Effects of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation

Alexander R. Gerson*, Jason C. L. Brown, Raymond Thomas, Mark A. Bernards and James F. Staples

Department of Biology, University of Western Ontario, London, Ontario, Canada, N6A 5B8

*Author for correspondence (e-mail: agerson2@uwo.ca)

SUMMARY

Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were fed one of four isocaloric, isolipemic diets containing 16, 22, 35 or 55 mg linoleic acid (18:2*n*-6) per gram. Mitochondrial properties were compared between hibernating and summer active states, and between diet groups. As in other studies, state 3 respiration was significantly reduced in hibernation, but only in animals fed the 22 mg g^{-1} 18:2 diet. In the other diet groups, there was no difference in state 3 respiration between the hibernating and summer active groups. In the 22 mg g^{-1} 18:2 diet group, there was no difference in mitochondrial proton conductance between hibernating and summer active animals, again in agreement with earlier studies. However, for all other diet groups, mitochondrial proton conductance was significantly reduced during hibernation. Mitochondrial phospholipid fatty acids changed significantly with hibernation, including increases in unsaturation indices and *n*-6/*n*-3, but no differences were found among diet groups. Mitochondrial proton conductance in hibernation showed a positive correlation with the content of linoleic acid (18:2) and arachidonic acid (20:4) in mitochondrial phospholipids. Lipid peroxidation was higher in mitochondria from hibernating animals, probably due to higher unsaturation, but there was no effect of dietary 18:2 on this pattern. Despite the dietary effects on mitochondrial metabolism, all animals hibernated with no differences in bout durations, body temperatures or whole-animal metabolic rates among the diet groups. The reduced mitochondrial proton leak in the 15, 35 and 55 mg g^{-1} 18:2 diet groups might compensate for the inability to suppress respiration, permitting whole-animal energy savings over the hibernation season.

Key words: hibernation, proton leak, mitochondria, polyunsaturated fatty acid, PUFA, oxidative phosphorylation, oxidative damage, reactive oxygen species.

INTRODUCTION

Hibernation is an energy-saving strategy employed by many small mammals to cope with the low temperatures and decreased food availability associated with winter. For ground squirrels (*Spermophilus* spp.), a hibernation season comprises a series of bouts and might last from late summer to mid-spring. Within any bout, core body temperature (T_b) can fall to close to freezing, and the metabolic rate (measured as mass-specific oxygen consumption; $\dot{V}_{O_2}$) can reduce to <5% of euthermic levels. This profound metabolic suppression is readily reversible during spontaneous arousals (which occur approximately every 7–12 days), when $\dot{V}_{O_2}$ and T_b increase to euthermic levels in a matter of hours. Ground squirrels typically fast throughout the hibernation season, and they rely primarily on endogenous fat stores, accumulated during the summer, for energy.

During the summer, ground squirrels preferentially consume plants rich in polyunsaturated fatty acids (PUFA), which are incorporated into depot fats and membrane phospholipids, including the inner mitochondrial membrane (Frank, 1994; Geiser et al., 1994; Harlow and Frank, 2001; McMurchie et al., 1983). Mammals cannot synthesize PUFA – with the exception of 20:3 under extreme dietary restriction (Hulbert, 2003) – and must therefore obtain them from the diet. Diets supplemented with linoleic acid (18:2*n*-6) result in a higher proportion of animals entering torpor, lower torpor T_b and longer bout duration during hibernation or daily torpor in chipmunks [(*Eutamias amoenus* (Geiser and Kenagy, 1987)], deer mice [*Peromyscus maniculatus* (Geiser, 1991)] and golden-mantled ground squirrels [*Spermophilus lateralis* (Frank, 1992)]. These studies suggest that diets low in PUFA result in depot fats and

membrane phospholipids that would solidify at low T_b so that energy must be expended to maintain T_b at higher levels. It is widely assumed that depot fats must be fluid in order to be mobilized (Aloia, 1988; Gunstone, 1996), and the fluidity of membrane phospholipids is crucial for the proper function of membrane-associated enzymes, diffusion within membranes and the maintenance of transmembrane gradients. The evidence suggests that hibernating animals have more unsaturated fatty acyl chains within their membrane phospholipids (Aloia, 1988), so supplementation of dietary PUFA might be advantageous by ensuring phospholipid fluidity at low T_b, enhancing the ability of animals to withstand very low $\dot{V}_{O_2}$ and T_b in hibernation, prolonging bout duration and permitting greater energy savings.

Several studies have shown significant reductions in state 3 (phosphorylating) respiration in liver mitochondria isolated from hibernating ground squirrels compared with that of squirrels in the summer active state (Barger et al., 2003; Martin et al., 1999; Muleme et al., 2006). This metabolic suppression is readily reversible during arousal (Muleme et al., 2006), suggesting that it is actively and acutely regulated. The regulatory mechanisms remain unknown, but it is clear that substrate oxidation is inhibited between complex II and complex IV of the electron-transport chain (Gehnrich and Aprille, 1988; Muleme et al., 2006). Remodeling the phospholipid environment of the inner mitochondrial membrane (IMM) can influence the activities of membrane-associated enzymes such as cytochrome *c* oxidase (COX) (Paradies et al., 1993), with little change in membrane fluidity (Kraffe et al., 2007). Dietary PUFA manipulation might, therefore, also affect hibernation-induced

alterations of IMM phospholipids and any associated mitochondrial metabolic suppression, thereby altering whole-animal patterns of hibernation. One of the goals of this study, therefore, was to study the effects of dietary PUFA manipulation on mitochondrial respiration in hibernation.

A leak of protons across the IMM partially uncouples substrate oxidation from ATP synthesis and is thought to account for up to 20% of the mammalian standard metabolic rate (Brand, 1990; Brand et al., 1999; Rolfe et al., 1999). The mechanisms responsible for proton leak remain poorly understood, but proton leak decreases as temperature decreases (Chamberlin, 2004). Proton leak also appears to be correlated with the unsaturation of IMM phospholipid fatty acyl constituents (Brookes et al., 1998). For example, hypothyroid rats have liver mitochondrial membranes containing more unsaturated phospholipid fatty acyl components (Pehowich, 1999) and are inherently less permeable to protons (Brand et al., 1992; Brand et al., 2003). Another goal of this study, therefore, was to examine the effects of dietary PUFA on mitochondrial proton leak in hibernation and to determine how this might relate to the whole-animal effects of PUFA on hibernation performance.

Diets very high in PUFA ($40–75\,mg\,g^{-1}$) result in elevated hibernation T_b and shorter bouts (Frank and Storey, 1996; Frank and Storey, 1995). Each double bond in a fatty acid chain is a possible site for attack by reactive oxygen species (ROS), resulting in peroxidation. This is especially relevant to membranes of mitochondria because ROS are produced inevitably during oxidative metabolism. Moreover, ROS appear to be produced at very high rates during arousal from hibernation (Tøien et al., 2001), so hibernators fed very high dietary PUFA might be at risk of massive peroxidative damage to proteins, nucleic acids and lipids (Frank and Storey, 1996; Frank and Storey, 1995). Another goal of this study, therefore, was to assess how dietary PUFA affects the level of mitochondrial oxidative damage in hibernators.

