (C22:6, $F_{(1,105)} = 3.3$, $p = 0.0736$, Figure 3A).

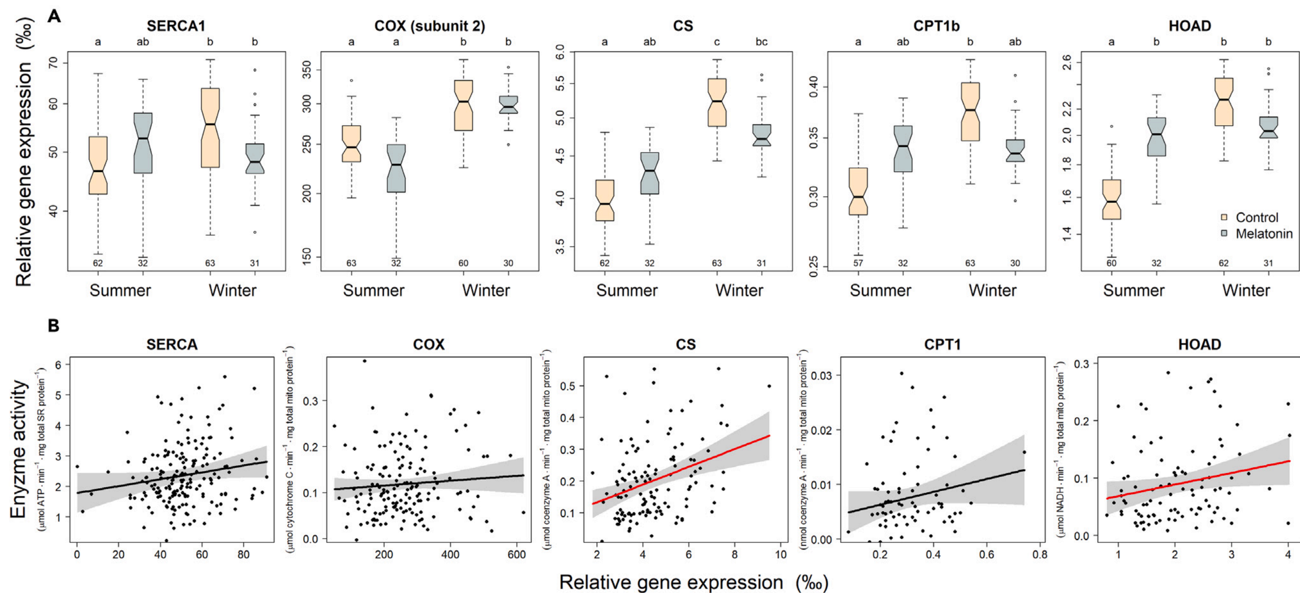

Figure 2. Relative gene expressions during summer and winter and their relations to enzyme activities

(A) Boxplots of relative gene expressions of key metabolic enzymes investigated in biopsy material sampled in February (Winter), and August (Summer). Bisque: Samples taken before implanting melatonin depots. Azure: Samples taken after implanting melatonin depots. Different letters above each graph indicate differences between groups with $p < 0.05$ (Tukey-like post-hoc multiple comparison), numbers above abscissas are sample sizes.

(B) Partial relations of relative gene expression, adjusted for logarithmized total RNA concentration, with the activity of the respective enzyme. Red regression lines indicate significant slopes with $p < 0.05$, shaded areas 95% confidence intervals of regression lines. For statistical details, see text. Note that SERCA activity represents total activity of all SERCA isoforms.

Mitochondrial enzymes

Cytochrome c oxidase. COX activity correlated significantly with the concentrations of two n-3 PUFA in mitochondrial PL, positive with EPA (partial effect of C20:5, $F_{(1,133)} = 6.8$, $p = 0.0102$, Figure 3B), and negative with ALA (C18:3, $F_{(1,132)} = 5.3$, $p = 0.0232$, Figure 3B)

Citrate synthase. The activity of CS was significantly associated only with the concentrations of EPA in mitochondrial PL (C20:5, $F_{(1,99)} = 4.1$, $p = 0.0445$, Figure 3C).

Carnitine palmitoyltransferase I. Again, only one of the six PUFA concentrations in mitochondrial PL quantified showed significant relations with CPT1, an enzyme of the β-oxidation pathway. The correlation with DHA was negative (C22:6, $F_{(1,46)} = 5.4$, $p = 0.0242$, Figure 3D).

Hydroxyacyl-coenzyme A dehydrogenase. For HOAD, the second enzyme of the β-oxidation pathway investigated, we found no statistically significant relations with the tested concentrations of PUFA in mitochondrial PL.

DISCUSSION

Our results demonstrate for the first time in a non-hibernating, large mammal profound cellular changes as part of the changes from a summer into a winter phenotype and vice versa, so far only known to occur in hibernators and daily heterotherms, but recently also reported for a non-hibernating bird.[47] Differential expression of key metabolic enzymes during summer and winter and particularly the seasonal changes of PUFA concentrations in PL increasing the activity of key metabolic enzymes during winter are the major findings of our study. Both molecular changes demonstrated may be prerequisites for a thrifty life at lower T_b during winter,[22,48] because they can compensate for Arrhenius effects,[49] that is a slower rate of biochemical reactions at lower temperatures catalyzed by the respective enzymes.

Control of molecular seasonal acclimatization

A potential cue governing the seasonal changes reported here could have been tissue temperature, as in ectotherms.[12,50] We took muscle biopsies for biochemical studies from superficial layers of the *Musculus semitendinosus* where peripheral T_b is known to be considerably lower during winter in large mammals living in seasonal environments, despite a better insulating winter fur.[22,37,38,51] In our study animals, subcutaneous T_b also changed throughout the year with lower winter values (annual amplitude about 1°C), and a course paralleling both ambient temperature and core T_b.[48] However, experimental flooding of the study animals with circulating melatonin from around summer solstice

