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Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions

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Summary

Changes in the properties of mitochondria from oxidative muscle of rainbow trout *Oncorhynchus mykiss* were examined during warm (5°C to 15°C) acclimation. Trout were studied shortly after the initial thermal change and after 8 weeks acclimation to 15°C. To identify potential mechanisms by which oxidative capacities change, the modifications of phospholipid composition, membrane proteins and functional capacities of red muscle mitochondria were examined. Marked functional changes of isolated muscle mitochondria during warm acclimation of rainbow trout were reflected by a host of modifications in phospholipid composition, but by few shifts in protein components. Shortly after transfer of trout from 5°C to 15°C, the maximal oxidative capacity of mitochondria measured at 15°C increased slightly, but rates at both assay temperatures (5°C and 15°C) decreased markedly after warm acclimation. The increase in capacity in short-term warm exposed trout was most pronounced when rates at 15°C were expressed relative to cytochrome *a* and c_1 levels. Non-phosphorylating (State 4) rates of oxygen uptake increased with short-term warm exposure before returning to initial levels after warm acclimation. Cytochrome *c* oxidase (CCO) activity in the mitochondrial preparations decreased with warm acclimation. The thermal sensitivity of the ADP affinity was markedly modified during short-term warm exposure, when the ADP/O ratio increased, but warm acclimation returned these values to those observed initially. ADP affinity increased after warm acclimation. Changes in the mitochondrial content of cytochromes and adenine nucleotide translocase (ANT) could not explain these patterns. On the other hand, changes in the proportions of the lipid classes and in the acyl chain composition of certain phospholipid classes mirror the modifications in functional properties. Short-term exposure to 15°C decreased the ratio of diacylphosphatidylethanolamine/diacylphosphatidylcholine (diacylPE/diacylPC), whereas warm acclimation led to restructuring of fatty acids (FA) and to increases of plasmalogen forms of PE and PC. Modification of overall membrane unsaturation did not appear to be the primary aim of restructuring membrane FA during warm acclimation, as total mitochondrial phospholipids and the major phospholipid classes only showed slight shifts of their acyl composition with warm acclimation. On the other hand, natural lysophosphatidylcholine (LysoPC) showed dramatic changes in FA content, as 16:0 and 18:1n-9 doubled whereas 22:6n-3 decreased from around 50% to 32% in warm acclimated trout. Similarly, in cardiolipin (CL), the levels of 16:0 and 18:1n-7 halved while 18:2n-6 increased to over 20% of the FA with warm acclimation. Given the central role of CL in modulating the activity of CCO, F_0F_1-ATPase and ANT, these changes suggest that specific compositional changes in CL are important modulators of mitochondrial capacities. The many structural changes in membrane lipids contrast with the limited modifications of the membrane protein components examined and support the concept of lipid structure modulating mitochondrial capacities.

Key words: mitochondria, thermal acclimation, oxidative muscle, cytochrome *c* oxidase, ADP affinity, thermal compensation, phospholipids, plasmalogens, fatty acids, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Due to its impact upon all levels of organisation, temperature is a crucial determinant of the performance of living systems. As temperature affects the kinetic energy of molecules, it modifies rates of diffusion, molecular interactions and macromolecular stability, membrane properties, and may

change the efficacy of metabolic regulation. In ectotherms, cellular function can acclimate to thermal change. Unlike the adaptive 'strategies' active during evolution, the mechanisms underlying cellular acclimatisation must be specified by the existing genotype. When thermally induced changes in metabolic demand persist, animals often remodel mitochondrial structure and function to better accommodate energetic needs. During cold acclimation, the commonly observed increases in muscle aerobic capacity occur at several levels: mitochondrial volume density, cristae surface density, and activity of enzymes in oxidative pathways. Increases in mitochondrial abundance may compensate for catalytic or diffusive limitations (for a review, see Guderley, 2004). Increases in cristae density can enhance the potential for oxidative phosphorylation without sacrificing volume needed for other organelles (St Pierre et al., 1998). Alterations in concentrations of enzymes can facilitate metabolic adjustments during thermal acclimation (Sidell, 1983), while increases in their specific catalytic capacity during cold acclimation may, at least partially, offset acute thermal effects on reaction rate. For example, cold acclimation of cyprinids increases the specific activity of succinate dehydrogenase, F_0F_1-ATPase and cytochrome *c* oxidase (CCO) in muscle (Hazel, 1972b; Itoi et al., 2003b; Wodtke, 1981a). Mitochondria from red muscle of rainbow trout show seasonal cycles of their apparent ADP affinity and its thermal sensitivity suggesting modifications of capacities of the adenine nucleotide translocase (ANT) (Blier and Guderley, 1993; Guderley and St Pierre, 1999). One factor these proteins have in common is that they occur in the hydrophobic environment of the inner mitochondrial membrane.

Lipids that constitute mitochondrial membranes should be regarded as structural partners of membrane proteins and as potential modulators of mitochondrial processes (Daum, 1985). Proper functioning of biomembranes becomes difficult below the transition temperature at which the liquid crystalline structure of the acyl core changes into a gel-like structure. Ectothermal organsims are thought to adapt their membranes to temperature decreases by increasing the acyl chain unsaturation of phospholipids to maintain membrane fluidity. This homeoviscous adaptation (HVA) also includes shifts in the proportions of phospholipid classes (phosphatidylethanolamine (PE) relative to phosphatidylcholine (PC) and cholesterol content (Hazel and Williams, 1990; Hazel, 1995; Crockett and Hazel, 1995). Modification of the capacity for oxidative phosphorylation during thermal acclimation is claimed to be due, at least in part, to HVA (Van Der Thillart and Modderkolk, 1978; Wodtke, 1981a; Wodtke, 1981b; Itoi et al., 2003a; Guderley, 2004). On the other hand, requirements for specific phospholipids in the vicinity of electron transport chain components (Hoch, 1992) suggest that changes in the FA composition of specific phospholipids may be central in the modulation of catalytic activity during thermal acclimation. Thus, due to the complexity of membrane lipid constituents (sterol, 8 main phospholipid classes and subclasses, and more than 50 fatty acids) and the elaborately subvided submembrane domains, specific effects may be obscured when examining the acyl composition of total phospholipids.

Particularly in the protein-dense mitochondrial membranes (Hazel and Williams, 1990), only a small number of lipid molecules is thought to separate membrane proteins or protein aggregates. In mammalian systems, mitochondrial membrane proteins can show specific requirements for phospholipid head group arrangements and FA in their proximity (Clandinin et al., 1985). Thus, even minor phospholipid classes can have an important functional impact on membrane-bound enzymes. Cardiolipin (CL), in particular, is specifically located in the inner mitochondrial membrane, and is a key factor in the control of CCO, F_0F_1-ATPase and ANT (Schlame et al., 2000). Localised changes of specific membrane phospholipids, like CL, may be critical in establishing the activities of membrane proteins and provide a powerful means of adjusting activity without requiring protein synthesis or modification of existing proteins during thermal acclimation. Thus, a detailed characterisation of membrane lipids is a prerequisite to understanding how heterogeneous mixtures of phospholipids interact with membrane proteins to alter biological functions. Although thermal acclimation leads to well characterised changes in the abundance of muscle mitochondria and mitochondrial oxidative capacities in temperate zone fishes (Guderley, 2004), changes of capacity of protein and phospholipid components have typically been studied separately. A few exceptions are provided by the classic studies (Hazel, 1972a; Hazel, 1972b; Van den Thillart and Modderkolk, 1978; Wodtke, 1981a; Wodtke, 1981b), in which changes in overall phospholipid composition brought about by cold acclimation were linked with changes in the activity of succinate dehydrogenase, State 3 respiration and CCO.

Little is known about the time course of thermal acclimation. Modifications in membrane structure during the time course of thermal acclimation of rainbow trout follow a defined sequence, as shown by the restructuring of phospholipid composition and molecular species in kidney plasma membrane (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b). After 8 h of cold acclimation (20°C to 5°C), the PC/PE ratio decreases. The proportions of saturated and monounsaturated FA change after 16–48·h and long-chain polyunsaturated FA only increase after 10–21 days of cold acclimation. Such a sequence of compositional changes in membrane phospholipids was suggested to explain the non-linear time course of mitochondrial oxidative capacity during warm and cold acclimation of rainbow trout (Bouchard and Guderley, 2003), but no study has established such a relationship.

The present study aims to evaluate at the same time, detailed modifications of phospholipid composition, levels of membrane proteins and functional capacities of red muscle mitochondria during warm acclimation (5°C to 15°C) of rainbow trout *Oncorhynchus mykiss*. After characterising 5°C-acclimated trout, we studied trout shortly (3 days) after transfer to 15°C and after 8 weeks acclimation to 15°C. By examining mitochondrial substrate oxidation at 5°C and 15°C, the

concentrations and activities of mitochondrial components, as well as the phospholipid composition of mitochondrial membranes we sought to identify potential mechanisms by which oxidative capacities change. We measured (1) the levels of adenine nucleotide translocase (ANT), (2) the concentrations of cytochromes *a*, *b*, *c* and c_1, (3) the apparent ADP affinity and (4) the activity of CCO. The content of cytochromes *a*, *b* and c_1 reflect the numbers of respiratory chain complexes: cytochrome *b* is present in Complexes II and III, c_1 occurs in Complex III, and cytochrome *a* is part of Complex IV. To evaluate the implication of the lipid environment in the time course of changes in mitochondrial oxidative capacities, we determined the proportions of each mitochondrial phospholipid class and subclass as well as their specific FA compositions and the sterol levels at each sampling time.