MATERIALS AND METHODS
Animal care
Thirteen-lined ground squirrels [*Spermophilus tridecemlineatus* (Mitchill)] were obtained from TLS Research (Bartlett, IL, USA). Upon arrival, animals were housed individually in standard rat cages with corncob bedding and paper towel for nesting material at 23°C on a natural light cycle adjusted weekly. The photoperiod ranged from 15.13 h:8.47 h L:D in June to 10.25 h:13.35 h L:D in October. The summer active group was housed under these conditions from April until August, whereas, in October, the hibernating group was placed in an environmental chamber (see below). Temperature telemeters were surgically implanted in animals from both groups, as described by Muleme and colleagues (Muleme et al., 2006), with

3–4 weeks of recovery. Each of these two groups was further subdivided randomly into four diet treatments receiving 16, 22, 35 or 55 mg linoleic acid (18:2n-6) per gram of diet. The diets were designed to mimic those of similar studies where dietary PUFA altered hibernation patterns and whole-animal metabolic rates (Geiser and Kenagy, 1987; Geiser, 1991; Frank, 1992; Frank and Storey, 1995; Frank and Storey, 1996) and are within the range of natural *S. lateralis* diets [as calculated by Munro and Thomas (Munro and Thomas, 2004)]. Custom diets were formulated by Purina Mills Test Diet division (Richmond, IN, USA) and supplied by Ren's Feed (Oakville, ON, Canada). All diets were isocaloric, and total fat was kept constant between diets at 9%, as determined by ether extraction. An analysis of dietary fatty acids was performed by Test Diet and is shown in Table 1. All animals were fed *ad libitum* for at least six weeks before sampling in the summer active group, and for the duration of the pre-hibernation fattening period (6–7 weeks) for the hibernating group. In *E. amoenus*, similar feeding durations were sufficient to change the composition of adipose tissue and cardiac mitochondrial phospholipids to reflect dietary fatty acids (Geiser et al., 1994).

Animals were weighed weekly during regular cage changes. Summer active animals were sampled during the period of the annual cycle when they gain mass. In the hibernating group, mass stabilization in late October indicated that the animals were ready to hibernate. The animals were moved to an environmental chamber, and the temperature was reduced $3°C\,day^{-1}$ from 23°C until the chamber reached 5°C; the light cycle was adjusted to 2 h:22 h L:D. After one week under these conditions, food was removed to induce hibernation. The animals were monitored daily and all animals entered hibernation within 4 days. Sampling began after a minimum of 4 weeks after hibernation began, allowing bout cycles to become regular. Before sampling, animals were placed in a respirometry chamber for 24–36 h to monitor $\dot{V}_{O_2}$. Moving hibernating animals sometimes induced arousal, and, in such instances, animals were monitored until they entered torpor again, then sampled 36–48 h into the subsequent torpor bout. T_b and $\dot{V}_{O_2}$ were measured using telemetry and flow-through respirometry, as described previously (Muleme et al., 2006). Hibernation T_b was calculated by averaging all body temperatures below 10°C recorded during the torpor phase of the final bout before sampling.

Mitochondrial isolation and respiration
Summer active animals were euthanized with an intraperitoneal Euthanyl injection ($270\,mg\,ml^{-1}$; $0.5\,ml\,100\,g^{-1}$), which has been shown to have no effect on mitochondrial energetics (Takaki et al., 1997). To prevent arousal, hibernating animals were euthanized by cervical dislocation. Liver mitochondria were isolated with gentle homogenization in isotonic buffers, followed by differential

Table 1. Characteristics of diets used in this study

Diet 18:2 ($mg\,g^{-1}$)	16	22	35	55
Diet component				
Linoleic acid	16.3	22.1	35.0	55.0
Linolenic acid	1.1	1.4	2.2	3.3
Arachidonic acid	0.3	0.2	0.2	0.1
Total n-3	2.0	1.6	1.6	1.7
Total saturated	33.8	31.1	24.7	14.5
Total monounsaturated	34.0	32.0	26.5	17.5
Energy from fat ($kJ\,g^{-1}$)	3.39	3.40	3.40	3.40
Total energy ($kJ\,g^{-1}$)	14.99	14.95	14.95	14.99

Unless otherwise indicated, values are $mg\,g^{-1}$ of total diet composition. Total fat content was kept constant at 9% among all diets.

centrifugation, as described previously (Muleme et al., 2006). A mitochondrial suspension that was not utilized for respiration and membrane potential measurements was frozen at –80°C for subsequent analysis of lipid peroxidation and phospholipid composition.

The oxygen consumption of mitochondrial suspensions was measured using a temperature-controlled polarographic O_2 meter (Rank Brothers, Dual Digital Model 20, Cambridge, UK), as described previously (Muleme et al., 2006). Mitochondria (0.25 mg protein) were added to the 2 ml chamber at the beginning of each assay. Rotenone (5 µmol l^{-1}) was used to inhibit complex I of the electron-transport chain (ETC), and succinate (6 mmol l^{-1}) was used as the respiratory substrate. Once stable state 2 respiration rates were recorded, ADP (0.1 mmol l^{-1}) was added to initiate state 3 respiration. When all ADP was phosphorylated, but excess succinate remained, respiration stabilized at lower levels, and this state 4 respiration was recorded.

Mitochondrial proton leak
The kinetics of proton leak were assessed by measuring mitochondrial membrane potential ($\Delta\Psi_m$; an estimate of proton motive force, ΔP) and mitochondrial respiration simultaneously, while titrating with malonate (see below). We used tetraphenylphosphonium (TPP$^+$; a lipophilic ion that distributes across phospholipid bilayers in proportion to $\Delta\Psi_m$) and a TPP$^+$-sensitive electrode (World Precision Instruments, Sarasota, FL, USA). By measuring the extra-mitochondrial TPP$^+$ concentration, we calculated mitochondrial $\Delta\Psi_m$ using a modified Nernst equation, as described by Nicholls and Ferguson (Nicholls and Ferguson, 1992). We employed a TPP$^+$ binding correction factor of 0.16 (Marcinkeviciute et al., 2000) and a mitochondrial volume of 0.001 ml mg^{-1} protein, as described by Barger and colleagues (Barger et al., 2003). These assays were performed at 37°C, a temperature where significant differences in mitochondrial respiration are evident between the hibernating and summer active states (Muleme et al., 2006). Preliminary experiments showed that measuring the kinetics of proton leak at lower temperatures required a much larger quantity of mitochondria that would have precluded other measurements. A Clark-type oxygen electrode was used to measure oxygen consumption (see previous section). Output from the TPP$^+$ electrode was measured using a pH meter (Accumet AB15) in mV mode. Mitochondria (approximately 1 mg protein) were added to 2 ml assay buffer containing rotenone (5 µmol l^{-1}), nigericin (80 ng ml^{-1}) and oligomycin (1 µg ml^{-1}). At this point, TPP$^+$ and reference electrodes were inserted into the reaction cell of the oxygen electrode and allowed to stabilize. Before each assay, TPP$^+$ electrodes were calibrated with five equal additions of TPP$^+$ that raised the TPP$^+$ concentration by 1 µmol l^{-1} each. The kinetics of proton leak were assessed by first initiating nonphosphorylating respiration by the addition of succinate (5 mmol l^{-1}). Under these conditions, all succinate-supported oxygen consumption can be attributed to proton leak. After stable readings were recorded, respiration was sequentially inhibited by five 5 µl additions of malonate (200 mmol l^{-1}), a non-competitive inhibitor of succinate dehydrogenase, yielding malonate concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 mmol l^{-1}. The TPP$^+$-sensitive electrode output and oxygen consumption were allowed to stabilize following each addition. Finally, carbonylcyanide-4-trifluoromethoxyphenyl-hydrazone (FCCP) was added to uncouple mitochondria in order to correct for electrode drift (Fig. 1).