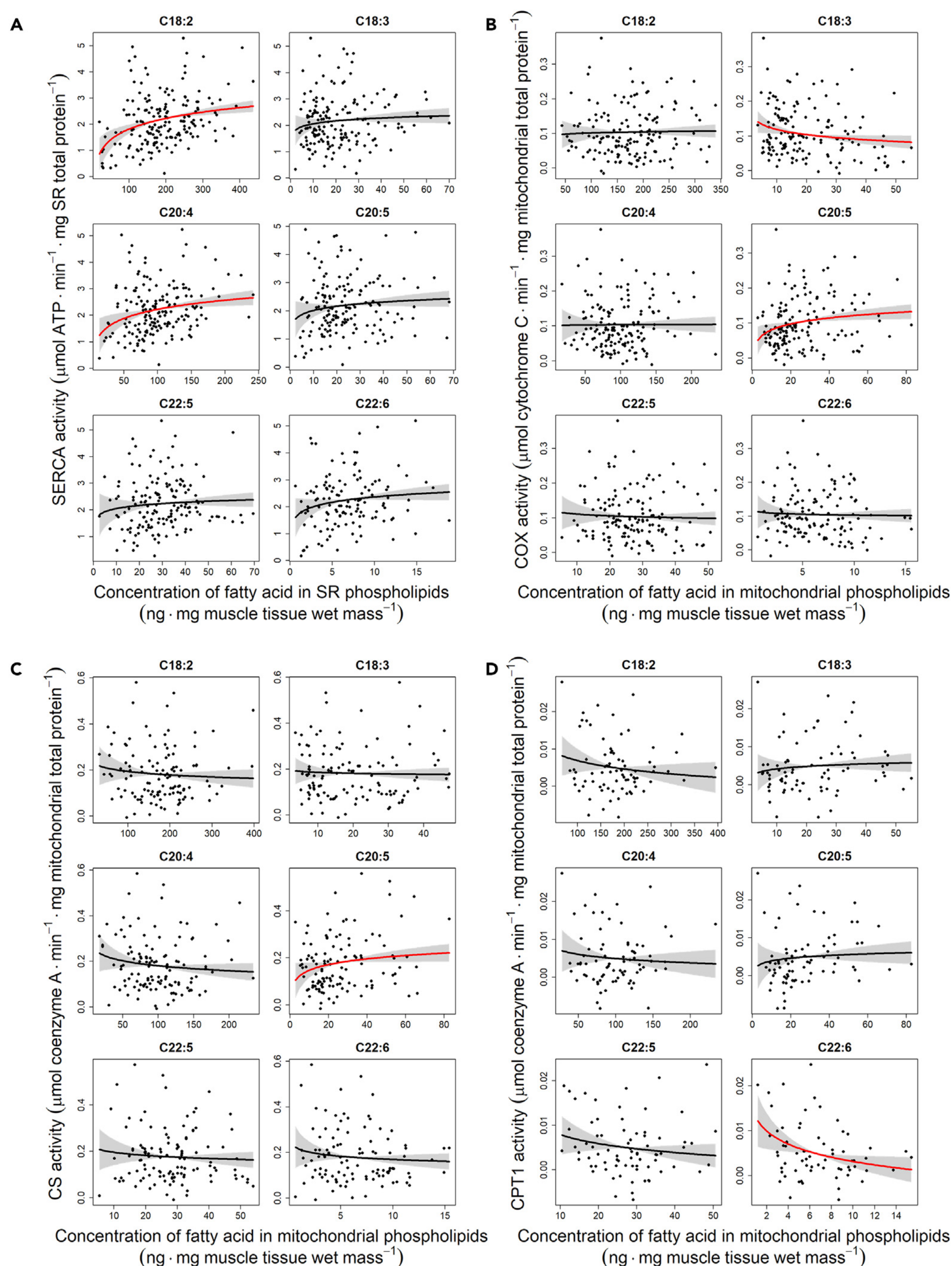

Figure 3. The relations of PUFA concentrations in phospholipids and enzyme activities

(A) SR phospholipids and the total activity of all SERCA isoforms, (B–D) mitochondrial phospholipids and the activity of COX (B), CS (C), and CPT1 (D). Values shown are adjusted for relative gene expression of the respective gene. Red regression lines indicate significant slopes with $p < 0.05$, shaded areas 95% confidence intervals of regression lines. For statistical details, see text.

onwards caused increases of the relative expression levels toward higher winter levels as early as August for all enzymes investigated, except COX, and of PUFA with lower summer PL concentrations (SR PL: LA, AA, EPA; mitochondrial PL: DHA), despite simultaneously peaking subcutaneous T_b.[48] A tendency back to lower levels already during winter was evident in all relative expression levels of genes, again except for COX, and in SR concentrations of LA and AA. Such earlier reversion to the summer phenotype of melatonin-treated animals has already been described for organismic traits of the study animals,[48] and seems to reflect photorefractoriness. The continuous administration of high amounts of melatonin mimics constant photoperiod that is known to dissociate the interaction between a circadian-based,

Table 1. Design of the experiment

	Precondition period			Experimental feeding of LA or ALA supplemented pellets																																
4 deer	a	r	a	*a*	*r*	***a***	*r*	*a*	*r*	*a*	*r*	***a***	*r*	*a*	*r*	a	a	**r**	a	r	a	r	a	**r**	a	r	a	*a*	*r*	***a***	*r*	*a*	*r*	*a*	*r*	***a***
4 deer	r	a	r	*a*	*a*	***r***	*a*	*r*	*a*	*r*	*a*	***r***	*a*	*r*	*a*	a	r	**a**	r	a	r	a	r	**a**	r	a	r	*a*	*a*	***r***	*a*	*r*	*a*	*r*	*a*	***r***
4 deer	*a*	*r*	*a*	a	r	**a**	r	a	r	a	r	**a**	r	a	r	*a*	*a*	***r***	*a*	*r*	*a*	*r*	*a*	***r***	*a*	*r*	*a*	a	r	**a**	r	a	r	a	r	**a**
4 deer	*r*	*a*	*r*	a	a	**r**	a	r	a	r	a	**r**	a	r	a	*a*	*r*	***a***	*r*	*a*	*r*	*a*	*r*	***a***	*r*	*a*	*r*	a	a	**r**	a	r	a	r	a	**r**
Month	M	A	M	J	J	**A**	S	O	N	D	J	**F**	M	A	M	J	J	**A**	S	O	N	D	J	**F**	M	A	M	J	J	**A**	S	O	N	D	J	**F**
Year	2017										2018												2019												2020	

a=*ad lib* feeding, r=restricted feeding; italics, shaded=pellets submerged in sunflower seed oil (high LA), standard font=pellets submerged in linseed oil (high ALA); bold=months of muscle biopsies. Double-line frame: Period with subcutaneous melatonin implants.

melatonin-dependent timer driving the initial photoperiodic response and a non-circadian-based timer driving circannual rhythmicity, thus leading to the apparent refractory state.[52] Altogether, the melatonin experiment clearly demonstrated photoperiodic control for all identified features of seasonal acclimatization at the molecular level, and the simultaneous recording of subcutaneous T_b excluded tissue temperature as the trigger for these changes.