Materials and methods

Animals and the time course of warm acclimation

Rainbow trout *Oncorhynchus mykiss* Walbaum were obtained from a fish hatchery (Ferme Piscicole Richard Boily, Île d'Orléans, Québec, Canada) on 19 March 2003, when the temperature in their outdoor holding ponds was around 5°C. Thermal acclimation occurred at the LARSA (Laboratoire Régional des Sciences Aquatiques) at Laval University in a 1000-litre tank. After their arrival, the fish were maintained at 5°C under the natural photoperiod. Fish were fed the same commercial food (Corey Aquafeeds, New Brunswick, Canada) used at the hatchery at maintenance rations, calculated for their size, number and tank temperatures, once a day.

Twelve fish were used to assess the oxidative capacities of these winter-acclimatised trout that had been held at 5°C for a week before beginning the study (cold acclimated). Then, water temperature was gradually raised to 15°C over 3 days, and eight specimens were studied during the subsequent 3 days (short-term warm exposed). Finally, 8 weeks after the beginning of the thermal change, when we assumed acclimation was complete (warm acclimated), 12 trout were used to assess final oxidative capacities.

Isolation of mitochondria

The fish were killed by a blow on the head, and the superficial lateral red muscle was immediately removed and minced. All the manipulations were carried out on ice except the centrifugations, which were performed at 4°C. Mitochondria were isolated and assayed (Guderley et al., 1997). To optimise the purity of mitochondrial pellets, additional steps were done during the differential centrifugation protocol. Briefly, after centrifuging the homogenate at 900 ***g*** for 10 min, the superficial lipid layer was removed. The remaining supernatant was recentrifuged at 900 ***g*** for 10 min. The supernatant obtained was considered free of unbroken cells or cell debris and was centrifuged at 9000 ***g***. The resulting pellet was rinsed once to remove $MgCl_2$ by resuspension in isolation buffer free of $MgCl_2$ and recentrifuged at 9000 ***g***. The mitochondrial pellet was resuspended in a volume of reaction buffer corresponding to one-tenth of the mass of muscle used.

Measurement of mitochondrial respiration

Oxygen consumption was measured in the reaction buffer at 5°C and 15°C. For each assay, malate was added to a final concentration of 0.37 mmol l^{-1} to spark the Krebs cycle, and pyruvate was added to a final concentration of 3.45 mmol l^{-1}. The maximal rate of oxidative phosphorylation (State 3) was obtained by the addition of ADP to a final concentration of 0.92 mmol l^{-1}. State 4 rates were measured after depletion of ADP, once oxygen uptake rates had stabilized. The ADP/O ratio was measured (Chance and Williams, 1956).

ADP affinity determinations

The apparent affinity of mitochondria for ADP (K_m) was determined polarographically as described (Guderley and St Pierre, 1999). This approach used the hexokinase reaction to maintain constant ADP levels so that oxidation rates could be obtained at low ADP levels. The assay medium was supplemented with glucose, $MgCl_2$ and hexokinase (yeast; Boehringer Mannheim Biochemicals, Montreal, Quebec, Canada) at final concentrations of 38 mmol l^{-1} glucose, 19 mmol l^{-1} $MgCl_2$ and 3 units l^{-1} hexokinase. Hexokinase exerts no control over mitochondrial respiration when the ratio of mg mitochondrial protein to units of hexokinase is less than 3 (Jacobus et al., 1982). In our study, these ratios were consistently less than 0.6. Similar ranges of excess hexokinase were present for the mitochondria from cold and warm acclimated trout as well as for short-term warm exposed animals. Pyruvate was the carbon substrate (3.45 mmol l^{-1}), with malate present at 0.37 mmol l^{-1}. Saturation curves for mitochondria were determined by sequential additions of ADP starting with the lowest concentration (approximately 3×10^{-3} mmol l^{-1} total ADP), followed by gradual additions of ADP to attain saturating concentrations. Oxygen uptake rates were determined for at least 90 s at each ADP concentration. Experiments were carried out at 5°C and 15°C. We standardised the quantity of mitochondrial protein at ~0.15 mg mitochondrial protein ml^{-1} assay medium to facilitate comparison between the sampling periods. The apparent K_m for ADP ($K_{m,app}$) and V_{max} (maximal velocity) were calculated using the Marquardt iterative search algorithm to fit the Michaelis–Menten equation using Nonlinear Regression analysis (StatGraphics Plus 5.1). The ADP solutions were calibrated spectrophotometrically using the pyruvate kinase and lactate dehydrogenase reactions (Bergmeyer, 1983).

Cytochromes and ANT concentrations

Cytochromes *a*, *b*, *c* and c_1 in the mitochondrial preparations were quantified by difference spectra. The electron transport chain components in 2% deoxycholate-dispersed mitochondria were reduced by 5 mmol l^{-1} ascorbate and the oxygen in the solution was eliminated by the addition of dithionite (Williams, 1964). The reduced samples were read against the samples

oxidised with 5 mmol l^{-1} ferricyanide. We used the solution to the simultaneous equations required to assess the individual cytochrome concentrations (Williams, 1964). Difference spectra were obtained using a double-beam UV/Vis spectrophotometer (Varian-Cary 210, Mississauga, Ontario, Canada). The concentration of adenine nucleotide translocase (ANT) was measured in mitochondrial suspensions by titration with its noncompetitive irreversible inhibitor, carboxyatractyloside (CAT) (Guderley et al., 2005). State 3 respiration was gradually inhibited and the inhibition was considered complete when addition of CAT had no further effect on oxygen uptake. The quantity of ANT in mitochondrial suspensions corresponded to the amount of CAT needed for inhibition.

Protein concentrations

The protein concentration in mitochondrial suspensions was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin (BSA) as the standard. Before analysis, an aliquot of each mitochondrial preparation was resuspended in the reaction buffer minus BSA and centrifuged at 9000 ***g*** at room temperature for 10 min. The supernatant was discarded and the pellet resuspended, washed and centrifuged twice more to remove the BSA. These preparations were conserved at –80°C until protein assays.

Cytochrome c oxidase activity

CCO activity was measured at 5°C and 15°C according to published methods (Bouchard and Guderley, 2003) except that mitochondrial suspensions were diluted in phosphate buffer without Triton-X (45 mmol l^{-1} KH_2PO_4 and 30 mmol l^{-1} K_2HPO_4, pH 6.8). We used an initial optimal cytochrome *c* concentration of 100 μmol l^{-1}. All assays were run in triplicate using fresh mitochondrial preparations. Activities were calculated using an extinction coefficient of 19.1 mmol l^{-1} cm^{-1} and are expressed as μmol cytochrome *c* transformed min^{-1} (first order reaction).

Membrane lipid analysis

The membrane lipids of mitochondrial suspensions were extracted according to the method described (Folch et al., 1957). Before lipid extraction, the aliquot of mitochondrial preparation was resuspended in the reaction buffer minus BSA and centrifuged at 9000 ***g*** at room temperature for 10 min. The supernatant was discarded and the pellet resuspended, washed in reaction buffer and centrifuged a further two times. The final extract was stored at –80°C under nitrogen after adding 0.01% w/v butylated hydroxytoluene (BHT, antioxidant).

Separation of polar lipids on silica gel microcolumns

An aliquot of the lipid extract was evaporated to dryness and lipids were recovered with three washings of 500 μl of $CHCl_3$/methanol (98:2, v/v) and deposited at the top of a silica gel micro-column (30 mm×5 mm i.d., packed with Kieselgel 60 (70–230 mesh, Merck, Darmstadt, Germany) previously heated at 450°C and deactivated with 6 weight% H_2O (Marty et al., 1992). Neutral lipids were eluted with 10 ml of $CHCl_3$/methanol (98:2, v/v) and stored at –20°C for later cholesterol analysis. The polar lipid fraction was recovered with 20 ml methanol and stored at –20°C for later phospholipid class separation by high performance liquid chromatography (HPLC) and FA composition analysis by gas chromatography (GC).

Cholesterol analysis

Cholesterol was analysed in a gas chromatograph (Chrompak 9002, Middelburg, The Netherlands) equipped with RTX65 (65% diphenyl, 35% dimethylpolysiloxane) fused silica capillary column (50 m×0.32 mm, 0.2 pm film thickness) using an on-column injection system and hydrogen as carrier gas, with a thermal gradient from 60°C to 280°C. Quantification of cholesterol was achieved by adding a known quantity of cholestane to samples.

Separation of membrane lipid classes and FA analysis

Separation of the phospholipid classes and subclasses followed Kraffe et al. (Kraffe et al., 2004) using two successive HPLC separations with two different mobile phases. This method allowed the separate analysis of plasmalogen (1-alkenyl-2-acyl-) and diacyl subclasses of PE and PC jointly with cardiolipin (CL), phosphatidylinositol (PI), phosphatidylserine (PS), and natural lysophosphatidylcholine (LysoPC). The remaining diacyl fraction of PE and PC (diacylPE and diacylPC) presented and discussed in this paper likely contained the 1-alkyl-2-acyl- form in addition to the 1,2-diacyl. Each fraction was collected and, after transesterification (methanol/BF_3), analysed by GC for FA composition. Fatty acid methyl esters (FAME) obtained were identified and quantified using both polar (CPWAX 52 CB, Varian, Middelburg, The Netherlands; 50 m×0.25 mm i.d.; 0.2 μm thickness) and nonpolar (CP-Sil 8 CB, Varian; 25 m×0.25 mm i.d.; 0.25 μm thickness) capillary columns and C23:0 FA as an internal standard. FA were expressed as molar percentage of the total FA content of each class or subclass. For plasmalogen ethanolamine (PlsmPE) and plasmalogen choline (PlsmPC) subclasses, the total percentage was adjusted to 50% to take into account the absence of alkenyl chains of the *sn*-1 position hydrolysed by the acid mobile phase.