The relationships between respiration and $\Delta\Psi_m$ were graphed and compared among diet groups and between hibernating and
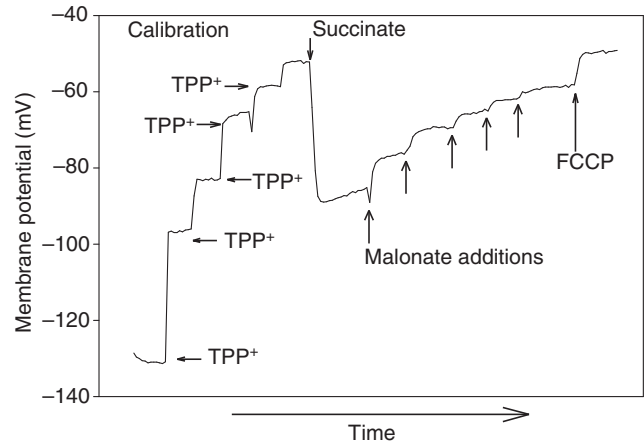

Fig. 1. Typical output from a TPP$^+$-sensitive electrode during calibration and the measurement of membrane potential of liver mitochondria isolated from a summer active ground squirrel. Mitochondria (1 mg protein) were incubated with rotenone (5 µmol l^{-1}), nigericin (80 ng ml^{-1}) and oligomycin (1 µg ml^{-1}) after which TPP$^+$ electrodes were calibrated by five additions of TPP$^+$, each of which raised [TPP$^+$] by 1 µmol l^{-1}. Addition of succinate initiated nonphosphorylating respiration, which was subsequently inhibited with five additions of malonate (0.5 µmol l^{-1}). Oxygen consumption was monitored simultaneously. FCCP was added at the conclusion of the assay to uncouple respiration completely, releasing unbound TPP$^+$ from the mitochondrial matrix and allowing for correction of electrode drift.

summer active groups, as described previously (Barger et al., 2003; Duong et al., 2006). The maximal membrane potential and non-phosphorylating respiration rates were compared between hibernating and summer active controls by a Student's t-test, and kinetic curves were compared by the overlap of standard error bars; the existence of no overlap was interpreted as a significant difference between curves. Proton conductance was calculated as described by Duong and colleagues (Duong et al., 2006), where a second-order polynomial was fitted to data for each individual in order to estimate the proton leak rate at a $\Delta\Psi_m$ of 130 mV, a value common to all treatments, except the 16 mg g^{-1} 18:2 diet summer active treatment, where some values were extrapolated to 130 mV when necessary. Two-way ANOVA was used to compare conductance among the diet treatments and between the summer active and hibernating groups.

Measurement of peroxidative damage
Mitochondrial samples, stored at –80°C, were used to determine the content of malondialdehyde (MDA), a secondary byproduct of lipid peroxidation. Samples were thawed only once, just before the assay to minimize the effects of freeze–thaw cycles on lipid peroxidation. A kit from OxisResearch (product number: MDA 586; Medicorp, Montreal QC, Canada) was used (Gérard-Monnier, 1997). Each mitochondrial sample (~2 mg protein) was incubated with *N*-methyl-2-phenylindole (concentration not reported by the kit manufacturer) and concentrated HCl at 45°C for 60 min. Probocol (10 µl, concentration not reported by the kit manufacturer) was added before incubation to prevent any further lipid peroxidation. Under these conditions, MDA in mitochondrial samples reacts with *N*-methyl-2-phenylindole, yielding a stable carbocyanine dye that has a maximum absorbance at 586 nm. After incubation, samples were centrifuged at 10,000 g for 10 min to obtain a clear supernatant, the absorbance of which was read at 586 nm.

COX activity

The activity of COX was measured using the CYTOCOX1 kit from Sigma-Aldrich. As COX is only found on the inner mitochondrial membrane, its activity can be used as an indicator of inner mitochondrial membrane surface area (Leary et al., 2003). The colorimetric assay is based on the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by COX. Mitochondria (approximately 0.4 mg protein) were sonicated, then added to a standard 1 ml cuvette with assay buffer (5 mmol l^{-1} Tris-HCl, pH 7.0, containing 125 mmol l^{-1} sucrose) to a final volume of 950 μl. To begin the reaction, 50 μl of ferrocytochrome c substrate solution (0.22 mmol l^{-1}) was added. The change in absorbance at 550 nm was read immediately. The activity of COX was calculated using an extinction coefficient of 21.84.

Phospholipid fatty acid analysis

Suspensions of isolated mitochondria (0.5–1.0 mg protein) were added to 15 ml 1:1 Folch solution (chloroform:methanol) to extract lipids. The samples were centrifuged at 1000g for 10 min and filtered through Whatman No. 1 filter paper. The residue was resuspended in 10 ml Folch 2:1, shaken and added to the Folch 1:1, after which 8 ml 6% KCl was added to separate polar compounds. Following a 10 min incubation at 70°C, the samples were allowed to cool and the aqueous layer was removed. The remaining organic layer was dried under nitrogen. Dried samples were resuspended either in 100 μl chloroform for separation or in 100 μl benzene:methanol 2:1 and frozen at –30°C under nitrogen for storage (Maillet and Weber, 2007).

Columns (Supelco, supelclean LC-NH$_2$ SPE 1 mL tubes, silica gel base, aminopropyl bonding) were conditioned with 2 ml hexane, after which the samples were added, followed by addition of 200 μl chloroform. Neutral lipids (NL) were separated by eluting with 1.8 ml chloroform:isopropanol (2:1); non-esterified fatty acids (NEFA) were eluted with the addition of 1.6 ml isopropyl ether:acetic acid (98:2). Phospholipids (PL) were eluted with 3 ml methanol and retained (Maillet and Weber, 2007). Both NL and NEFA were discarded.

PL were transesterified by the addition of 500 μl of BF$_3$ solution (14% in methanol), followed by a 24 h incubation at 60°C. The BF$_3$ was rinsed from the sample by three separate additions of 1 ml purified reverse-osmosis H$_2$O and 1 ml ethyl ether. After each addition, the aqueous layer was removed and discarded. The samples were evaporated under nitrogen, resuspended in 200 μl dichloromethane and transferred to chromatography vials for quantification.

Phospholipids were quantified with a Varian model CP-3800 gas chromatograph with a CP-Sil 5 low bleed MS (WCOT silica, 30 m×0.25 mm i.d.) column coupled to a Varian Saturn 2000 mass spectrophotometer (GC–MS) and flame ionization detectors to identify specific fatty acids. Samples (1 μl) were injected into the gas chromatograph and subsequently the GC–MS for analysis. Helium was used as the carrier gas (1.2 ml min^{-1}). The temperature program was as follows: 70°C held for 2 min, then increased to 240°C at 5°C min^{-1}, after which 240°C was maintained for 4 min.

The temperature was then increased to 300°C at 40°C min^{-1} to clear the column. Peak area counts were determined by calculating the integral of each peak using Varian Star Workstation software; the amount of different fatty acids in each sample was calculated using standard curves. The percentage of each fatty acid relative to total fatty acids detected in each sample was calculated and expressed as a mol%. Unsaturation index was calculated as in Eqn 1, where the mol% was multiplied by the number of double bonds in a fatty acid then summed for all fatty acids and this value was divided by 100 (Hong et al., 2002):

$$\text{Unsaturation index} = \Sigma(\text{mol\%})(\text{no. of double bonds}) / 100. \quad (1)$$

Statistics

Mitochondrial respiration rates were compared within diet groups using two-way repeated measures ANOVA with metabolic state (i.e. summer active or hibernating) and assay temperature as factors. Levels of MDA, and PL composition, were compared using two-way ANOVA with diet and metabolic state as factors. Student–Newman–Keuls pairwise comparisons were used when significant differences were found among diet groups. Hibernation T_b, $\dot{V}_{O_2}$ and bout duration were compared among diet groups using one-way ANOVA. Significant differences were determined if P values were ≤0.05. Pearson's product moment was used to explore potential correlations between the mol% of each fatty acid class detected and conductance at 130 mV, state 3 and state 4 respiration rates, Q_{10} and COX activity.

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

RESULTS
Whole animal data, mitochondrial respiration and COX activity

Hibernation T_b, bout duration and $\dot{V}_{O_2}$ (either summer active or hibernation) did not differ among the diet groups; however, hibernation did significantly reduce $\dot{V}_{O_2}$ by more than 95% (Table 2).