In line with the view of an active regulation of the PL composition in different tissues,[53] we can further exclude direct effects of experimental feeding of EFA on the seasonal changes of PUFA concentrations in PL. Average daily intake of EFA during the 13 days prior to a biopsy did not influence the concentration of LA in PL and only weakly the concentration of ALA. The accumulation of ALA in PL with increasing intake and the lack of a similar trend for dietary LA was surprising because the opposite was to be expected according to a bias in our EFA supplementation. Despite adding equal amounts of sunflower seed and linseed oil the food pellets contained relatively more LA than ALA, because the base pellet contained about four times more LA than ALA. Immersing the base pellet in sunflower seed oil resulted in an LA content a third higher compared to the immersion in linseed oil with a quantity of oils of 10% w/w, and a half higher when immersing in 5% w/w. Conversely, the ALA content after immersing the base pellet in linseed oil was 20 times higher compared to immersion in sunflower seed oil when immersing in 10% w/w, and still 10 times higher when adding oils with 5% w/w. As a result, the variation of daily intake created by experimental manipulation of pellet availability and seasonal variation of voluntary food intake[44] was in the range of 2–333 $g \cdot day^{-1}$ for LA but only in the range of 0.3–137 $g \cdot day^{-1}$ for ALA.

Another source of error was the availability of natural forage. Intake from vegetation in the enclosure peaks in autumn.[44] A preferred food of red deer in autumn are beech and oak nuts. These seeds are rich in LA[54] and the higher winter concentrations of LA in PL may, at first glance, result from a large intake of beech and oak nuts during autumn, particularly in the second year of study when we had a mass production of seeds by the beech and oak trees in our study enclosure.[48] Nevertheless, this scenario is unlikely, because the same trend of higher winter values was found for DHA, a derivate of ALA which is a typical component of chloroplasts and thus green plant parts.[54] ALA from natural food is therefore far less available during autumn and winter. The most convincing result rebutting natural food intake as cause for higher winter values of LA however, is again provided by the melatonin experiment. Increased concentrations of LA in SR PL approaching the higher winter values were present already in August after melatonin treatment. Therefore, photoperiodic induction of biochemical pathways responsible for the remodeling of the acyl composition of PL is the most likely explanation for the phase advance of seasonal molecular changes caused by the experimental administration of melatonin from around the summer solstice onwards.

Functional aspects of seasonal changes of expressions and activities of key metabolic enzymes

All enzymes investigated had higher expression levels in winter. The same can be assumed for other enzymes not analyzed but responsible for chain elongation and desaturation of LA and ALA because of higher winter concentrations of AA and EPA in SR PL, and of DHA in mitochondrial PL. At first glance, increased expression of key metabolic enzymes during winter is surprising, given that the metabolism of red deer is simultaneously reduced with resting heart rates of about only half of the summer level, a feature rendered possible by tolerance of lower T_b and lower locomotor activity.[22,39,44,48] However, a higher capacity of the metabolic machinery does not necessarily translate into higher energy expenditure but may be a prerequisite for a life at lower T_b during winter. With higher expression levels, the organism may compensate for the reduction of enzymatic activity due to Arrhenius effects. Higher expression levels of key enzymes may be necessary to maintain sufficient muscle capability despite lower muscle temperature, for example for flight reactions. A similar phenomenon has been described for red deer with respect to gut function. Important parts of the digestive system like the rumen, liver and kidney shrink during winter, most likely to minimize the energetic costs of maintaining unneeded organ tissue due to reduced appetite and food intake.[44] Nevertheless, the capacity for active peptide and glucose transport across the small intestine epithelium is even higher during winter indicating augmented expression of the responsible transporter proteins to exploit the lower amount of feed consumed more efficiently.[44]

The detected correlations between enzyme activities and relative gene expressions were rather weak, presumably due to biological and methodological factors known to influence these correlations (reviewed in[55]). Transcription and translation do not demonstrate a simple linear relationship. Mechanisms such as *cis*-acting and *trans*-acting generate a range of translational outcomes that affect the enhancement or

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repression of protein synthesis from a given copy number of mRNA molecules. Thus, many factors may contribute to uncouple transcription and translation under certain conditions or even in a consistent manner.[55] In our data, there are at least three contributing issues: (1) both quantities are measured with different units. Gene expression values are concentrations of copies of mRNA of a target gene relative to the concentrations of copies of a reference gene whereas assays determining enzyme activities measure maximal possible activities. (2) For SERCA and CPT only a single isoform, and for COX only a single subunit was quantified with our mRNA analyses, whereas the preparations used for measuring enzyme activities contained more isoforms. For instance, although SERCA1 is the dominating form in fast-twitch skeletal muscle,[21] and the dominating fiber type of the sampled *Musculus semitendinosus* is indeed fast-twitch,[56–58] we undoubtedly had some degree of other isoforms in the SR preparations such as SERCA2a. Similarly, relative expression levels of CPT1b and of subunit 2 of COX are also only proxies of the protein concentrations of CPT1 and COX in the preparations of mitochondria. Finally, (3) in the case of CPT1 both relative gene expression and enzyme activity may have been too low for detecting an association.

Functional aspects of seasonal changes of the acyl composition of phospholipids

For all enzymes tested, except for HOAD, we found some of the expected correlations between changes of the PUFA concentrations in PL and enzyme activity.