Calculation of amounts of phospholipid classes

The quantities of each class and subclass of phospholipid were determined from their respective FA spectrum obtained by GC. To obtain the molar content of each analysed fraction, a correction factor was applied to their respective total FA molar contents: ×1 for PlsmPE and PlsmPC fractions and for the natural lysoPC fraction; ×1/2 for the diacylPE, diacylPC, PS and PI fractions, and ×1/4 for the CL fraction.

Statistical analysis

Statistical comparisons and linear regressions were carried out with StatGraphics Plus 5.1 (Sigma Plus Inc., Toulouse, France). One-way analysis of variance (ANOVA) followed by

a posteriori Tukey multiple comparisons was used for intergroup comparisons of the impact of warm acclimation on mitochondrial characteristics of trout. Differences were considered significant when $P \leqslant 0.05$.

Results

Overall characteristics of the trout

The externally measurable status of the trout – body mass, fork length and Fulton's condition factor – did not change significantly during warm acclimation (Table 1).

Mitochondrial concentrations of cytochromes and ANT

After short-term warm exposure, cytochrome levels were unchanged (Fig. 1). Warm acclimation decreased the levels of cytochromes *b* and *c*, but left those of cytochromes *a* and c_1 unchanged. The protein-specific levels of ANT exceeded those of the cytochromes and were slightly, but significantly, higher ($P<0.05$) in short-term warm exposed trout compared to cold or warm acclimated trout.

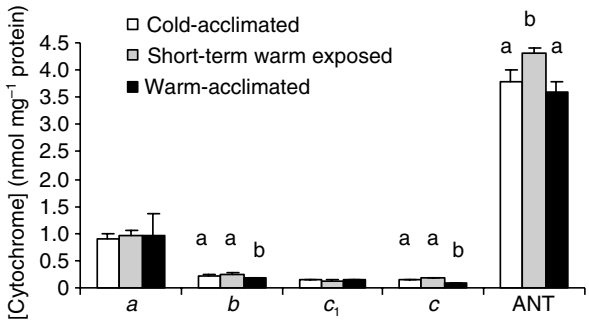

Fig. 1. Concentration of mitochondrial cytochromes in oxidative muscle during warm acclimation. Cytochrome and ANT concentrations are normalised to the protein content in the mitochondrial preparations. Values are means ± s.e.m. (N=9 for cold acclimated trout, N=8 for short-term warm exposed trout and N=11 for warm acclimated trout). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* test, $P \leqslant 0.05$).

Mitochondrial maximal oxidative capacities during warm acclimation

The isolation procedure yielded mitochondria with respiratory control ratios (RCR; State 3/State 4) ranging from 3 to 9 throughout the experiment. Maximal rates of pyruvate oxidation (nmol O min^{-1} mg^{-1} mitochondrial protein) changed markedly during warm acclimation. Rates were stable after short-term warm exposure and dropped markedly with warm acclimation at both assay temperatures ($P<0.05$) (Fig. 2). State 4 rates (nmol O min^{-1} mg^{-1} mitochondrial protein) showed a biphasic pattern, with rates, particularly at 15°C, increasing during short-term warm exposure and returning to initial values with warm acclimation (Table 2). These patterns led the mean RCR values to decrease from 5 to 3.3 with warm acclimation. The thermal sensitivity (Q_{10}) of maximal rates of pyruvate oxidation did not change between cold acclimated and short-term warm exposed trout (mean values of 1.36±0.12 and 1.49±0.07, respectively), but increased in warm acclimated trout (mean value of 1.84±0.07, $P<0.05$). The phosphorylation capacity of mitochondria, expressed by the molar ratio between added ADP and consumed oxygen (ADP/O), reached significantly higher values in mitochondria of short-term warm exposed trout at both assay temperatures compared to cold and warm acclimated trout: 5°C (mean values of 2.8±0.1 for cold and warm acclimated trout; 3.3±0.2 for short-term warm exposed) and 15°C (3.1±0.1 for cold and warm aclimated trout; 3.8±0.1 for short-term warm exposed).

Table 1. *General characteristics of experimental trout*

	Body mass (g)	Length (cm)	Condition factor (mass/length^3) ×100
Cold acclimated	414.9±23.1	33.3±0.6	1.12±0.03
Short-term warm exposed	401.8±16.9	33.7±0.6	1.05±0.02
Warm acclimated	441.0±25.7	34.7±0.6	1.05±0.03

Values are means ± s.e.m. (N=11 for cold acclimated trout, N=8 for short-term warm exposed and N=12 for warm acclimated trout). No significant differences were found between acclimation states (ANOVA and *a posteriori* tests).

The denominator typically used to standardise mitochondrial rates is the amount of protein (mg) in the mitochondrial preparation. This reflects protein located both in the matrix and in the membrane. As the adjustments of membrane composition that are a major focus of our study would primarily affect membrane-bound complexes, we examined oxidative capacities relative to the concentrations of cytochromes and ANT, all located in the mitochondrial membrane. For State 3 rates measured at 5°C expressed over cytochrome *a*, c_1 and ANT, the same pattern was observed as with protein specific rates: stability during short-term warm exposure and a decrease with warm acclimation (Fig. 2). On the other hand, at 15°C, use of cytochromes *a* and c_1 as the denominator revealed a biphasic response, with State 3 rates increasing during short-term warm exposure (significant only for cytochrome c_1) and then decreasing during warm acclimation. State 4 rates expressed over the cytochromes and ANT changed less with warm acclimation than protein-specific State 4 rates (Table 2).

Changes in cytochrome c oxidase activity during warm acclimation

The time course of warm acclimation changed the activity of CCO in a similar fashion as the protein-specific capacities of isolated mitochondria (Fig. 3). CCO activity in mitochondrial suspensions increased slightly, but not significantly, in short-term warm exposed trout when assayed at 5°C. Warm acclimation significantly decreased CCO activity compared to cold acclimated or short-term warm exposed trout ($P<0.05$) at both assay temperatures. The same

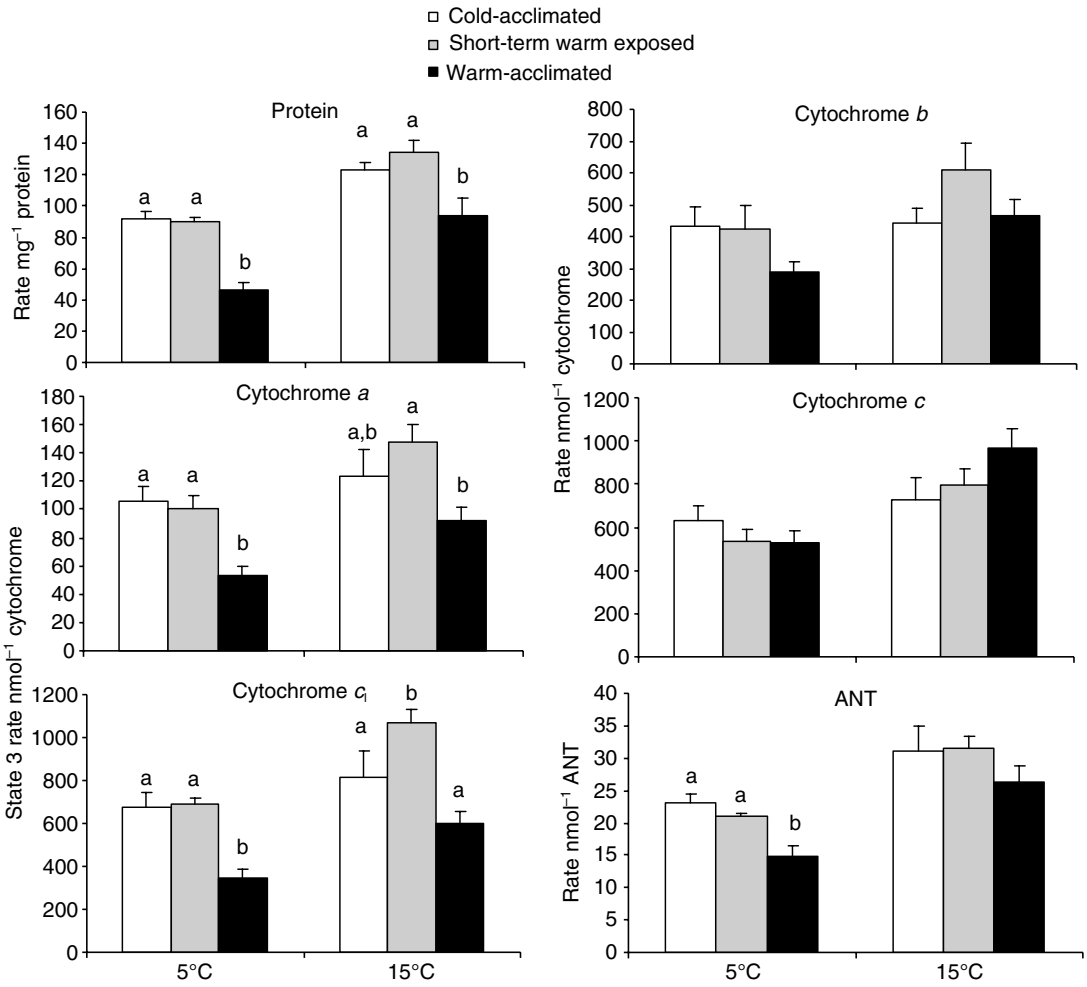

Fig. 2. Time course of changes in maximal rates of pyruvate oxidation (nmol O min^{-1}) by mitochondria from oxidative muscle during warm acclimation. State 3 rates are expressed as mg^{-1} mitochondrial protein, nmol^{-1} cytochrome and nmol^{-1} ANT. Values are means ± s.e.m. (N=11 for cold acclimated trout, N=8 for short-term warm exposed trout and N=11 for warm acclimated trout). Different superscript letters indicate rates at a given assay temperature that differ between acclimation states (ANOVA and *a posteriori* test, $P \leqslant 0.05$).

pattern was found when CCO activity was expressed over cytochrome *a* levels. The Q_{10} for CCO remained between 1.4 and 1.6 during our experiment. Throughout the experiment, only 4–5% of mitochondrial CCO activity was used during maximal rates of pyruvate oxidation (State 3/CCO activity expressed in mU).