When fed the 22 mg g^{-1} 18:2 diet, state 3 respiration was significantly reduced by 41% and 35% in hibernators compared with summer active controls when measured at 37°C and 31°C, respectively (Fig. 2B). When measured at 25°C or 10°C, however, state 3 respiration did not differ significantly between the summer active and hibernating conditions. There were no significant differences in state 3 respiration between summer active and hibernation in any of the other three diet groups regardless of the assay temperature (Fig. 2A,C,D). No significant differences in state 4 respiration were found between the summer active and hibernating conditions, regardless of diet or assay temperature (Fig. 3). Q_{10} values for respiration were approximated between 37°C and 10°C. These values did not differ between the hibernating and summer active states for either state 3 (hibernation 2.09, summer 1.94) or state 4 (hibernation 2.10, summer 1.93), and there was no

Table 2. The effect of dietary PUFA on body temperature, pre-hibernation body mass, mass-specific metabolic rate ($\dot{V}_{O_2}$) and bout duration

Diet 18:2 (mg g^{-1})	Torpid T_b (°C)	Mass (g)	Bout duration (days)	Torpid $\dot{V}_{O_2}$ (μmol O$_2$ h^{-1} g^{-1})	Summer active $\dot{V}_{O_2}$ (μmol O$_2$ h^{-1} g^{-1})	Sample size (SA/HIB)
16	5.0±0.2	210.0±6.0	7.4±1.0	2.4±0.5	71.6±6.3	6/7
22	4.8±0.1	225.0±4.7	7.7±0.4	2.8±1.6	102.1±27.4	6/6
35	6.3±1.3	224.8±11.7	6.6±1.0	1.8±0.6	57.2±7.8	5/5
55	5.7±1.0	233.7±2.9	7.7±0.8	1.9±0.6	67.5±10.3	4/6

Values are means ± s.e.m. No significant differences were found among diet groups. SA, summer active; HIB, hibernating.
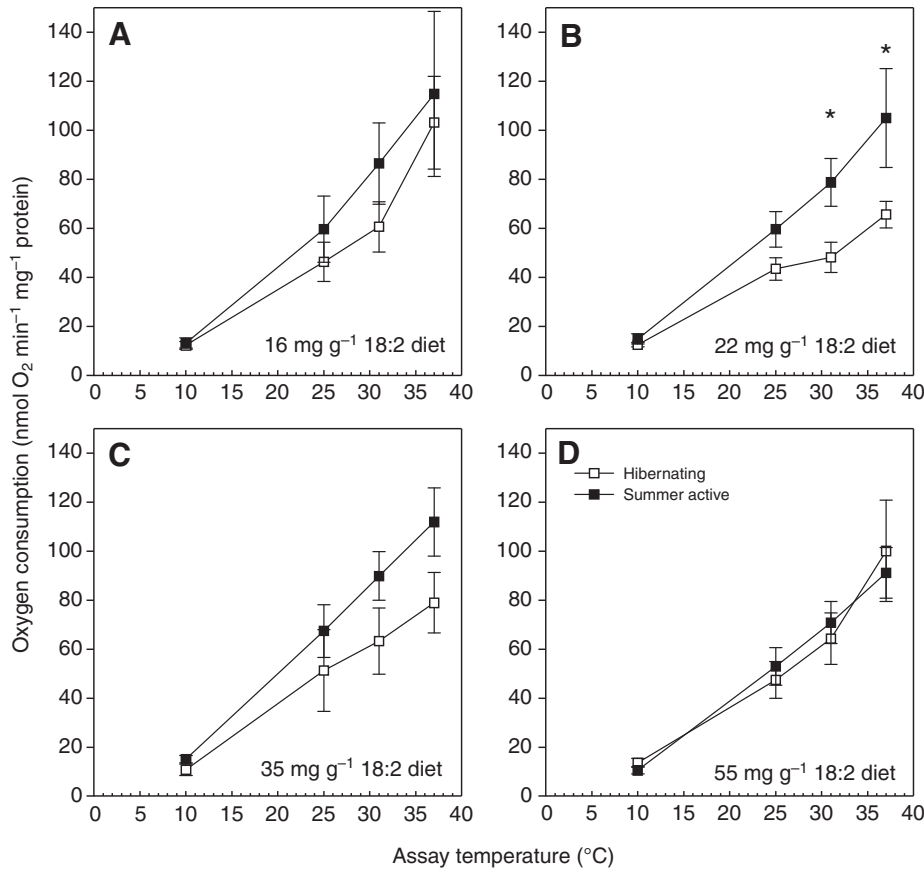
Fig. 2. The effect of temperature and dietary PUFA on liver mitochondrial state 3 respiration rates from hibernating and summer active *Spermophilus tridecemlineatus*. (A) 16 mg g^{-1} 18:2 (hibernating *N*=8, summer active *N*=6), (B) 22 mg g^{-1}18:2 (hibernating *N*=8, summer active *N*=6), (C) 35 mg g^{-1} 18:2 (hibernating *N*=8, summer active *N*=5) and (D) 55 mg g^{-1} 18:2 (hibernating *N*=6, summer active *N*=4). * indicates a significant difference between hibernation and summer active at the same assay temperature. Values are means ± s.e.m.
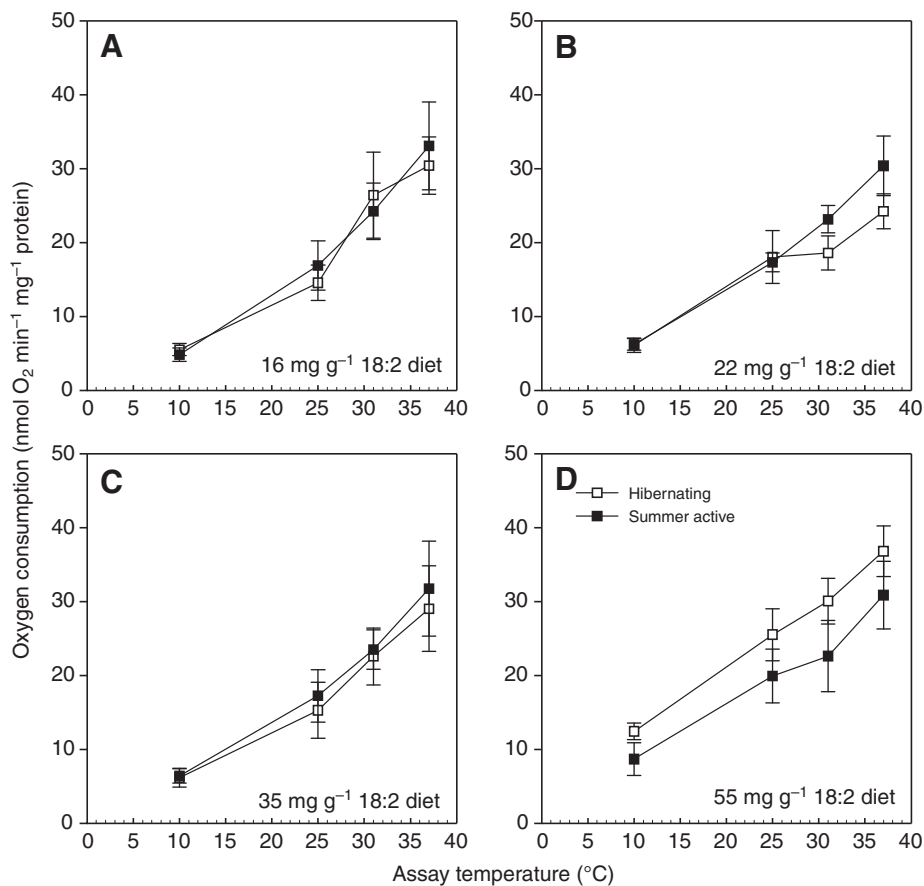