The activity of SERCA increased with higher concentrations of LA and AA in PL independent of the higher expression of SERCA1 in winter samples. While these relationships between both n-6 PUFA in SR PL with SERCA activity were in line with results from studies of hibernators, we did not also find the reported negative correlation with the concentration of DHA in SR PL,[14,15,25,59,60] instead a borderline significant positive relation. An explanation for this result could be a cone-shaped relation between the DHA content of SR and the activity of SERCA. The DHA concentration found in red deer SR were with on average of 6.9 ng•mg muscle wet tissue mass^{-1} (standard deviation (s.d.) 3.72ng), or 0.8% of total FA mass in SR PL (s.d. 0.45%), a magnitude lower than the values reported for hibernators.[14,15,25,59,60] Our estimates of the relative contributions of seasonally differing SERCA expression levels and FA concentrations in PL delivered a summer to winter increase of 0.5% when only different mRNA levels were modeled, but of 8.8% and 6%, respectively, when only LA or AA levels were modeled (for details how these values were assessed, see "quantification and statistical analysis"). However, for a valid interpretation of these estimates, considerate must be noted that the total activity of all SERCA isoforms present were measured in each sample, but only the expression level of a single isoform, SERCA1. If the expression of the second isoform occurring in skeletal muscle, SERCA2a, did not correlate well and positively with the expression of SERCA1, our estimates would be the more misleading, the higher the SERCA2a concentration was in a sample. However, as SERCA1 is the dominating isoform in skeletal muscle,[21] we believe it is safe to say that the overwhelming proportion of the total increase of SERCA activity from summer to winter was caused by the increase of n-6 PUFA in SR PL in our study animals.

The activity of mitochondrial enzymes identified were all a power of 10 or less lower than the activity of SERCA. Nevertheless, some of the expected influences of seasonal changes of n-3 PUFA concentrations in mitochondrial PL could be detected, although with very low effect sizes. The activity of the two indicators of mitochondrial ATP production investigated, COX and CS, increased with increasing concentration of EPA in mitochondrial PL. However, the estimated increase of CS activity from summer to winter attributable to EPA was only 2.7%, whereas the estimated increase attributable to higher CS mRNA was 9.4%. These numbers were similar for COX though even lower. Relationships of COX and CS activities with other PUFA concentrations in mitochondrial PL were either absent, or enigmatic such as the weak but statistically significant decrease of COX activity with the concentration of ALA in mitochondrial PL. In contrast to COX and CS, none of the positive relationships reported in the literature between concentrations of n-3 PUFA in mitochondrial PL could be found for the two investigated key enzymes of β-oxidation CPT1 and HOAD.[26–29] There was only one statistically significant relationship, the unexpected and thus also enigmatic negative correlation between the activity of CPT1 and the concentration of DHA in mitochondrial PL. While various molecular mechanism responsible for an influence of the membrane environment are discussed for transmembrane proteins like SERCA and COX,[13,61,62] it is less clear how a functional relationship between the PL environment and the not membrane crossing CS can exist. However, CS is located in the inner mitochondrial matrix. Such so-called 'matrix enzymes' are typically placed near the inner mitochondrial membrane,[63] or are even bound to it, as for CS.[64] This may explain why we found an association between EPA concentrations in mitochondrial PL and the activity of CS. However, in contrast to SERCA, the winter increase in activity of all mitochondrial enzymes investigated seemed to the larger degree be due to increased expression of these proteins and only to a lesser extent because of an altered bilayer environment.

Potential mechanism of seasonal molecular changes

The mechanism potentially underlying the changes reported here in the acyl composition of PL, and how these modifications could influence the activity of investigated enzymes, are reviewed in.[13] In a nutshell, remodeling of the PL FA composition is primarily accomplished by a diacylation/reacylation process known as the Lands cycle.[65–67] This biochemical pathway is responsible for the remodeling of about 50% of *de novo* synthesized phosphatidylcholine, the most abundant PL in mammals.[68] Of particular interest here are acyltransferases with preferences for those FA changing in PL from summer to winter. Candidates are lysophosphatidylcholine acyltransferase 3,[68] or glycerol-3-phosphate acyltransferase 1.[69,70]

The influence of an altered PL environment on membrane-bound enzymes presumably arises from the energy required to deform the surrounding bilayer during protein conformational changes. This energy is different according to altered biophysical properties of the membrane with respect to, for instance, a mismatch between the hydrophobic transmembrane domain of an enzyme and membrane thickness, the lateral pressure profile of the bilayer exerted to an enzyme, and membrane fluidity (the "homeoviscous adaptation" hypothesis[11,12]).

Conclusions

In the present study we demonstrate for the first time in a non-hibernating large mammal extensive seasonal changes of the expressions of key metabolic enzymes and of the PUFA content of phospholipids with effects on the activity of important metabolic enzymes, particularly SERCA. These results are of striking similarity to what has so far only been described for hibernators and small mammals exhibiting daily torpor during winter. Given the overwhelming evidence for comprehensive seasonal changes on the organismic level in large mammals,[48,71] we can, based on the results of this study, safely assume that similar fundamental differences between the summer and winter phenotype of seasonal organism exist in many biochemical properties of cells, and are controlled by photoperiod. This may also hold true for humans. Annual rhythmic changes of the PUFA content of human mucosa PL are known to exist,[72] albeit with individual phase relations indicating "free-running" of the underlying circannual rhythm due to weak or absent Zeitgebers in our modern lifestyle.[73]

Limitations of the study

The restricted muscle tissue mass obtainable via biopsy without harming the study animals was a problem for our study. We aimed to sample at least 200mg to be partitioned for mRNA and biochemical analyses. Some samples had to be discarded because of too much contamination with tendon, blood, or fat. Many samples contained less than the desired amount of muscle tissue impairing the reproducibility of our biochemical analyses and thus the number of utilizable results. For the mRNA analyses, we tried to correct for a potential bias caused by small sample mass by including the total amount of mRNA in a sample in our statistical models.[74]