ADP affinity

During the time course of warm acclimation, mitochondria from red muscle changed their apparent ADP affinity ($K_{m,app}$), as well as its thermal sensitivity (Table 3). At 5°C, the highest value for the $K_{m,app}$ was obtained for cold acclimated trout with short-term warm exposed and warm acclimated trout having significantly lower values. On the other hand, at 15°C, short-term warm exposed trout had a higher $K_{m,app}$ than trout in the other groups. Thus, while both cold and warm acclimated trout show a 50% decrease of $K_{m,app}$ with an increase in assay temperature, for short-term warm exposed trout the apparent K_m values did not differ between 5 and 15°C (*t*-test, P>0.05).

At the assay temperature of 5°C, the V_{max} (maximal velocity) calculated by iterative fitting of the Michaelis–Menten equation was close to the values of State 3 respiration (Table 3) and showed the same changes during the acclimation protocol. Thus, the ADP-dependence of mitochondrial oxidative capacities at 5°C was well described by the Michaelis–Menten equation. At 15°C, the calculated V_{max} was considerably lower than the measured State 3 rates.

Mitochondrial compositions of membrane phospholipid classes and subclasses during the time course of warm acclimation

Throughout the study, glycerophospholipids (PE, PC, PS, PI, CL and LysoPC) were the predominant phospholipid classes in mitochondria. Plasmalogen forms were only found in PE (PlsmPE) and PC (PlsmPC), those in PE being more prominent. Sphingomyelin was always found in trace amounts, indicating that the mitochondrial membranes were not significantly contaminated by other cellular membranes.

Table 2. *Time course of changes in state 4 rates of pyruvate oxidation by mitochondria from oxidative muscle during warm acclimation*

State 4 rate	Cold acclimated	Short-term warm exposed	Warm acclimated
5°C			
Protein	19.6±2.1[a,b]	24.6±0.8[a]	17.7±0.9[b]
Cytochrome *a*	27.8±4.2	28.5±4.0	18.8±2.0
Cytochrome *b*	93.2±7.3	97.9±16.6	101.8±8.2
Cytochrome c_1	170.6±20.6[a]	185.8±15.0[a]	124.6±8.2[b]
Cytochrome *c*	158.4±20.0	153.1±22.5	221.9±26.4
ANT	5.7±0.6	5.8±0.2	5.0±0.4
15°C			
Protein	29.5±4.9[a]	42.2±2.3[b]	33.0±2.5[a,b]
Cytochrome *a*	31.0±1.6	36.7±2.9	35.2±4.8
Cytochrome *b*	117.4±26.3	168.3±36.2	192.0±18.7
Cytochrome c_1	223.3±58.8	315.4±36.9	250.3±20.2
Cytochrome *c*	192.7±43.7	273.0±52.0	455.2±44.3
ANT	8.5±2.3	9.9±0.6	10.9±1.0

State 4 rates of pyruvate oxidation are expressed as nmol O min^{-1} mg^{-1} mitochondrial protein, or nmol O min^{-1} $nmol^{-1}$ cytochrome or ANT. Values are means ± s.e.m. (N=11 for cold acclimated trout, N=8 for short-term warm exposed trout and N=11 for warm acclimated trout). Different superscript letters indicate rates at a given assay temperature that differ with acclimation state (ANOVA and *a posteriori* tests, $P \leqslant 0.05$)

During warm acclimation, the relative levels of the phospholipid classes and subclasses changed (Fig. 4), with particularly marked changes in diacylPE, diacylPC, PlsmPE, PlsmPC and LysoPC. The proportion of the diacyl form of PE declined during warm acclimation from 33.95% in cold acclimated trout to 22.94% in warm acclimated trout (P<0.05). With short-term warm exposure, diacylPE levels decreased, but not significantly, to 30.14%. The changes in PlsmPE showed an inverse pattern over the time course of the experiment compared to diacylPE. Indeed, while values of PlsmPE remained stable in short-term warm exposure, levels doubled after 8 weeks of warm acclimation. Thus, warm acclimated trout mitochondria have the highest proportion of plasmalogen in PE (35.6%) while this value was only 14.4 and 16.3%, respectively in cold acclimated and short-term warm exposed trout. Although proportions of diacylPC were similar between cold and warm acclimated trout, the level of diacylPC rose significantly in short-term warm exposed trout concomitant with a decrease, albeit not significant, of lysoPC. PlsmPC content relative to total phospholipids varied in the same way as PlsmPE, remaining stable in short-term warm exposure and doubling in warm acclimation. Although the proportion of plasmalogens in PC was markedly lower than that in PE, the proportion of plasmalogens in PC also reached the highest level in warm acclimated trout (2.7%). The proportions of CL, PS and PI did not vary significantly between acclimation groups. However, LysoPC increased in mitochondria from warm acclimated trout (P<0.05).

Cholesterol and total phospholipid contents of mitochondrial membranes

Membranes from trout red muscle mitochondria were primarily composed of phospholipids, with approximately tenfold higher levels of phospholipids than cholesterol (Table 4). Cholesterol, expressed relative to mitochondrial protein, did not change with acclimation status (approximately 0.05 μmol mg^{-1} mitochondrial proteins) while total phospholipids were stable between cold acclimated and short-term warm exposed trout but decreased in warm acclimated trout. Consequently, the relative levels of cholesterol and phospholipids (molar ratio) were higher in mitochondrial membranes from warm acclimated trout (P<0.05) (Table 4).

Fatty acyl chain composition of phospholipid classes and subclasses during warm acclimation

Changes in acyl chain composition during the time course of warm acclimation differed when examined for the total phospholipids (Table 5) or for the specific classes (Tables 6–8). The acyl chain composition of plasmalogen subclasses of PE and PC also changed during warm acclimation, suggesting that FA of these subclasses may influence membrane dynamics. For the overall phospholipids, saturated fatty acids (SFA) were dominated by 16:0 while 22:6n-3 was the main unsaturated FA. The other major FA were 18:0, 18:1n-9, 18:2n-6, 20:4n-6, 20:5n-3 and 22:5n-3. The time course of warm acclimation did not greatly affect the FA composition of total phospholipids. When FA levels differed, it was the mitochondria of warm acclimated trout that were distinct from those obtained from the other states. The levels of 22:6n-3 rose slightly, but not significantly, before returning to initial values with warm acclimation. 20:5n-3 decreased with warm acclimation, whereas 18:1n-9 and 18:2n-6 rose. The n-3/n-6 ratio was decreased (P<0.05), monounsaturated fatty acids (MUFA) were increased (P<0.05), saturated were decreased (but not significantly) while polyunsaturated (PUFA) were stable at the different stages of acclimation. Dimethylacetals (DMA), obtained from the fatty aldehydes bound as vinyl ethers to the *sn*-1 position of plasmalogen phospholipids, increased in long-

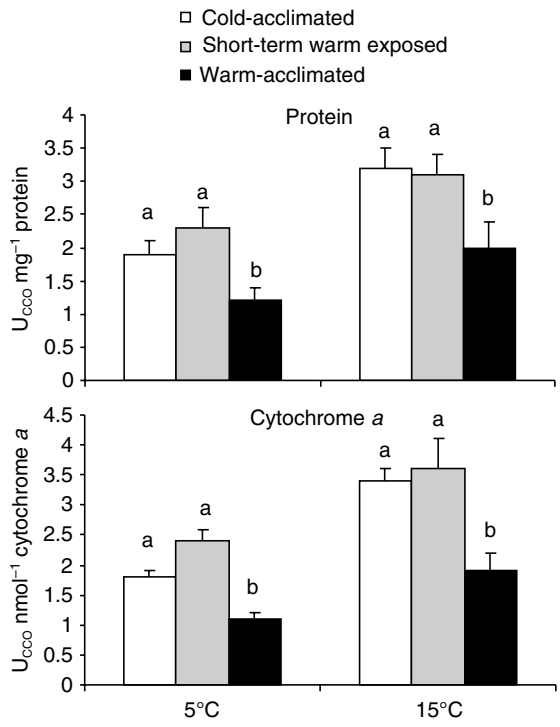

Fig. 3. Time course of changes in cytochrome *c* oxidase activity during warm acclimation, expressed in U_{CCO} mg^{-1} mitochondrial protein and U_{CCO} nmol^{-1} cytochrome *a* (U_{CCO}=μmol cytochrome *c* reduced min^{-1}). Values are means ± s.e.m. (*N*=8 for cold acclimated trout, *N*=7 for short-term warm exposed trout and *N*=12 for warm acclimated trout). Different superscript letters indicate rates at a given assay temperature that differ between acclimation states (ANOVA and *a posteriori* test, $P \leqslant 0.05$).