Fig. 3. The effect of temperature and dietary PUFA on liver mitochondrial state 4 respiration rates from hibernating and summer active *Spermophilus tridecemlineatus*. (A) 16 mg g^{-1} 18:2 diet (hibernating *N*=8, summer active *N*=6), (B) 22 mg g^{-1} 18:2 diet (hibernating *N*=8, summer active *N*=6) (C) 35 mg g^{-1} 18:2 diet (hibernating *N*=8, summer active *N*=5) and (D) 55 mg g^{-1} 18:2 diet (hibernating *N*=6, summer active *N*=4). No significant differences were evident. Values are means ± s.e.m.
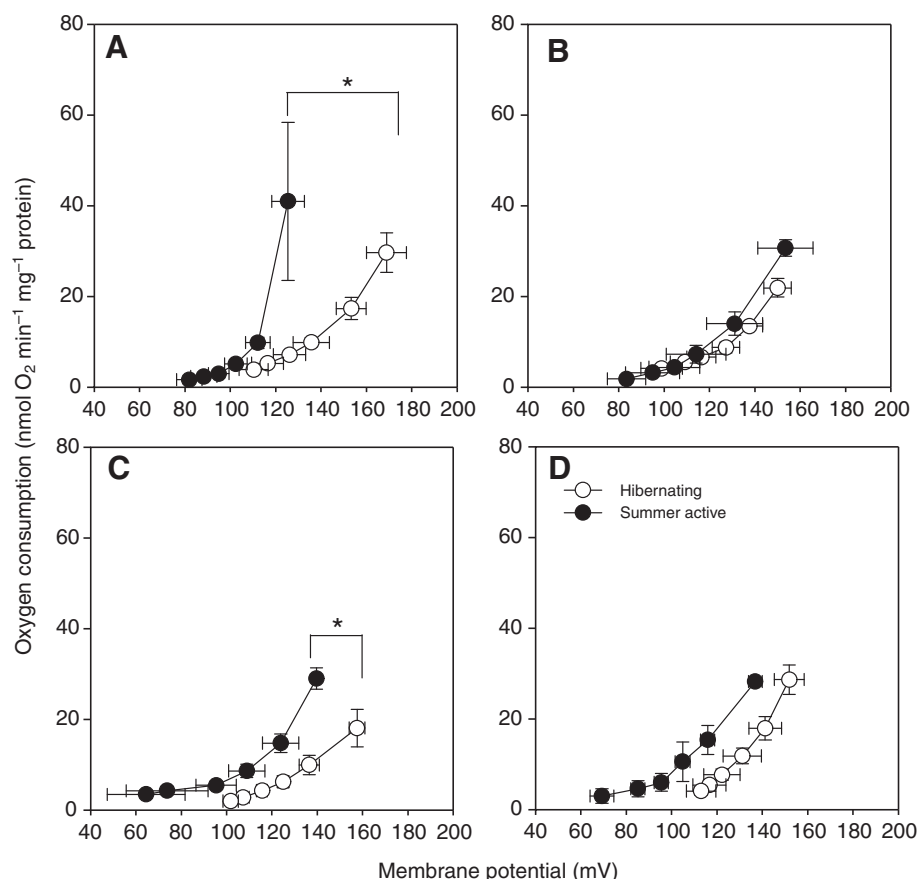
Fig. 4. The effect of hibernation and dietary 18:2 on the kinetics of proton leak in liver mitochondria. * indicates significant differences in maximal membrane potential (no malonate; the upper-right point of each curve). (A) 16 mg g^{-1} 18:2 diet (hibernating N=8, summer active N=6), (B) 22 mg g^{-1} 18:2 diet (hibernating N=8, summer active N=6), (C) 35 mg g^{-1} 18:2 diet (hibernating N=8, summer active N=5) and (D) 55 mg g^{-1} 18:2 diet (hibernating N=6, summer active N=4). Values are means ± s.e.m.

diet effect. Within each metabolic state (summer active and hibernating), no significant differences among diet groups in state 3 or state 4 respiration were found.

Table 3 shows COX activities of mitochondria from summer active and hibernating animals of each diet group. No significant differences occurred among diet groups. There was, however, a significant sixfold increase in COX activity with hibernation in the 55 mg g^{-1} 18:2 diet group.

Kinetics of proton leak

Proton leak kinetic curves (using respiration rates measured relative to mitochondrial protein) for hibernators were shifted down and to the right when compared with summer active animals fed the same diet, except the 22 mg g^{-1} 18:2 diet group (Fig. 4). Moreover, at a common membrane potential of 130 mV, liver mitochondria from hibernators had lower proton conductance than summer active controls in the 16, 35 and 55 mg g^{-1} 18:2 diet groups; no difference was found in the 22 mg g^{-1} 18:2 group (Fig. 5). When comparing proton leak among diet groups within hibernators and summer active controls, no significant differences were found. Within diet groups, maximal nonphosphorylating respiration rates (Fig. 4, uppermost point in curves) did not differ significantly between the summer active and hibernating states, but the maximum membrane potential was significantly higher

Table 3. The effect of dietary PUFA and hibernation on mitochondrial cytochrome c oxidase (COX) activity

Diet 18:2 (mg g^{-1})	16	22	35	55
Summer active	2.07±0.86 (6)	1.43±0.95 (6)	1.50±0.74 (5)	0.65±0.15 (4)
Hibernating	2.35±0.66 (8)	2.06±0.33 (8)	2.86±1.44 (8)	3.97±0.56* (6)

No significant differences were found among diet groups within summer active or hibernating groups. Hibernation resulted in a significant increase ($P<0.05$) in COX activity in the 55 mg 18:2 g^{-1} diet group (indicated by *). Values are means ± s.e.m. expressed as mU mg^{-1} protein. Sample size is indicated in parentheses.

Table 4. The effect of dietary PUFA and hibernation on malondialdehyde (MDA) levels in isolated liver mitochondria

Diet 18:2 (mg g^{-1})	16	22	35	55
Summer active	1.22±0.32 (6)	1.36±0.19 (6)	1.07±0.20 (5)	0.92±0.19 (4)
Hibernating*	2.99±0.58 (8)	2.72±0.46 (8)	2.70±0.30 (8)	2.46±0.618 (6)

Diet had no significant effect on the level of lipid peroxidation, but hibernating animals had significantly higher MDA levels (indicated by *; $P<0.05$). Values are means ± s.e.m. Values are µM MDA mg^{-1} protein. Sample size is indicated in parentheses.
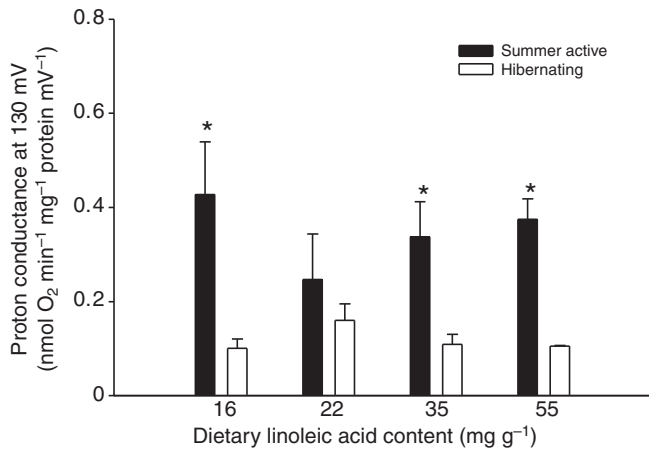
Fig. 5. Estimated liver mitochondrial proton conductance at a membrane potential of 130 mV. Proton conductance was determined as proton leak rate mV^{-1} at 130 mV. Two-way ANOVA showed a significant difference between summer active and hibernating animals (indicated by *). No significant differences were found among diet groups within the summer active and hibernating states. Values are means ± s.e.m. Number of individuals is the same as for Fig. 4.

in hibernation for the 16 and 35 mg g^{-1} 18:2 diets, and tended to be higher for the 55 mg g^{-1} 18:2 diet. However, in the 22 mg g^{-1} 18:2 diet, these membrane potential values were virtually

indistinguishable between the two states. The patterns described above were maintained when proton leak was measured using respiration rates standardized relative to COX activity (as an estimate of IMM surface area; data not shown).

Lipid peroxidation and mitochondrial phospholipid fatty acids
The levels of MDA were not affected by dietary 18:2, but hibernation resulted in an approximately twofold increase in this measure of lipid peroxidative damage (Table 4).