However, the two major limitations of our study are: (1) the available data did not allow to elucidate potential mechanism of membrane remodeling, and (2) whether and how an altered FA composition of PL affects muscular heat production. The latter may be of particular importance for large mammals like red deer. There is a strong negative correlation between body size and the capacity of non-shivering thermogenesis (NST) through brown adipose tissue (BAT), predicting no thermal benefit of BAT in species above a body mass of 10kg.[75,76] Moreover, red deer may even lack functional UCP1, like many large eutherians.[77] However, NST also occurs in skeletal muscle as a by-product of SERCA activity.[78–80] ATP hydrolyzation by SERCA, necessary to transport Ca^{++} across the SR membrane, inevitably produces heat.[81] This heat production is augmented by the phenomenon of "slippage", the release of Ca^{++} ions bound to SERCA back into the cytosol instead into the SR lumen.[82,83] Slippage can be further augmented by the small peptide sarcolipin (reviewed in[84]), a member of the so-called "regulins" of SERCA.[85] With increasing futile cycling of Ca^{++} by SERCA an increasing amount of energy from ATP hydrolyzation is converted into heat. Therefore, a higher thermogenic capacity of muscle during winter could be achieved with more uncoupling of SERCA. The seasonal change of FA composition of PL may well contribute to the degree of uncoupling of SERCA, as suggested by Paran et al.[86] If so, a higher maximal SERCA activity is required to compensate for a higher degree of uncoupling in order to maintain sufficient Ca^{++} transport capacity in winter acclimatized muscle. Our results suggest that this is to some degree achieved by higher SERCA expression in winter, but mostly by boosted SERCA activity when surrounded by PL enriched with n-6 PUFA. However, for unraveling the role of membrane remodeling for muscular NST, in addition to maximum SERCA activity, it is necessary to measure the associated heat production, either directly, or indirectly by simultaneous measurement of Ca^{++} transport, and to identify the uncoupling agent.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

Conceptualization, W.A.; Methodology, all authors; Validation, W.A., A.H., and S.S.; Formal analysis, W.A. and K.G.; Investigation, K.G. and W.A.; Resources, all authors; Data Curation, K.G. and W.A.; Writing—Original Draft, K.G. and W.A.; Writing—Review and Editing, all authors; Visualization, W.A. and K.G.; Supervision, W.A.; Project Administration, W.A.; Funding Acquisition, W.A. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Skeletal muscle tissue	Repeated biopsies from *Musculus semitendiosus* of 16 red deer, this study	N/A
Chemicals, peptides, and recombinant proteins		
Tris (hydroxymethyl) aminomethan	Sigma	1.08387.500
DTT	Sigma	43819_1G
Imidazole	Sigma	I2399_100G
$MgCl_2$	Sigma	H26700_100G
NaN_3	VWR	1.06688_100G
PEP	Sigma	P7127_5G
ATP	Sigma	A3377_10G
EGTA	Sigma	03777_10G
Thapsigargin	Sigma	T9033_0,5MG
Lactate dehydrogenase	Sigma	L2500_5KU
NADH	Sigma	N4505_100MG
Calcium ionophore (A23187)	Sigma	C7522_1MG
$CaCl_2$	Sigma	21114_1L
Pyruvate kinase	Sigma	P1506-5KU
KCl	VWR	1.04936.0250
Sucrose	Sigma	84100.250G
Complete EDTA free protease inhibitor cocktail	Roche	11873580001
Bicine buffer	VWR	0149_100G
Palmitoyl CoA	Sigma	P9716
Carnitine	Sigma	C0283_1G
Bradford reagent	Sigma	B6916
BSA standard	Sigma	P0834
Triton X-100	Sigma	T8787
Chloroform	Sigma	32211_2,5L
Hexane	Sigma	32293_2,5L
2-propanol	Sigma	190764_2,5L
Diethyl ether	Sigma	32203_2,5L
NaCl (final 0.9%) in H_2O	VWR	1.06404.0500
Sulfuric acid	Sigma	1.01833.2500
Methanol	Sigma	34860_2,5L
13 FAME standards Supelco chain length 14-22	Sigma	Customer Mix
Tween	Sigma	P9416_50ML
RNAlater	Invitrogen	AM7021
Melatonin	Alfa Aesar	AAJ6245206
Silicone elastomer membrane	Dow Corning	7-4107
Silastic medical adhesive silicone	Dow Corning	Type A
Critical commercial assays		
Citrate synthase assay kit	Sigma	CS0720

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
HADH kit	Sigma	SPAACO01
Cytochrome C oxidase assay kit	Sigma	CYTOCOX1
CPT1 assay	Sigma	CS0720
RNeasy fibrous tissue mini kit	Qiagen, Hilden, Germany	N/A
Applied biosystems™ high-capacity cDNA reverse transcription kit	ThermoFisher Scientific	N/A
Deposited data		
Raw data	Phaidra	https://doi.org/10.34876/3x4p-8910
Oligonucleotides		
Primer for SERCA1 forward: CGG CCT CTG AGA TGG TGC TG, Reverse: CCG TCC GTC ACC AGG TTC AC, Primer: TCC GCT ACC TCA TCT CCT CCA ACG	EUROFINS	N/A
Primer for citrate synthase forward: ACA GGG CAA GGC GAA GAA CC, reverse: AGC TGT GCT AAC ACG CCC AG, primer: CAT CTC CGT CAT GCC GTA GTA CTG CA	EUROFINS	N/A
Primer for CPT1 forward: CAG CAC CTC CCG GAC AGC, reverse: GCC CAC TCC ACT CTT CCC C, primer: ATG TTG GAG GTC TTT GTT CAG GTG GTG C	EUROFINS	N/A
Primer for COX2 forward: CCG AAT GTT AGT CTC CTC TGA AG, reverse: GCA TTG TCC GTA ATA TAG ACC TG, primer: TCT TGA GCC GTA CCC TCT CTA GGA CT	EUROFINS	N/A
Primer for HOAD forward: GCC GCT GAA CAC ACA ATC, reverse: AGG CAC TGG GTT GAA GAA AT, primer: TCA CAA GCC TAG CCA ATT CCA CCA	EUROFINS	N/A
Primer for GAPDH rorward: CAA TGC CTC CTG CAC CAC CA, reverse: GAC ACG TTG GGG GTA GGC AC, primer: ATG ATC ACT TCG GCA TCG TGG AGG	EUROFINS	N/A
Software and algorithms		
R version 4.2.2	R Core Team, 2022	https://cran.r-project.org/bin/windows/base/
Droplet Digital™ PCR QuantaSoft software, version 1.2	Bio Rad	https://www.bio-rad.com/de-at/life-science/digital-pcr/qx200-droplet-digital-pcr-system/quantasoft-software-regulatory-edition
GC PostRun software lab solution light version, 5.99	Shimadzu	https://www.shimadzu.de/labsolutions-lcgc
Other		
Feeding station	Schauer, Prambachkirchen, Austria	N/A
Ear tag for red deer	Dalton, Bocholt, Germany	N/A
Red deer pellet	Raiffeisenverband Salzburg	Garant Trophy STTM Luzerne Apple
Linseed oil	Vetripharm	389026
Sunflower seed oil	Aro	AAA0000973894
Droplet digital PCR droplet reader	BioRad	N/A
Teflon glass mixer	VWR	432-0201
Centrifuge Heraeus Multifuge X1R	Thermo	75004250
Ultracentrifuge Optima L-100 XP	Beckman Coulter	LXP 10 H 26
Spectrophotometer	Metrohm Inula	Hitachi U-1900