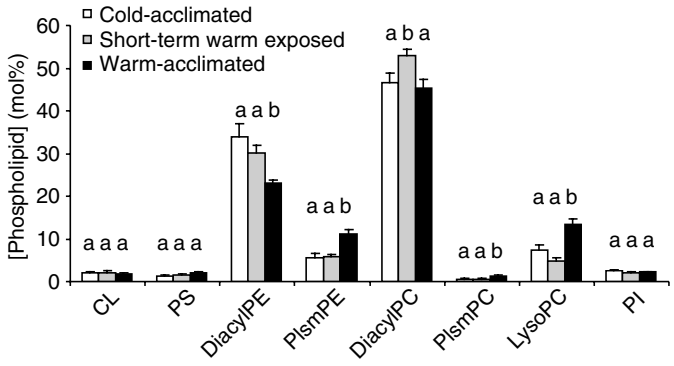

Fig. 4. Time course of changes in proportion of phospholipid classes and subclasses during warm acclimation. Values are means ± s.e.m. (*N*=4 for each acclimation state). Different superscript letters indicate values for a class or subclass that differ between acclimation states (ANOVA and *a posteriori* test, $P \leqslant 0.05$). Phospholipids are expressed as mol% of total moles of glycerophospholipids, calculated as indicated in Materials and methods. Abbreviations are defined in List of abbreviations.

term warm acclimated trout ($P<0.05$). A modest, but not statistically significant, decrease in unsaturation index (UI) was noted for warm acclimated trout.

Changes in the FA compositions of the two prominent mitochondrial phospholipid classes, PE and PC, in both their diacyl and plasmalogen forms (Tables 6 and 7), were similar in magnitude to those in the total phospholipids. For diacylPC, the more unsaturated FA (20:5n-3 and 22:6n-3) decreased in warm acclimation ($P<0.05$). Correspondingly, 18:1n-9 increased with the same time course. SFA, dominated by 16:0, increased in short-term warm exposed trout, only to fall to initial levels in warm acclimated trout. The plasmalogen form of PC showed fairly constant levels of individual FA. However, warm acclimation increased PUFA and decreased SFA with significant differences between warm and cold acclimated trout. The FA composition of diacylPE differed from that of diacylPC in that 18:0 was also a major SFA and in that 22:6n-3 and 20:5n-3 were respectively higher and lower than in diacylPC. In diacylPE, all statistically significant effects distinguished warm acclimated trout from the other groups, except for 22:6n-3, for which warm acclimated trout only differed from warm exposed trout. Among SFA, 16:0 increased slightly and 18:0 decreased slightly leading to constant total SFA in diacylPE during warm acclimation. As observed for diacylPC, 20:5n-3 and 22:6n-3 decreased in warm acclimated trout ($P<0.05$), whereas 18:1n-9 tended to increase. The levels of 22:5n-3 became higher than those of 20:5n-3 in mitochondria from warm acclimated trout. Interestingly, 22:5n-3 is the sole PUFA that increased significantly during warm

Table 3. *Time course of the apparent affinity for ADP ($K_{m,app}$) and the maximal velocity (V_{max}) for oxygen consumption by mitochondria during warm acclimation*

	5°C		15°C	
	K_m	V_{max}	K_m	V_{max}
Cold acclimated	0.12±0.007^a	72.5±5.4^a	0.06±0.003^a	69.2±8.1^a
Short-term warm exposed	0.07±0.006^b	72.1±2.7^a	0.097±0.016^b	15.6±1.1^b
Warm acclimated	0.03±0.003^c	31.6±5.1^b	0.015±0.002^c	21.8±2.2^b

Values are means ± s.e.m. (*N*=8 for cold acclimated, *N*=7 for short term warm exposed and *N*=12 for warm-acclimated trout). Different superscript letters indicate rates at a given assay temperature that differ with acclimation state (ANOVA and *a posteriori* tests, $P \leqslant 0.05$).

K_m values are expressed in mmol l^{-1} ADP, V_{max} values as nmol atom O min^{-1} mg^{-1} mitochondrial protein.

Table 4. *Time course of changes in content of cholesterol and total phospholipids in mitochondria during warm acclimation*

	Cold acclimated	Short-term warm exposed	Warm acclimated
Total phospholipids (μmol mg^{-1} protein)	0.52±0.03[a,b]	0.52±0.03[a]	0.37±0.06[b]
Cholesterol (μmol mg^{-1} protein)	0.051±0.006	0.051±0.003	0.053±0.003
Cholesterol/phospholipids (μmol μmol^{-1})	0.10±0.02[a]	0.10±0.01[a]	0.14±0.01[b]

Values are means ± s.e.m. (N=3 for cold acclimated, N=4 for short-term warm exposed and N=3 for warm acclimated trout). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* test, $P \leqslant 0.05$).

acclimation. These PUFA showed much the same patterns of change in PlsmPE.

More dramatic modifications during the time course of warm acclimation were noted when considering the FA composition of the minor phospholipid classes CL and LysoPC (Table 8).

Table 5. *FA composition of total phospholipids during the time course of warm acclimation*

	Total phospholipids		
Fatty acids	Cold acclimated	Short-term warm exposed	Warm acclimated
16:0	22.2±2.4	22.5±0.7	19.4±1.0
18:0	4.6±0.5	4.1±0.2	5.2±0.3
16:1n-7	1.0±0.1[a,b]	0.9±0.1[a]	1.3±0.1[b]
18:1n-9	6.6±0.1[a]	6.1±0.4[a]	9.6±0.8[b]
18:1n-7	1.6±0.1	1.6±0.1	1.5±0.1
20:1n-9	1.0±0.2[a]	1.0±0.1[a,b]	0.6±0.1[b]
18:2n-6	2.9±0.1[a]	2.8±0.3[a]	4.1±0.4[b]
20:4n-6	1.4±0.1	1.3±0.1	1.6±0.1
20:5n-3	7.5±0.7[a]	6.1±0.2[a,b]	5.0±0.3[b]
22:4n-6	0.3±0.1	0.2±0.1	0.3±0.1
22:5n-6	0.7±0.1	0.6±0.1	0.7±0.1
22:5n-3	2.5±0.2[a,b]	2.1±0.1[a]	2.8±0.2[b]
22:6n-3	39.6±2.3[a,b]	42.3±0.9[a]	37.9±1.1[b]
Others*	5.9±0.4	5.7±0.3	5.3±0.4
DMA**	2.6±0.7[a]	3.0±0.4[a]	5.3±0.4[b]
Total SFA	28.6±2.3	28.1±0.8	25.7±1.0
Total MUFA	11.6±0.3[a]	11.1±0.5[a]	14.3±0.8[b]
Total PUFA	57.1±1.4	57.7±1.0	57.8±1.3
n-3/n-6	8.6±0.5[a]	9.3±0.6[a]	6.4±0.5[b]
UI	316.9±10.0	322.8±6.1	300.5±7.5

Results are expressed as mol%. Values are means ± s.e.m. (N=4 for each acclimation state). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* tests, $P \leqslant 0.05$).

*Others: total of 19 fatty acids detectable (14:0, 15:0, 17:0, 16:1n-5, 18:1n-5, 18:3n-3, 18:3n-6, 20:1n-11, 20:1n-7, 20:2n-6, 20:3n-6, 20:4n-3, 21:5n-3, 22:1n-11, 22:1n-9, 24:1n-11, 24:1n-9, 24:5n-3, 24:6n-3), none of which were more than 1.0%.

**Total of dimetylacetals (mainly 16:0DMA and 18:0DMA).

Abbreviations are defined in the List of abbreviations.

Effectively, warm acclimation caused more marked alterations in acyl composition of these phospholipids than for PC and PE, with many FA being halved or doubled in their levels. In CL, the decrease in 16:0 and monounsaturated FA, mainly 18:1n-7, was accompanied by a marked increase in 18:2n-6 during warm acclimation. The 18:2n-6, appeared to be an important fatty acid in CL compared to other phospholipids, and accounted for more than 20 mol% of total FA of CL in warm acclimated trout. In contrast, 22:6n-3, which was the most abundant fatty acid in CL, stayed constant during time course of warm acclimation. For LysoPC, the same PUFA varied as in diacylPC, diacylPE and PlsmPE, but the changes were more dramatic. Marked decreases in 22:6n-3 (from 53.4% to 32.4%) and in 20:5n-3 in warm acclimated trout (P<0.05) were accompanied by virtual doubling of 18:1n-9 and 16:0 (P<0.05). Few modifications in FA composition were noted for PI and PS (data not shown).

Discussion

To identify potential mechanisms by which oxidative capacities of mitochondria from trout oxidative muscle changed during transfer from 5°C to 15°C, the present study examined the modifications of phospholipid composition (phospholipid classes and the associated fatty acids), levels of membrane proteins and functional capacities on the same experimental preparations. The changes in functional capacities could not be explained by changes in the concentration of mitochondrial membrane proteins, but rather by specific modifications in the phospholipid bilayer. To assess our conclusions, we will compare our results for the functional capacities, protein composition and phospholipid composition with published studies that had generally described these characteristics separately.