Table 5 shows the phospholipid fatty acids quantified from isolated mitochondria. Only a limited quantity of mitochondria remained after the other assays, so only the six most abundant fatty acids were in sufficient concentrations to be quantified by gas chromatography. As a result, the number of samples that yielded sufficient data was small, especially for the summer active animals. Palmitic acid (16:0) did not differ among diet groups for either hibernating or summer active animals. Stearic acid (18:0) decreased with hibernation, whereas oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4) showed significant increases with hibernation, but no differences among diets were found. Docosahexaenoic acid (22:6) showed significant increases with hibernation, but only in the 16 and 55 mg g^{-1} 18:2 diet groups. The unsaturation index was ~40% higher in the hibernation group, but there was no significant diet effect. Also, hibernation resulted in an increase in the n-6/n-3 ratio, with no significant differences among diet groups. Conductance at 130 mV correlated positively with levels of both linoleic acid (18:2; P=0.023, r^2=0.28) and arachidonic acid
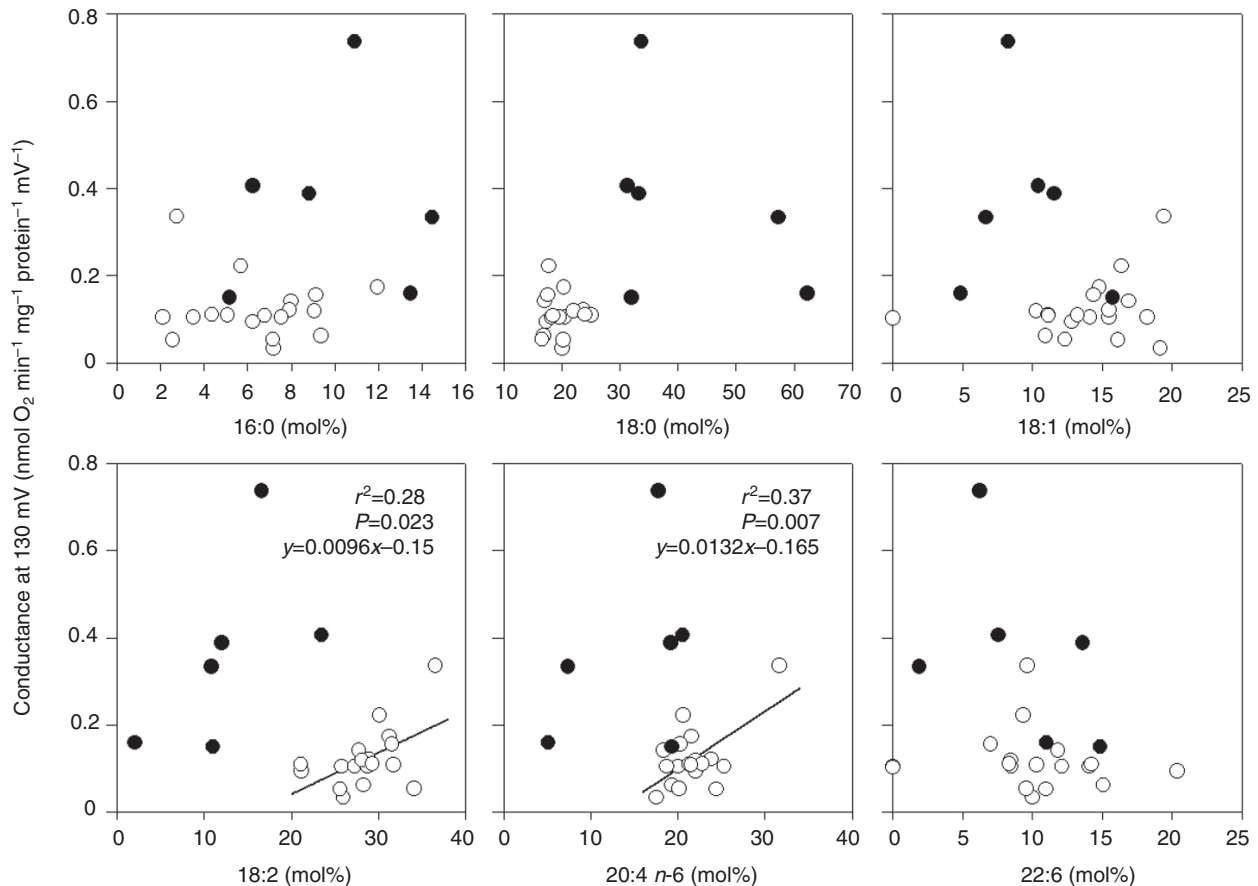

Fig. 6. Correlations between mitochondrial membrane conductance and the quantity of a specific mitochondrial phospholipid fatty acid. Unfilled circles indicate samples from hibernating animals; filled circles indicate samples from summer active animals. Lines represent a statistically significant relationship within hibernators.

(20:4; $P=0.007$, $r^2=0.37$) in mitochondrial phospholipids in hibernators but not in the summer active group (Fig. 6). There were no significant correlations between the phospholipid content of any fatty acid and state 3 and state 4 respiration rates, Q_{10} or COX activity (data not shown).

DISCUSSION

Although dietary 18:2 did not alter hibernation characteristics at the whole-animal level, mitochondrial function was affected by both diet and metabolic state. The 16, 35 and 55 mg g^{-1} 18:2 diets prevented the suppression of state 3 respiration in hibernation but resulted in reduced proton leak. The mechanism by which diet affected mitochondrial bioenergetics is not readily apparent because mitochondrial phospholipid fatty acid composition appeared to be affected more by metabolic state than dietary 18:2. The increase in PL fatty acid unsaturation in hibernation paralleled increased lipid peroxidation, although levels of this oxidative damage did not differ significantly among diet groups.

Whole-animal characteristics

Previous studies showed that diets containing either minimal (as low as 2 mg g^{-1}) or very high (up to 59 mg g^{-1}) linoleic acid resulted in fewer animals entering torpor or prevented torpor altogether (Frank, 1992; Frank and Storey, 1995). Additionally, Geiser and Kenagy (Geiser and Kenagy, 1987) demonstrated that diets enriched with unsaturated fatty acids resulted in lower torpor $\dot{V}_{O_2}$ and T_b in chipmunks (*Eutamias amoenus*). In the present study, no significant differences were found among diet groups for hibernating T_b, bout duration or $\dot{V}_{O_2}$. The 55 mg g^{-1} 18:2 diet in the present study was comparable to the very high PUFA diets used by Frank and Storey (Frank and Storey, 1995), which did inhibit hibernation in *S. lateralis*. Despite the large number of studies performed on the effects of PUFA on hibernation, none has been performed on *S. tridecemlineatus*, but rather on *S. richardsonii* and *S. lateralis*, so the range of dietary linoleic acid that alters whole-animal hibernation characteristics remains unknown for this species. Expanding the range of dietary 18:2 and/or decreasing the ambient temperature used to induce hibernation in future experiments could reveal PUFA effects on whole-animal hibernation in *S. tridecemlineatus*. Although no whole-animal differences were found, dietary PUFA did affect mitochondrial function in hibernation.

Mitochondrial respiration

The suppression of state 3 respiration in hibernation (Fig. 2B) reported in this study is comparable to that reported in other studies
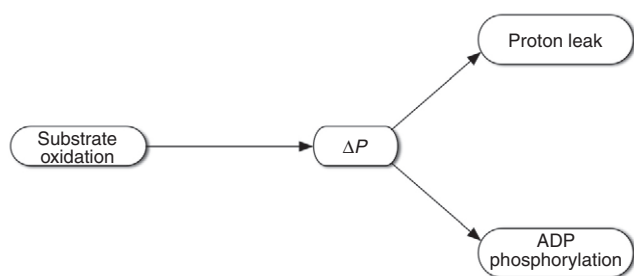

Fig. 7. Substrate oxidation generates the proton motive force (ΔP), which can then be utilized to power reactions related to the phosphorylation of ADP under state 3 conditions or to drive the leak of protons through the inner mitochondrial membrane under conditions of low ATP turnover (state 4).