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Spectrophotometer Multiskan GO	Thermo	N/A
96-well flat-bottom microplates	Nunc	CLS 3635
Solid phase extraction (SPE) cartridges Supelclean™ LC-NH2 SPE Supelco	Sigma	52636U
ZebronTM ZB-WAX capillary column 30 m x I.D. 0.25 mm with 0.25 μm	Phenomenex	7HG-G007-11
Blowpipe	Teledart GmbH&Co. KG, Westheim/Pfalz, Germany	B16
Medetomidine -HCL 2%, magistral formulation	Richter Pharma AG, Wels, Austria	N/A
Tiletamine-zolazepam	Virbac, Glattbrugg, Switzerland	Zoletil 100
Eye lubricant	Laevosan, Linz, Austria	Oleovit
16-gauge catheter	B.Braun Austria GesmbH, Maria Enzersdorf, Austria	Vasofix Safety, Ø 1.7mm, length 50 mm
Infusion NaCl 0.9%,	B.Braun Austria GesmbH, Maria Enzersdorf, Austria	N/A
Atipamezole	Richter Pharma AG, Wels, Austria	Narco Stop
biopsy needle	Climed GmbH, Vienna, Austria	KBM 6/10 muscle biopsy set
synthetic absorbable surgical suture material	SMI AG, Hünningen, Belgium	Surgicryl PGA,
Meloxicam	Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein; Germany	Metacam

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Walter Arnold (walter.arnold@vetmeduni.ac.at)

Materials availability

This study did not generate new unique materials.

Data and code availability

- All data have been deposited at Phaidra and are publicly available as of the date of publication. DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethical approval

The animal study protocol was approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna, in accordance with the University's guidelines for Good Scientific Practice and authorized by the Austrian Federal Ministry of Education, Science and Research (BMWFW-68.205/0021-WF/V/3b/2017).

Experimental animals and feeding

The study was conducted from March 2017 until March 2020 in a 45 hectar (ha, 45,000 m^2) large enclosure adjacent to the Research Institute for Wildlife Ecology, Vienna (48.22°N, 16.28°E, 360m above sea level), consisting of ∼39 ha mixed beech and oak forest and a ∼6 ha large meadow. A herd of red deer (*Cervus elaphus*), adult females, their progeny from several years and an adult stag, roamed freely in this enclosure.

Sixteen adult females, 2–9 years old at the beginning of the study, were selected as experimental animals. They received, in addition to natural forage available in the enclosure, pelleted food at an automated feeding station (Schauer, Prambachkirchen, Austria). Preceding the study, we trained the experimental animals to use this station regularly. A one-way gate system with swinging doors ensured that only a single animal could enter the feeding station at a time, thus preventing disturbance of food intake by conspecifics. An antenna mounted next to the

feeding trough recognized the transponder located in the ear tag of each experimental animal (Dalton, Bocholt, Germany). Thereafter, the station delivered the programmed type of food for a given individual in portions of 50g with a 30s pause between portion deliveries, registered the number of portions delivered, and stopped delivery if a programmed maximum amount per day was reached. Animals usually emptied the feeding trough before leaving the feeding station. Therefore, a reliable quantification of pellet consumption per visit was possible. Further, animals were weighed during each visit of the feeding station to the closest kg with a balance located in the floor in front of the feeding trough.

Experimental animals were randomly assigned to two feeding groups of eight individuals each, balanced by body mass (group A: 131 kg $\pm$ standard error of the mean [s.e.m.] 5.85; Group B: 137 kg $\pm$ 4.99; t-test: $t = -0.63$, $p = 0.54$), and social rank. Social rank was determined prior to the study by the outcome of 2904 observed agonistic encounters between experimental females (methodological details in[87]).

We supplied in the feeding station a commercially available red deer pellet (Garant Trophy STTM Luzerne Apple, Raiffeisenverband Salzburg), supplemented with either ALA or LA, by immersing pellets in 10% w/w linseed oil, or sunflower seed oil, respectively. After treatment, both types of pellets remained nearly iso-caloric (after immersion in linseed oil, content of crude fat 23.6%, of energy 17.4 $kJ \cdot g^{-1}$; after immersion in sunflower seed oil 21.8% and 16.7 $kJ \cdot g^{-1}$, respectively; energy contents calculated from Weender analysis and energy values given in refs. 88,89), but differed substantially in the content of EFA. Crude fat of pellets immersed in linseed oil consisted of 19.3% LA and 48.6% ALA, whereas crude fat of pellets immersed in sunflower seed oil consisted of 57.3% LA and 2.3% ALA (determined by gas chromatography, as described below). After one year of feeding these pellets, many experimental animals developed signs of obesity. We therefore reduced the amount of oils added to 5% w/w for the rest of the experiment. This reduced the content of crude fat in linseed oil immersed pellets to 7.9%, the crude fat content of LA to 27.7% and of ALA to 36.0%, whereas sunflower seed oil immersed pellets now contained 7.8% crude fat, with 52.9% LA of crude fat, and 3.6% ALA, respectively.

To ensure that each animal received both LA and ALA supplementation but remained at the same diet for a complete annual cycle, type of oil supplementation was switched every year in June (Table 1). Further, we applied a feeding regime reflecting periods of high and low food supply. We regularly alternated monthly periods of *ad lib.* and restricted feeding (Table 1). During periods of restricted feeding, an animal received a daily pellet ration of 20% of the amount of pellets consumed per day during the preceding *ad lib.* period. We knew from a previous study[39] that this level of food restriction does not cause excessive loss of body mass and does not spoil the motivation to visit the feeding station. For a summary of the design of the feeding experiment and sampling schedule, see Table 1.