Mitochondrial properties

Role of membrane proteins

Warm acclimation led to the expected shifts in mitochondrial oxidative capacities. Transfer of trout from 5°C to 15°C led to a slight increase in mitochondrial maximal oxidative capacity measured at 15°C in short-term warm exposed trout followed by a marked drop after warm acclimation at both assay temperatures The increase in capacity in short-term warm exposed trout was most pronounced for State 3 respiration rates expressed relative to cytochrome a and c_1 levels. Non-phosphorylating (State 4) rates of oxygen uptake increased with

Table 6. *Fatty acid composition of diacyl and plasmalogen forms of PC during the time course of warm acclimation*

	DiacylPC*			PlsmPC**		
Fatty acids	Cold acclimated	Short-term warm exposed	Warm acclimated	Cold acclimated	Short-term warm exposed	Warm acclimated
14:0	1.9±0.2[a]	1.6±0.2[a,b]	1.2±0.2[b]	Trace	Trace	Trace
16:0	26.7±0.7[a]	28.7±0.4[b]	25.5±0.3[a]	9.9±1.5	9.9±0.7	6.9±0.7
18:0	1.2±0.1[a,b]	1.1±0.1[a]	1.7±0.1[b]	10.0±2.0	6.0±1.0	5.7±0.2
16:1n-7	1.7±0.1	1.5±0.1	1.7±0.3	Trace	Trace	Trace
18:1n-9	7.0±0.2[a]	6.5±0.3[a]	11.4±1.1[b]	6.1±0.4	4.5±0.9	5.9±0.8
18:1n-7	1.2±0.1	1.2±0.1	1.4±0.1	Trace	Trace	Trace
20:1n-9	0.3±0.1	0.4±0.1	0.4±0.1	0.6±0.5	0.4±0.3	0.2±0.1
18:2n-6	2.1±0.1[a]	1.7±0.1[a]	3.0±0.2[b]	2.0±1.1	0.4±0.3	2.4±0.6
20:4n-6	1.4±0.1	1.3±0.1	1.6±0.1	2.5±1.5	1.5±1.1	0.5±0.3
20:5n-3	9.7±1.3[a]	8.5±0.3[a]	6.5±0.6[b]	1.0±0.6	0.9±0.4	2.4±0.7
22:4n-6	0.1±0.1	0.1±0.0	0.1±0.1	Trace	Trace	Trace
22:5n-6	0.5±0.1	0.4±0.1	0.5±0.1	Trace	Trace	Trace
22:5n-3	1.6±0.2	1.3±0.1	1.7±0.1	0.5±0.4	0.9±0.6	1.1±0.2
22:6n-3	40.8±0.5[a,b]	41.4±0.5[a]	38.5±1.3[b]	14.4±2.1	17.8±2.8	18.7±1.6
Others***	3.8±0.4	4.1±0.2	4.4±0.4	3.2±1.9	6.9±1.5	5.2±2.0
Total SFA	30.4±0.7[a]	32.0±0.1[b]	28.9±0.4[a]	19.8±1.4[a]	17.5±1.1[a,b]	13.8±1.5[b]
Total MUFA	12.1±0.1[a]	11.9±0.5[a]	17.2±1.3[b]	7.9±1.4	5.2±0.9	6.8±0.6
Total PUFA	57.5±0.6[a]	56.2±0.5[a,b]	53.9±1.7[b]	22.3±2.4[a]	27.3±1.8[a,b]	29.4±0.5[b]

Results are expressed as mol%. Values are means ± s.e.m. (N=4 for each acclimation state). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* tests, $P \leqslant 0.05$).

*DiacylPC refer to fatty acids at both *sn*-1 and *sn*-2 positions of the diacyl form.

**PlsmPC refer to *sn*-2 fatty acyl chains of the plasmalogen form. The total percent was adjusted to 50% to take into account the absence of the alkenyl chains of the *sn*-1 position hydrolyzed by the acid mobile phase as described in Materials and methods.

***Others: total of 18 fatty acids detectable (15:0, 17:0, 16:1n-5, 18:1n-5, 18:3n-3, 18:3n-6, 20:1n-11, 20:1n-7, 20:2n-6, 20:3n-6, 20:4n-3, 21:5n-3, 22:1n-11, 22:1n-9, 24:1n-11, 24:1n-9, 24:5n-3, 24:6n-3), none of which were more than 1.0% in any subclass.

Abbreviations are defined in the List of abbreviations.

transfer from 5°C to 15°C before returning to initial levels after long-term thermal acclimation. These functional characteristics of muscle mitochondria and their modification during warm acclimation confirmed the biphasic responses previously found for State 3 and 4 rates (Bouchard and Guderley, 2003). Maximal rates of oxygen uptake accounted for approximately 4% of the maximal capacity of CCO, as observed for Arctic char and rainbow trout muscle mitochondria (Blier and Lemieux, 2001; Bouchard and Guderley, 2003). The thermal sensitivity of the ADP affinity was dramatically modified during short-term warm exposure, when the ADP/O ratio increased, but warm acclimation returned these values to those observed in cold acclimated trout. In addition, an increase in ADP affinity was observed after warm acclimation. A loss of apparent ADP affinity with a decrease in temperature characterises rainbow trout mitochondria over much of their annual cycle (Blier and Guderley, 1993; Guderley and St Pierre, 1999), but thermal independence of the apparent ADP K_m (as for the short-term warm acclimated trout) occurs at both the coldest and the warmest periods of the year (Guderley and St Pierre, 1999). As the changes in mitochondrial functional properties we observed were similar to those previously determined, causal mechanisms uncovered are also likely to apply broadly.

Trout oxidative muscle mitochondria had similar cytochrome and ANT concentrations and relative levels as muscle mitochondria from carp, cane toads, chickens, guinea pig and rats (Williams, 1968; Wodtke, 1981a; Guderley et al., 2005). The increase of ANT during short-term warm exposure resembles the changes observed previously in trout muscle mitochondria (Bouchard and Guderley, 2003). Changes in the relative levels or concentrations of electron transport chain complexes could modify the catalytic capacity of the mitochondria (Sidell, 1983). Differences in cytochrome ratios and ANT in muscle mitochondria from rats, cane toads and bearded dragon lizards may contribute to interspecific variability in oxidative capacities (Guderley et al., 2005). However, the relative levels of cytochromes and ANT were quite constant during warm acclimation of trout, although warm acclimation of trout decreased the levels of cytochromes *b* and *c*. Cytochrome *b* is situated in Complexes II and III. If the portion associated with Complex III became less abundant,

Table 7. *Fatty acid composition of diacyl and plasmalogen forms of PE during the time course of warm acclimation*

	DiacylPE*			PlsmPE**		
Fatty acids	Cold acclimated	Short-term warm exposed	Warm acclimated	Cold acclimated	Short-term warm exposed	Warm acclimated
16:0	13.7±0.4[a]	13.1±0.3[a]	10.9±0.7[b]	6.2±1.7	5.0±0.5	4.5±0.5
18:0	7.1±0.5[a]	6.8±0.3[a]	9.7±0.8[b]	3.2±1.0	3.1±0.2	3.8±0.4
16:1n-7	0.5±0.1	0.4±0.1	0.4±0.1	0.2±0.1	0.4±0.1	0.5±0.1
18:1n-9	5.6±0.2	5.6±0.3	7.6±0.9	3.9±0.5[a]	5.4±0.4[a,b]	7.0±0.8[b]
18:1n-7	2.0±0.1[a]	2.0±0.1[a]	1.7±0.1[b]	0.5±0.1[a]	0.8±0.1[b]	0.8±0.1[b]
20:1n-9	2.0±0.2[a]	2.1±0.1[a]	1.4±0.1[b]	0.4±0.1	0.7±0.1	0.4±0.1
18:2n-6	2.8±0.1	2.5±0.2	3.1±0.4	1.4±0.3	1.3±0.2	1.7±0.2
20:4n-6	1.0±0.1[a]	1.1±0.1[a]	1.3±0.1[b]	1.0±0.1[a]	1.0±0.1[a]	1.4±0.1[b]
20:5n-3	4.2±0.3[a]	3.8±0.1[a]	3.1±0.2[b]	3.4±0.1	3.0±0.1	2.9±0.2
22:4n-6	0.6±0.1[a]	0.6±0.1[a]	1.1±0.1[b]	0.2±0.1[a]	0.2±0.1[a]	0.5±0.1[b]
22:5n-6	0.9±0.1	0.9±0.1	1.0±0.1	0.5±0.1	0.4±0.1	0.5±0.1
22:5n-3	4.0±0.2[a]	4.1±0.2[a]	6.1±0.4[b]	1.9±0.1[a]	1.7±0.1[a]	2.7±0.2[b]
22:6n-3	50.2±0.6[a,b]	51.5±0.3[a]	47.8±1.2[b]	25.3±1.8[a]	24.7±0.4[a]	21.0±1.4[b]
Others***	4.6±0.2	5.0±0.1	4.4±0.2	1.6±0.3	2.1±0.3	2.2±0.4
Total SFA	21.7±0.4	20.7±0.4	21.3±1.4	9.6±2.4	8.5±0.5	8.6±0.9
Total MUFA	10.9±0.5	11.2±0.4	11.9±1.2	5.9±0.8[a]	8.4±0.3[b]	9.8±0.9[b]
Total PUFA	67.4±0.8	68.2±0.5	66.8±1.8	34.5±1.9	33.1±0.6	31.7±1.5

Results are expressed as mol%. Values are means ± s.e.m. (N=4 for each acclimation state). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* tests, $P \leqslant 0.05$).

*DiacylPE refer to fatty acids at both *sn*-1 and *sn*-2 positions of the diacyl form.

**PlsmPE refer to *sn*-2 fatty acyl chains of the plasmalogen form. The total percent was adjusted to 50% to take into account the absence of the alkenyl chains of the *sn*-1 position hydrolyzed by the acid mobile phase as described in Materials and methods.

***Others: total of 19 fatty acids detectable (14:0, 15:0, 17:0, 16:1n-5, 18:1n-5, 18:3n-3, 18:3n-6, 20:1n-11, 20:1n-7, 20:2n-6, 20:3n-6, 20:4n-3, 21:5n-3, 22:1n-11, 22:1n-9, 24:1n-11, 24:1n-9, 24:5n-3, 24:6n-3), none of which were more than 1.0% in any subclass.