(Barger et al., 2003; Martin et al., 1999; Muleme et al., 2006). This respiratory suppression was prevented by the 16, 35 and 55 mg g^{-1} 18:2 diets. In state 3 respiration, ΔP is utilized primarily to phosphorylate ADP, so reduction of state 3 could result from inhibition of the phosphorylation system (any reaction that generates or utilizes ATP), the substrate oxidation system (any reaction responsible for generating ΔP), or both (Fig. 7). There appears to be significant suppression of ETC activity between complexes II and IV in hibernation (Barger et al., 2003; Gehnrich and Aprille, 1988; Muleme et al., 2006) and daily torpor (Brown et al., 2007). Complex II is inhibited by oxaloacetate (Gutman, 1978), and this mechanism might play an important role in hibernation mitochondrial respiratory suppression (Fedotcheva, 1985). Although liver mitochondrial adenine nucleotide transporter (ANT) content does not change in hibernation (Barger et al., 2003) or mammalian daily torpor (Brown et al., 2007), Lerner and colleagues (Lerner et al., 1972) found ANT to be inhibited by myristoyl-CoA (14:0) and oleoyl-CoA (18:1) at concentrations of 30 μmol l^{-1}, inhibiting ADP transport into the mitochondria. However, Planter and colleagues (Planter et al., 1972) found both myristic and oleic acids to be decreased in the plasma of hibernating *S. tridecemlineatus*, making ANT inhibition an unlikely candidate for respiratory inhibition. Nonetheless, it would be interesting to study the effects of dietary PUFA on the levels of these potential inhibitors and the activities of these enzyme complexes in hibernation. Additionally, cellular membranes have been shown to change acutely between hibernation and arousal (Pehowich, 1994); if this occurs in mitochondria as well, it could have profound consequences for oxidative phosphorylation. Dietary PUFA might constrain the degree to which membranes can be acutely remodeled.

An increase in phospholipid unsaturation is assumed to increase membrane fluidity, permitting continued function at low temperatures. In our study, however, the temperature sensitivity of mitochondrial respiration (from Q_{10} values) did not differ between the summer active and hibernating states despite significant differences in mitochondrial phospholipid fatty acid unsaturation. It is possible that 10°C is not low enough to interfere with IMM function. Alternatively, Kraffe and colleagues (Kraffe et al., 2007) propose that very slight saturation changes might alter the activity of specific membrane-associated enzymes, with minimal effect on fluidity. The membrane phospholipid environment might influence the function of succinate dehydrogenase (SDH), COX and ANT, all of which could alter substrate oxidation, proton leak, or both (Hulbert, 2003; Paradies et al., 1993; Vik et al., 1980). The expression of COX subunits appears to be upregulated in hibernation (Hittel and Storey, 2002), corresponding with an increase in IMM surface area (Malatesta et al., 2001). Although our data demonstrate sustained or increased COX activity in hibernation (Table 3), these measurements were made under optimal conditions and might not reflect the conditions within the IMM during hibernation. Upregulation of COX might be necessary to facilitate the extremely high $\dot{V}_{O_2}$ associated with arousal from hibernation, which is initiated at very low T_b. Reductions in ANT activity and/or content would reduce the exchange of ADP and ATP across the IMM, inhibiting ATP synthesis. ANT has also been implicated in proton cycling (Brand et al., 2005), dissipating the proton gradient across the IMM. However, a decrease in ANT content in hibernation (Barger et al., 2003) or mammalian daily torpor (Brown et al., 2007) is not supported by the available evidence.

Despite the lack of mitochondrial metabolic suppression seen in the 16, 35 and 55 mg g^{-1} 18:2 diet groups, the animals still hibernated, and $\dot{V}_{O_2}$ in hibernation was not affected by diet. Moreover, no

significant differences between summer active and hibernating state 3 respiration rates were found at either 25°C or 10°C. The evidence suggests that active regulated metabolic suppression is more important at high T_b. For example, during the entrance phase of a hibernation bout, below a T_b of 25°C, passive thermal effects seem to be responsible for the majority of reduction in whole-animal $\dot{V}_{O_2}$ (Heldmaier and Elvert, 2004). It would be interesting to examine the effects of dietary 18:2 on T_b and $\dot{V}_{O_2}$ during entry into hibernation to see whether the inability to suppress mitochondrial respiration prolongs this phase of a hibernation bout.

Kinetics of proton leak

Proton leak across the IMM typically displays a non-ohmic relationship, and this is reflected in our study: at high $\Delta\Psi_m$, oxygen consumption increases exponentially (Fig. 4). However, the proton leak curves of the hibernators tend to be more linear: the curves are very similar to curves of summer active animals of the same diet at low $\Delta\Psi_m$, but, at high $\Delta\Psi_m$, the curves separate, with summer active curves generally increasing at a greater rate than those from hibernators. This pattern suggests decreased sensitivity of nonphosphorylating respiration to increases in membrane potential with hibernation, especially in the 16 and 35 mg g^{-1} 18:2 diet groups.

Further analysis showed that hibernation reduced proton leak (i.e. a reduced oxygen consumption at a common membrane potential), except in one diet group (22 mg g^{-1} 18:2 diet), where state 3 respiration was suppressed in hibernation. The 16, 35 and 55 mg g^{-1} 18:2 diet groups all had reduced proton conductance in hibernation, and higher maximal $\Delta\Psi_m$, but no significant reduction in state 3 respiration compared with the summer active state. Barger and colleagues (Barger et al., 2003) found a trend towards reduced proton leak during hibernation in *S. parryii* (fed Mazuri rodent pellets at 13 mg 18:2 g^{-1}, supplemented with sunflower seeds that are high in unsaturates, including 18:2), although there was not a significant reduction. Our data suggest that proton leak is suppressed in hibernation only in cases where substrate oxidation is not (i.e. the 16, 35 and 55 mg g^{-1} 18:2 diet groups).

A suppression of substrate oxidation could decrease ΔP, the driving force for proton leak, as seen in the mitochondria of overwintering frogs (St-Pierre et al., 2000). If downregulation of substrate oxidation is not possible owing to the effects of dietary PUFA, reducing proton leak (i.e. the 'demand' for ΔP) could also result in significant energy savings under conditions of low ATP turnover. Low proton permeability would tend to increase ΔP, which would reduce ETC activity and substrate oxidation. Indeed, the maximal $\Delta\Psi_m$ values from the 16 mg g^{-1} 18:2 and 55 mg g^{-1} 18:2 diet groups were significantly higher in hibernation, despite equivalent respiration rates (Fig. 4), whereas the 22 mg g^{-1} 18:2 diet group had marginally lower (although not statistically significant) $\Delta\Psi_m$ and non-phosphorylating respiration rates in hibernation. Therefore, at the mitochondrial level, hibernators might conserve energy by controlling supply and/or demand for ΔP depending on dietary PUFA. This might also explain why whole-animal characteristics were not affected by dietary 18:2 – animals fed different diets used different mitochondrial mechanisms to reduce liver metabolism. It is possible that diets with more extreme levels of PUFA would result in even less control over the suppression of substrate oxidation, and compensatory mechanisms for regulating proton leak might not be sufficient to reduce mitochondrial metabolism, resulting in the higher hibernation $\dot{V}_{O_2}$ values reported in other studies (Frank and Storey, 1995; Geiser, 1991; Geiser and Kenagy, 1987).

Mitochondrial proton leak might be altered either by altering the properties of the membrane that govern leak per unit surface area or by simply changing the total surface area of the IMM. The activities of COX indicate a trend towards increased IMM surface area in the hibernators overall, with a significant increase in the hibernating group fed the 55 mg g^{-1} 18:2 diet. Proton conductance from this group, however, was significantly lower than its summer active counterpart. This suggests that the decrease in proton leak in hibernation is due to changes in the inherent properties of the membrane, such as phospholipid composition (see below).

All proton leak measurements were performed at 37°C in this study. In isolated rat liver mitochondria, a drop in assay temperature from 37°C to 4°C decreased proton leak substantially (Dufour et al., 1996). Although daily torpor significantly increases proton leak in liver mitochondria isolated from *Phodopus sungorus*, reducing the assay temperature also decreases leak (Brown et al., 2007). In rats, a decrease in assay temperature from 25°C to 4°C results in an increase in the control coefficients of the phosphorylation system relative to substrate oxidation and proton leak (Dufour et al., 1996). Comparable data are not available for hibernators or daily heterotherms; future studies will compare these quantities between the summer active and hibernating states over the range of T_b experienced in a hibernation bout.