METHOD DETAILS

Surgical procedures

All surgical procedures were performed under general anesthesia. For administering anesthesia drugs (intramuscularly (IM) via hand injection or blowpipe, KIT B16L, Teledart GmbH&Co. KG, Westheim/Pfalz, Germany), deer were called into a crate. The animals were trained preceding the study to approach on call and to tolerate handling by positive reinforcement. Anesthesia was induced with medetomidine (0.08–0.1 $mg \cdot kg^{-1}$; medetomidine-HCL 2%, magistral formulation, Richter Pharma AG, Vienna, Austria) and tiletamine-zolazepam (2.5–3 $mg \cdot kg^{-1}$; Zoletil 100, Virbac, Glat$T_b$rugg, Switzerland) Once immobilization was achieved, deer were positioned in lateral recumbency, their eyes lubricated (Oleovit Augensalbe, Laevosan, Linz, Austria) and covered by a blindfold. Oxygen was administered via nasal insufflation (3$l \cdot min^{-1}$) and a 16-gauge catheter (Vasofix Safety, Ø 1.7mm, length 50 mm, B. Braun Austria GesmbH, Maria Enzersdorf, Austria) was inserted into a jugular vein to provide a constant rate of crystalloid infusion (6 $ml \cdot kg^{-1} \cdot h^{-1}$; NaCl 0.9%, B.Braun Melsungen AG, Melsungen, Germany) throughout the procedure. Vital parameters and clinical parameters relevant during anesthesia (e.g., heart rate, peripheral oxygen hemoglobin saturation using pulse oximetry (SpO_2), respiratory rate and end-tidal carbon dioxide ($PE'CO_2$) concentration, rectal T_b, color of mucosa, reflexes) were continuously monitored. Anesthesia was terminated 60 minutes after induction by antagonizing medetomidine with atipamezole (5mg atipamezole$\cdot$mg medetomidine^{-1}; Narco Stop, Richter Pharma AG, Wels, Austria) administered IM. Each animal was kept under observation until fully recovered and observed over 24 hours for any signs of re-narcotization.

We sampled skeletal muscle tissue from every experimental animal four times a year via biopsy, twice in August and twice in February, with a two-week period in between. Samples were always taken between 10am and 4pm. Between subsequent biopsy events, feeding regimes were changed for each individual from *ad lib.* to restricted feeding, or *vice versa*. Muscle biopsies were taken from the *Musculus semitendiosus*, alternately from the left and right hind leg. The biopsy site was prepared according to standard surgical procedures and covered by sterile surgical drapes. A small incision was made into the skin's surface. Muscle tissue was extracted by biopsy needle punch (KBM 6/10 muscle biopsy set, Climed GmbH, Vienna, Austria). Samples were snap-frozen in liquid nitrogen and stored at −80°C for a maximum of 3 months before analysis. The muscle fascia and skin were closed by single button suture technique with synthetic absorbable surgical suture material (Surgicryl, Surgicryl PGA, SMI AG, Hünningen, Belgium). Additional preemptive post-surgical analgesia (0.5 $mg \cdot kg^{-1}$ Meloxicam; Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein; Germany) was provided IM. After the biopsy, animals were monitored, and the biopsy site was controlled daily.

Experimental application of melatonin

Each study animal received between May 27, 2019 and July 2, 2019 a subcutaneously implanted melatonin depot. Muscle samples taken before implantation from an individual were used as controls for samples taken after this individual received the implant (Table 1). Each depot (4 × 4.5cm) contained 1g melatonin (Alfa Aesar, AAJ6245206) enclosed in a silicone membrane (Dow Corning Silicone elastomer membrane,

7-4107), sealed with a silicone adhesive (Dow Corning, Silastic Medical Adhesive Silicone, Type A). Depots were surgically inserted into the subcutis under anesthesia, as described above, craniolateral of the *Manubrium sterni*.

We designed the implants to achieve levels of circulating melatonin in the experimental animals that were clearly beyond the peak winter values of 118pg$\cdot$ml^{-1} blood, reported for Iberian red deer (*Cervus elaphus hispanicus*)[46] for at least three months. We determined the necessary mass of the melatonin depot and the size of the implant from estimated total blood volume and assuming a half-life of subcutaneously administered melatonin in the blood of 30min, as reported for sheep.[90] The total blood plasma volume is in ruminants ~5% of the body mass,[91] corresponding to approximately 58% of the total blood volume.[92] Our experimental animals had a mean body mass of 149 kg before implantation of melatonin depots. Therefore, the estimated total blood volume was on average 12.84l.

Before administration, we determined *in vitro* the diffusion rate of melatonin through the silicone membrane. Four test implants were submerged for 39 days in a bath of Ringer's solution and placed at 37°C in a desiccator, and weighed on days 1, 18, and 39. A linear decrease of mass was found (final mass = initial mass – 0.68 mg$\cdot$day^{-1}, R^2 = 0.9895). With this *in vitro* data and the above outlined assumptions, the estimated concentration of circulating melatonin caused by the implants was 354 pg$\cdot$ml^{-1} blood over a period of at least three months.

Cell fractionation

Muscle biopsies, homogenized in ice-cold buffer (100mM Tris, 250mM sucrose, 600mM KCl, 0.5mM DTT, 4°C) using a Teflon glass mixer, were centrifuged at 1000g for 20min to remove cell debris and nuclear fraction. The supernatant was subjected to another centrifugation step (15000g for 20min) to remove subcellular components such as mitochondria, lysosomes and peroxisomes. The pellet was stored in 1M bicine buffer for analyses of activities of mitochondrial enzymes. Subsequently, after adding 600mM KCl to the supernatant and an additional centrifugation step at 200000 g at 4°C for 30min, the pellet containing SR microsomes with SERCA protein embedded in the microsomal membranes was re-suspended in 200μl of 250mM sucrose, flash-frozen in liquid nitrogen and stored at −80°C.