Abbreviations are defined in the List of abbreviations.

it could reduce the capacity for pyruvate oxidation. As cytochrome *c* is a substrate for Complexes III and IV, reductions in its availability could decrease mitochondrial oxidative capacity (Lesnefsky et al., 2001). On the other hand, the constant levels of cytochrome *a*, a component of Complex IV (CCO), suggest that the concentrations of Complex IV were maintained in warm acclimated trout. Carp acclimated to 30°C also maintain constant levels of cytochrome *a* in muscle mitochondria and decrease levels of $c+c_1$ (Wodtke, 1981a). Since warm acclimation caused parallel decreases in mitochondrial oxidative capacity and CCO activity, and since cytochrome *a* levels did not change, we conclude that the decreases in oxidative capacity were not caused by a change in the concentration of CCO.

Role of membrane lipid composition

Cholesterol. Our study evaluated potential means by which mitochondrial membrane lipid composition sets mitochondrial oxidative capacities during thermal change. To ensure proper functioning of biomembranes, the physical and chemical environment of the membrane must be regulated to maintain activity of membrane-associated proteins. Among membrane components, changes in cholesterol are suggested to play such a role during thermal acclimation (Crockett and Hazel, 1995).

The basic stoichiometry of mitochondrial membranes from trout red muscle, in particular the phospholipid to protein ratio, was in close agreement with data from carp red muscle (Wodtke, 1981b), and similar to the values obtained for mitochondria from pig heart and rat liver (Comte et al., 1976; Hovius et al., 1990). The ratio of cholesterol to protein was in the range of published values. Thermal acclimation led to substantial changes in overall mitochondrial lipid composition, but left the ratio of cholesterol to protein unchanged. Specifically, the ratio of total phospholipids to protein was significantly decreased in mitochondrial membranes from warm acclimated trout. As suggested (Wodtke, 1981b), these modifications were most likely due to a lower proportion of inner membranes in mitochondria of warm acclimated trout. Indeed, although less frequently observed during thermal acclimation than changes in mitochondrial volume density, an increased mitochondrial cristae density occurs during winter acclimatisation in oxidative muscle of rainbow trout (St Pierre et al., 1998). Since cholesterol seems to be mainly associated with the outer mitochondrial membrane (Daum, 1985;

Table 8. *Fatty acid composition of cardiolipin (CL) and natural lysophosphatidylcholine (LysoPC) during time course acclimation*

	CL			LysoPC		
Fatty acids	Cold acclimated	Short-term warm exposed	Warm acclimated	Cold acclimated	Short-term warm exposed	Warm acclimated
16:0	8.9±0.7[a]	7.4±0.5[a]	3.2±0.2[b]	17.4±3.3[a]	18.9±2.2[a]	31.2±1.4[b]
18:0	2.0±0.4	1.3±0.1	2.2±0.4	2.4±0.5	5.2±1.5	4.2±13.
16:1n-7	1.2±0.1[a]	1.8±0.1[a]	3.2±0.4[b]	0.8±0.1	0.6±0.3	1.3±0.1
18:1n-9	7.4±0.2[a]	7.0±0.3[a]	6.1±0.2[b]	5.7±0.4[a]	7.0±0.6[a]	11.9±0.8[b]
18:1n-7	6.4±0.5[a]	6.3±0.3[a]	2.4±0.2[b]	1.0±0.1	1.1±0.2	1.4±0.1
20:1n-9	2.4±0.2[a]	2.1±0.2[ab]	1.7±0.2[b]	0.5±0.1	0.4±0.2	0.2±0.1
22:1n-11	3.1±0.5	3.3±0.4	2.2±0.2	Trace	Trace	Trace
18:2n-6	13.4±0.5[a]	14.4±0.5[a]	21.4±0.6[b]	2.0±0.3	2.2±0.6	3.5±0.3
18:3n-3	1.2±0.4[a]	1.7±0.1[a]	3.1±0.2[b]	Trace	Trace	Trace
20:4n-6	0.5±0.1	0.3±0.1	0.5±0.2	1.5±0.2	1.7±0.1	1.5±0.1
20:5n-3	1.2±0.1	1.0±0.1	1.2±0.1	9.7±0.7[a]	7.9±0.6[ab]	6.3±0.8[b]
22:4n-6	Trace	Trace	Trace	Trace	Trace	Trace
22:5n-6	0.6±0.1	0.5±0.1	0.6±0.1	0.8±0.1	0.8±0.2	0.7±0.1
22:5n-3	2.1±0.1[a]	1.4±0.1[b]	1.7±0.1[c]	2.0±0.1[a]	1.3±0.1[b]	1.7±0.2[ab]
22:6n-3	44.8±0.9	44.5±1.2	43.3±0.8	53.4±2.3[a]	50.2±4.5[a]	32.3±1.9[b]
Others***	5.5±0.4	5.9±0.2	5.7±0.4	1.3±0.1	1.1±0.9	2.1±0.6
Total SFA	12.3±1.3[a]	9.7±0.7[b]	6.8±0.4[c]	21.1±2.8[a]	25.3±4.1[a]	37.0±2.5[b]
Total MUFA	20.4±0.7[a]	21.3±0.6[a]	15.9±0.2[b]	8.6±0.6[a]	9.7±1.2[a]	15.3±0.6[b]
Total PUFA	67.2±0.7[a]	69.0±1.0[a]	77.3±0.6[b]	70.3±2.4[a]	65.0±1.8[a]	47.6±0.5[b]

Results are expressed as mol%. Values are means ± s.e.m. (N=4 for each acclimation state). Different superscript letters indicate values that differ between acclimation states (ANOVA and *a posteriori* tests, $P \leqslant 0.05$).

***Others: total of 18 fatty acids detectable (14:0, 15:0, 17:0, 16:1n-5, 18:1n-5, 18:3n-3, 18:3n-6, 20:1n-11, 20:1n-7, 20:2n-6, 20:3n-6, 20:4n-3, 21:5n-3, 22:1n-9, 24:1n-11, 24:1n-9, 24:5n-3, 24:6n-3), none of which were more than 1.0%.

Abbreviations are defined in the List of abbreviations.

Echegoyen et al., 1993), specific modifications of the proportion of cholesterol in the outer mitochondrial membrane with warm acclimation seem unlikely.

Phospholipid classes and subclasses restructuring. Phospholipids that constitute mitochondrial membranes should be regarded as structural partners of membrane proteins and as potential modulators of mitochondrial processes (Daum, 1985). Among mechanisms of membrane remodeling during thermal acclimation, alterations in phospholipid headgroup composition are a common response of poikiloterms (Hazel and Carpenter, 1985; Hazel, 1988; Hazel and Landrey, 1988a). In trout muscle mitochondria, the proportion of PE and PC, in particular the diacyl forms, responded the most to thermal change. These two phospholipid subclasses represented more than 78 mol% of total phospholipids. These rapid changes after short-term temperature change have been described in various cell membranes and have led to the concept that phospholipid headgroup alterations are central for regulation of physical and functional properties of cell membranes during the initial stages of thermal change (Hazel and Carpenter, 1985; Hazel, 1995). As reported (Hazel and Landrey, 1988a) and reviewed (Lee, 1991), these alterations in membrane lipid composition during warm acclimation are not consistent with HVA because a reduction in the numbers of PE should decrease, not increase, membrane order. The increase in lysoPC in warm acclimated trout also would not favour homeoviscous adaptation, as lysoPC increases membrane permeability when incorporated into PC bilayers (Kumar et al., 1988). On the other hand, as PE has a smaller headgroup than PC, decreased proportions of PE are believed to offset direct effects of high temperature on phospholipid volume and maintain an appropriate balance between bilayer-stabilising (lamellar phase forming, like PC) and -destabilising (hexagonal phase forming, like PE) lipids. Several lines of evidence suggest the PC/PE ratio is a primary determinant of membrane function (Hazel, 1995). The rapid adjustments of PE and PC found in mitochondrial membranes suggest that maintaining membrane status, even temporarily, within the time course of warm acclimation is critical.

Our study demonstrated the novel finding that diacyl and plasmalogen subclasses of PE and PC do not vary in parallel. Indeed, the proportion of plasmalogens of both phosphatides rose only in warm acclimated trout while the diacyl forms of PE and PC changed with short-term warm exposure, with

diacylPC increasing and diacylPE decreasing. Since temperature-induced changes in PE and PC were different for plasmalogen and diacyl forms, care should be taken when considering the 'PC/PE ratio' in membranes. From the present study, differences are evident since diacylPC/diacylPE ratios increased during warm acclimation (from 1.4 in cold acclimated trout to 1.8 for short-term warm exposed trout and 2.0 in warm acclimated) while diacylPC+PlsmPC/diacylPE+PlsmPE ratios increased after short-term warm exposure and decreased slightly with warm acclimation (1.2, 1.5 and 1.4, respectively, in cold acclimated, short-term warm exposed and warm acclimated trout). Few data exist concerning modifications of plasmalogen levels in biological membranes during thermal acclimation. However, the increase in total plasmalogen in warm acclimated trout is consistent with data from carp red muscle mitochondria (Wodtke, 1981b), goldfish brain (Roots and Johnston, 1968; Chang and Roots, 1985) and marine invertebrates (Dembitsky, 1979). To the best of our knowledge, ours are the first data on the time course of modifications in plasmalogen levels during thermal acclimation. The functional significance of these changes in membrane plasmalogens is unclear. Plasmalogens are glycerophospholipids characterised by an alk-l′-enylether bond in position *sn*-1 instead of an acyl bond in diacyl homologue. It has been suggested that the lack of the carbonyl oxygen in position *sn*-1 affects the hydrophilicity of the headgroup and allows stronger intermolecular hydrogen-bonding between headgroups. These properties would favour the formation of the non-lamellar structures, reflecting the propensity of ethanolamine plasmalogen to adopt the inverse hexagonal phase (Lohner, 1996). Plasmalogens were also reported to reduce transmembrane solute fluxes at high growth temperatures (Hazel and Williams, 1990). This could help minimise proton leak across mitochondrial membranes at elevated temperature. *In vitro*, the higher susceptibility of the vinyl-ether bond to oxidative attack led to the hypothesis that plasmalogens may act as anti-oxidants, protecting cells from oxidative stress (Brosche and Platt, 1998; Maeba and Ueta, 2003; Brites et al., 2004). Generation of free radical species of oxygen during mitochondrial electron transport chain can damage mitochondrial membranes and hinder function by generating lipid peroxides from unsaturated FA (Chang and Roots, 1985; Genova et al., 2004). For example, peroxidation of mitochondrial CL in aged rats is followed by losses of peroxidised CL and parallel decreases in CCO activity (Paradies et al., 1997). Warming exacerbates the formation of reactive oxygen species (ROS) in marine invertebrates (Keller et al., 2004). Beside other mechanisms (i.e. higher activities of antioxidant enzymes), increases in PlsmPE in trout mitochondria during warm acclimation may compensate for thermally enhanced rates of lipid peroxidation reactions at warm temperatures. Interestingly, changes in the proportion of PE (no distinctions being made between plasmalogens and the corresponding diacyl) during thermal acclimation are largely restricted to the inner membrane of rainbow trout liver mitochondria (Miranda and Hazel, 1996). Such an asymmetrical distribution of PlsmPE in the inner membrane of mitochondria would facilitate that interaction with free radical species.