It is not clear why the values of $\Delta\Psi_m$ calculated in this study are lower than those reported for *S. undulatus*, where TPMP$^+$ was used (Barger et al., 2003). These lower values might relate to the lower binding correction factor for TPP$^+$ (0.16) compared with TPMP$^+$ (0.40). The values reported in this study, however, are similar to those from dwarf Siberian hamster liver mitochondria measured using the same techniques (Brown et al., 2007). Brustovetsky and colleagues (Brustovetsky et al., 1993) claim that, in *S. undulatus*, the liver mitochondrial volume decreases in hibernation (although the actual volume change was not quantified), whereas Martin and colleagues (Martin et al., 1999) reported no volume change with hibernation in *S. lateralis*. Volume changes are thought to have minimal impact when TPP$^+$ is used to estimate $\Delta\Psi_m$ (Rottenberg, 1984). If, however, the liver mitochondrial volume is reduced during hibernation in *S. tridecemlineatus*, the membrane potential would be elevated, exaggerating the trends shown in this study (Fig. 4).

Mitochondrial phospholipid composition and oxidative damage

Mitochondrial phospholipid fatty acids from hibernating animals were significantly more unsaturated than those from summer active animals for each diet group (Table 5). Presumably this would help to maintain function at the low hibernation T_b, but this also might result in decreased proton leak, as in hypothyroid rats (Pehowich, 1999). Unsaturation index and *n*-6/*n*-3 ratios have been correlated with proton leak under various treatments (Pehowich, 1999; Ramsey et al., 2005) and between mammalian species (Porter et al., 1996). We found significant positive correlations between mitochondrial membrane conductance at 130 mV and both linoleic acid (18:2) and arachidonic acid (20:4) in hibernation, regardless of dietary 18:2 (Fig. 6). It is difficult to attribute any mechanistic significance to these correlations, however, as dietary manipulations that have been shown to alter these fatty acids significantly in rat liver mitochondrial phospholipids do not change the kinetics of proton leak (Ramsey et al., 2005).

Brookes and colleagues (Brookes et al., 1997) suggested that membrane lipids alone do not regulate proton permeability and proposed that nonspecific leak occurs at the protein–lipid interface in intact mitochondria. Other evidence suggests that the IMM phospholipid composition is directly related to the function of certain enzymes. In a study on temperature acclimatization in trout, Kraffe

Table 5. Mitochondrial phospholipid fatty acid composition, unsaturation index and *n*-6/*n*-3 ratios for all diet groups for both summer active (SA) and hibernating (HIB) animals

Diet 18:2 (mg g⁻¹)		16	22	35	55
Fatty acid					
16:0	SA	8.52±2.87 (2)	5.17 (1)	11.15±4.66 (2)	10.36±2.87 (2)
	HIB	4.52±1.44 (8)	6.30±1.44 (8)	6.05±1.54 (7)	6.94±2.03 (4)
18:0	SA*	32.10±4.45	32.06	47.76±29.00	44.31±4.45
	HIB	19.60±2.23	20.91±2.37	20.34±2.38	19.71±4.44
18:1	SA*	10.11±1.74	15.77	8.23±6.68	8.56±1.74
	HIB	15.16±0.87	14.98±0.87	12.15±0.93†	10.57±1.23†
18:2	SA*	19.96±3.58	11.03	7.04±9.93	17.16±3.58
	HIB	26.37±1.79	28.34±1.79	31.96±1.91†	29.24±2.53†
20:4	SA*	19.02±3.33	19.30	12.18±14.01	13.99±3.33
	HIB	22.05±1.66	23.29±1.66	21.62±1.78	23.61±2.36
22:6	SA	6.85±2.12	14.89	12.31±2.5	4.75±2.13
	HIB	12.31±1.07†	11.66±1.23	9.20±1.23	12.04±1.74†
Unsat. index	SA*	1.67±0.17	2.04	1.45±0.98	1.27±0.48
	HIB	2.29±0.07	2.17±0.13	2.09±0.09	2.17±0.51
n-6/*n*-3	SA*	5.67±0.16	2.03	1.47±1.64	2.87±1.50
	HIB	4.24±0.46	4.68±0.66	5.83±0.39	4.79±0.81

* indicates a significant difference ($P<0.05$) between summer active and hibernation. Within each fatty acid class, † indicates diet groups that differ significantly ($P<0.05$) between SA and HIB. Values are means ± s.e.m. except in the summer active group, where values are means ± range as sample size did not permit the calculation of s.e.m. Values are expressed in units of mol%. Sample sizes are indicated in parentheses for the 16:0 class.

and colleagues. (Kraffe et al., 2007) found that control over state 4 respiration could be attributed to alterations in specific fatty acyl components, although no differences in unsaturation index were found between mitochondrial lipids from cold- and warm-acclimatized animals, suggesting that differences in membrane composition affect enzyme function rather than membrane fluidity. Our data might support this notion as dietary 18:2 altered mitochondrial function in hibernation, with no significant effects on unsaturation index. Phospholipid headgroups also have a significant effect on membrane fluidity and the function of membrane-bound enzymes (Hochachka and Somero, 2002). Liver mitochondrial phospholipid classes are known to differ between hibernation and euthermia in *Jaculus orientalis*; in particular, cardiolipin appears to decrease in the 'heavy' mitochondrial fraction during hibernation (Mountassif et al., 2007). Decreases in cardiolipin are associated with suppression of mitochondrial respiration in estivating snails (Stuart et al., 1998). Unfortunately, owing to a limited quantity of mitochondria, it was not possible to analyze head-group classes in this study.

To our knowledge, this is the first study to compare directly the effect of diet and hibernation on lipid oxidative damage. No differences in MDA were found among diet groups, possibly because the levels of phospholipid fatty acid unsaturation between diet groups were very similar. However, mitochondria from hibernating animals showed significantly higher levels of MDA than summer active mitochondria, probably as a result of both increased mitochondrial PL fatty acid unsaturation and high oxidative stress during arousal (Tøien et al., 2001).

Our data suggest that hibernation had a greater affect than dietary PUFA in remodeling mitochondrial membranes. Nonetheless, PUFA did affect proton leak and the suppression of state 3 respiration in hibernation. Future studies should examine the relationship between IMM phospholipid composition (fatty acids and head-group class) and the activity of SDH, COX and ANT. It would also be desirable to study the significance of mitochondrial function to hibernation metabolic suppression by examining mitochondrial function at higher levels of organization. For example Nobes and colleagues (Nobes et al., 1990) assessed mitochondrial proton leak in intact hepatocytes isolated from rats in different thyroid states. Unfortunately, Staples and Hochacka (Staples and Hochacka, 1997) found no difference in oxygen consumption in hepatocytes isolated from hibernating, summer active, aroused or summer-cold-acclimatized *S. lateralis*. This might mean that the hibernating mitochondrial phenotype does not affect metabolism at the cellular level. More likely, however, the results of Staples and Hochacka (Staples and Hochacka, 1997) reflect an artifact of the lengthy hepatocyte isolation procedure, resulting in the 'arousal' of cellular and perhaps mitochondrial metabolism.

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ANT	adenine nucleotide transporter
ATP	adenosine triphosphate
COX	cytochrome *c* oxidase
ETC	electron-transport chain
FCCP	carbonylcyanide-4-trifluoromethoxyphenylhydrazone
GC–MS	gas chromatography–mass spectrometry
IMM	inner mitochondrial membrane
MDA	malondialdehyde
NEFA	non-esterified fatty acid
NL	neutral lipid
PL	phospholipid
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SDH	succinate dehydrogenase
T_b	core body temperature
TCA	tricarboxylic acid
TPMP⁺	triphenylmethylphosphonium ion
TPP⁺	tetraphenylphosphonium ion
$\dot{V}_{O_2}$	mass-specific metabolic rate
ΔP	mitochondrial proton motive force
$\Delta\Psi_m$	mitochondrial membrane potential

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