Measurement of enzyme activities

ATPase activity in the SR preparation was measured by a standard coupled enzyme assay according to the method previously described by Simonides and van Hardeveld.[93] The rate of ATP hydrolysis was calculated from spectrophotometric recording of NADH oxidation at 340nm (ε = 6.25 mM$^{-1}\cdot$cm^{-1}) in a standard reaction mixture containing 5mM imidazole (pH 6.9), 100mM KCl, 10mM MgCl2, 10mM NaN3, 0.5mM DTT, 1mM PEP, 5mM ATP, 125μl EGTA, 10mM CaCl2, 1μl pyruvate kinase, 1.5μl lactate dehydrogenase, 300mM NADH and 2mM calcium ionophore (A23187) at 37°C. The difference between ATPase activities recorded before and after adding 100nM thapsigargin, a specific inhibitor of all isoforms of the calcium pump,[94] was attributed to total activity of all SERCA isoforms in the preparation.

Activities of the mitochondrial enzymes CS, COX, CPT1, and HOAD were measured according to respective standard kinetic assays, initiated by addition of the mitochondria preparation.[27,28,95,96] CS and CPT1 activities were determined via spectrophotometric recording of the release of coenzyme A (CoASH) at λ = 412nm. COX activity was determined by oxidation of cytochrome C ($\Delta\varepsilon$ = 21.84 mM$^{-1}\cdot$cm^{-1}), HOAD activity by oxidation of NADH ($\Delta\varepsilon$ = 6.25 mM^{-1} cm^{-1}), both via spectrophotometric recording at λ = 340nm and λ = 550nm, respectively.

Enzyme activities are expressed for SERCA as μmol ATP hydrolyzed$\cdot$min^{-1}, for mitochondrial enzymes as nmol CoASH released$\cdot$min^{-1}, or as nmol CytC or NADH oxidized$\cdot$min^{-1}, respectively. To allow comparison of samples, all enzyme activities were standardized to mg total protein in a sample determined with the Bradford method.[97]

Analyses of phospholipids

Total lipids were extracted from the isolated SR microsomes or mitochondria following the procedure of Folch et al.[98] Briefly, after the addition of chloroform-methanol (2:1) and vortexing for 10 min, NaCl (final 0.9%) in H_2O was added, vortexed again, and centrifuged at 3000rpm for 5min. For the isolation of total PL by anion exchange chromatography according to Kaluzny et al.,[99] solid phase extraction (SPE) cartridges Supelclean™ LC-NH2 SPE (Supelco-Sigma/Aldrich) were pre-equilibrated with hexane ahead of sample application and eluted with methanol.

FA methyl esters of the SR and mitochondria were identified by comparing the sample retention times with those of standards (mixture of 13 FAME standards of chain length 14 to 22 (Sigma-Aldrich)). We detected 10 different FA with chain lengths of 16–22 and 0–6 double bonds. Further, we determined the quantity of each of the 10 FA in a sample by integration of the peak area using the GC PostRun Software (Shimadzu) and comparing it to the areas of analytical standards of known quantity applied ahead of each batch of samples analyzed. Concentrations of single FA are expressed as ng$\cdot$mg wet tissue mass^{-1}, obtained from muscle biopsy.

Quantification of mRNA

Approximately 20mg of muscle tissue from each biopsy were stored in RNAlater (Invitrogen, AM7021). These samples were kept at 4°C for at least 24 hours to allow the buffer to penetrate the tissue. Subsequent storage was then at −80°C until RNA was extracted with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was reverse-transcribed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Gene expression levels for all genes were analyzed from cDNA via Droplet Digital PCR (ddPCR™). Expression levels were estimated using probe-based assays on a QX200™ Droplet Reader (Bio-Rad) and analyzed using the Bio-Rad Droplet Digital™ PCR QuantaSoft software. Expression levels are given as the relative ratio of the concentration (copies$\cdot\mu$l^{-1}) of the assay target gene over the concentration of the reference gene glyceraldehyde-3-phosphate dehydrogenase.

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The logarithmized total RNA concentration found increased with the available wet tissue mass of muscle samples ($F_{(1,189)}$=8.1, $p = 0.0049$), as expected.[74] To account for the variability of relative expression values due to RNA concentrations in samples, we included the logarithmized total RNA concentration as co-variate in statistical models containing relative expression values as predictors. For all enzyme mRNA tested, the logarithmized total RNA concentration indeed correlated negatively with relative expression values.

No published assays were available for the analyzed SERCA1, CPT1b (main isoforms in skeletal muscle[21,100]), HOAD, COX (subunit 2) and CS. Therefore, we designed suitable primers and probes from the reference sequence NM_001204393.1 with the assistance of the NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs were first tested on high quality cDNA extracts with the resulting products amplified and Sanger sequenced. Sequences were then blasted against the NCBI nucleotide databank (blast.ncbi.nlm.nih.gov[101]) to verify amplification of the target gene.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses were carried out using R version 4.2.2.[102] Membrane FA concentrations, or of activities of enzymes presented, are means of duplicate measurements. We discarded all values with a coefficient of variation >0.2 of duplicate measurements. Further, we omitted outliers detected with $p < 0.05$ with Rosner's test from the R-package EnvStats.[103]

Data were analyzed with linear mixed models (lme)[104] with individual as random factor to account for repeated measurements. We transformed responses and/or predictors when this resulted in a decline of AIC>2. Normal distribution of residuals from statistical models was verified visually using histograms and qq plots. We report F statistics and p values from type III Anova. To test for differences in response variables between levels of predictors, we used Anova and Tukey-like post-hoc multiple comparison tests with the ghlt function (multcomp package[105]). For visualizing regressions, we used the R-package visreg.[106]

To estimate the relative contributions of higher winter expression of an enzyme and of seasonally different FA composition of PL on enzyme activity, we modified the statistical models underlying Figure 3. For each significant relation shown in Figure 3 we calculated one model without the predictor mRNA level of the respective enzyme, and a second model without the predictor FA concentration in PL. Using average values of the predictors in summer and winter, the first model predicted an average summer to winter difference in the activity of an enzyme when neglecting an influence of the concentration of the respective FA in PL. The second model predicted the magnitude of this difference, when neglecting the influence of seasonally different expression levels of the respective enzyme.