FA restructuring

Modification of phospholipid FA composition is the most commonly observed cellular response to thermal change. Ectotherms are thought to adapt their temperature-sensitive membranes by increasing lipid unsaturation in response to low temperature (Hazel and Williams, 1990). This cellular response has been extensively related to compensation of membrane function and modification of activities of membrane-bound enzymes (Van Der Thillart and Modderkolk, 1978; Wodtke, 1981a; Wodtke, 1981b; Itoi et al., 2003a; Guderley, 2004). In the present study, the levels of the cytochromes or ANT could not explain the decrease in mitochondrial capacity or CCO activity during warm acclimation. These functional modifications could result from HVA acting through changes in the mitochondrial membrane FA composition. However, only modest variations in the UI or in the ratio of unsaturated to saturated FA in the total phospholipids occurred during warm acclimation. This suggests that the remarkable modulations in mitochondrial oxidative capacities, CCO activity and ADP affinity occur through mechanisms that may not significantly alter fluidity. This agrees with a compelling body of evidence indicating that many aspects of membrane organisation can influence function more than simple changes in lipid order (for reviews, see Lee, 1991; Hazel, 1995).

At the level of individual FA, inverse variations of the main unsaturated FA in the mitochondrial membranes occurred. In particular, the PUFA, 20:5n-3 and 22:6n-3, decreased during warm acclimation while 18:1n-9 and 18:2n-6 increased. These modifications are not dramatic but agree with the shift of mitochondrial FA composition of total phospholipids from rainbow trout red muscle with seasonal acclimatisation (Guderley et al., 1997) and with the increase of 18:2n-6 and decrease of 22:6n-3 and 20:5n-3 in total lipids from red muscle mitochondria of warm acclimated carp (Wodtke, 1981b) and warm acclimated goldfish (Van Der Thillart and Modderkolk, 1978). They are also consistent with reports for sea bass liver and heart mitochondria (Trigari et al., 1992). Because *cis* double bonds introduce a kink into the acyl chain, unsaturated FA pack less compactly and thus offset, to a significant degree, the increase in membrane lipid order caused by a drop in temperature. However, considerations of modulation of lipid order cannot explain why trout and other winter-active poikilotherms accumulate PUFA rather than monoenes in their membrane lipids at low temperature (Hazel and Williams, 1990), since monoenoic FA are superior to PUFA with respect to the magnitude (expressed on a per double bond basis) of the changes in fluidity they produce and the lower metabolic cost of their production (Stubbs and Smith, 1984; Rabinovich and Ripatti, 1991; Williams and Hazel, 1993). As suggested (Lee, 1991; Hazel, 1995), modification of overall membrane unsaturation may not be the primary aim of restructuring of membrane FA during thermal adaptation.

Only a small number of lipid molecules are thought to separate membrane proteins or protein aggregates, particularly in the protein dense mitochondrial membranes (Hazel and Williams, 1990). The lipid–protein associations required for appropriate protein conformations may demand specificity of acyl chain length and unsaturation more than an appropriate overall fluidity. Thus, modifications in specific phospholipid classes or subclasses may account for the modifications of mitochondrial activity, CCO activity and ADP affinity. Warm acclimation did not lead to the same FA changes in all phospholipid classes. Indeed, while warm acclimation led to mitigated FA changes of total phospholipids, changes largely reflecting the FA composition of diacylPE and diacylPC, which together represented ~80% of the phospholipids, warm acclimation caused marked alterations in LysoPC and CL, two minor phospholipid classes. In addition, while warm acclimation led to similar FA modifications for diacylPC, diacylPE, PlsmPC and PlsmPE, LysoPC and CL presented distinct FA alterations. In LysoPC, 16:0 rose with warm acclimation, while it decreased in PE and PC. In CL, both 18:1n-7 and 18:1n-9 decreased with warm acclimation (the former more than the latter), whereas they changed little or increased in PE and PC. Few analyses of FA composition of phospholipid classes are available for fish mitochondria, and even fewer consider minor classes. To the best of our knowledge, this study represents the first comprehensive report on changes of the FA composition of natural lysoPC with thermal acclimation. Although the function of lysoPC during thermal acclimation remains unclear, many functions in biological membranes have been attributed to it (Watanabe et al., 2006). LysoPC may regulate mediated membrane processes in highly curved bilayer domains (Kumar et al., 1988). The shorter lysoPC FA moities in warm acclimated trout may also constrain membrane curvature (Rigoni et al., 2005).

The remarkable specificity of FA modifications in CL with thermal acclimation is suggestive of interactions with membrane proteins. CL only occurs in mitochondria and plays an important role in the micro-environment of mitochondrial enzymes involved in oxidative phosphorylation, including cytochrome *c* oxidase, cytochrome bc_1 complex, ATPase, ANT and NADH reductase (Paradies et al., 2002; Schlame et al., 2000). The influence of cardiolipin on the micro-environment of mitochondrial complexes requires certain types of fatty acids (Schlame et al., 2005) and, in mammals, alteration of CL acyl composition modulates the activity of these membrane complexes (Yamaoka et al., 1988; Berger et al., 1993; Watkins et al., 1998). Warm acclimation of rainbow trout led to similar changes in the acyl composition of CL of muscle mitochondria as in carp even if the acyl chain composition differs between these species (Wodtke, 1981b). As in our study, changes in CCO activity during thermal adaptation of carp accompanied FA modifications of this annular phospholipid (Wodtke, 1981a). We therefore propose that the specific reduction of CCO activity in warm acclimation is due, at least in part, to an increase in 18:2n-6 in CL, concomitant with a decrease in 18:1n-7 and 16:0.

Parallels between membrane lipid composition and mitochondrial capacities

During thermal acclimation, phospholipids from trout muscle changed their proportions and FA composition following a time course similar to that shown for plasma membranes from trout kidney (Hazel and Landrey, 1988a; Hazel and Landrey, 1988b). Remodelling of phospholipid headgroups (diacyl forms) was the earliest detectable response, while FA compositions primarily changed with prolonged acclimation. Logically, the functional properties that changed during short-term warm exposure probably reflected the proportions of phospholipid classes, whereas those changing with warm acclimation likely followed both changes in phospholipid proportions and FA composition. Short-term warm exposure enhanced State 3 and 4 rates and changed the thermal sensitivity of the apparent ADP affinity. The adjustment of the diacylPE/diacylPC ratio during short-term warm exposure should modify the tendency of the bilayer to form reversed hexagonal phases, thus modifying the activity of membrane proteins. ANT activity in rats is strongly correlated with the membrane content of hexagonal phase-forming lipids (PE/PC) (Mak et al., 1983). Replacing PC with PE increases ADP/ATP exchange in liposomes (Krämer and Klingerberg, 1980). As ANT may help set mitochondrial affinity for ADP, the inverted thermal sensitivity of the ADP affinity after short-term warm exposure is consistent with such effects. The changes in acyl chain composition of specific phospholipids during warm acclimation coincided with the marked reduction of oxidative capacity and CCO activity. This reduction in capacity and CCO activity is unlikely to be due to removal of cardiolipin from the mitochondrial inner membrane (Stuart et al., 1998; Paradies et al., 2000; Schlame et al., 2000). Rather, the striking parallel between the modifications of FA composition of CL and oxidative capacities/CCO activities (Fig. 5) suggest that regulation by membrane lipids occurs, at least in part, *via* the acyl chain composition of CL. As ANT activity also depends on interactions with CL (Paradies et al., 1994; Schlame et al., 2000), the increased ADP affinity after warm acclimation may reflect the changed FA composition of CL.

Conclusion

Much as observed for plasma membrane from trout kidney, thermal acclimation brings rapid adjustments of diacyl phospholipid classes of mitochondrial membranes and slower changes in their specific FA composition. The levels of lysoPC and the plasmalogens, particularly in PE but also in PC, increased with warm acclimation. The parallel response times of mitochondrial properties suggest that proportions of diacylPE and diacylPC strongly affect State 4 rates and the thermal sensitivity of the ADP affinity, whereas the FA composition of specific phospholipids, particularly CL, sets the maximal oxidative capacities, the CCO activity and the strength of the ADP affinity. Specific FA modifications of lysoPC may also be of functional importance. Our data revealed new players during membrane modifications with

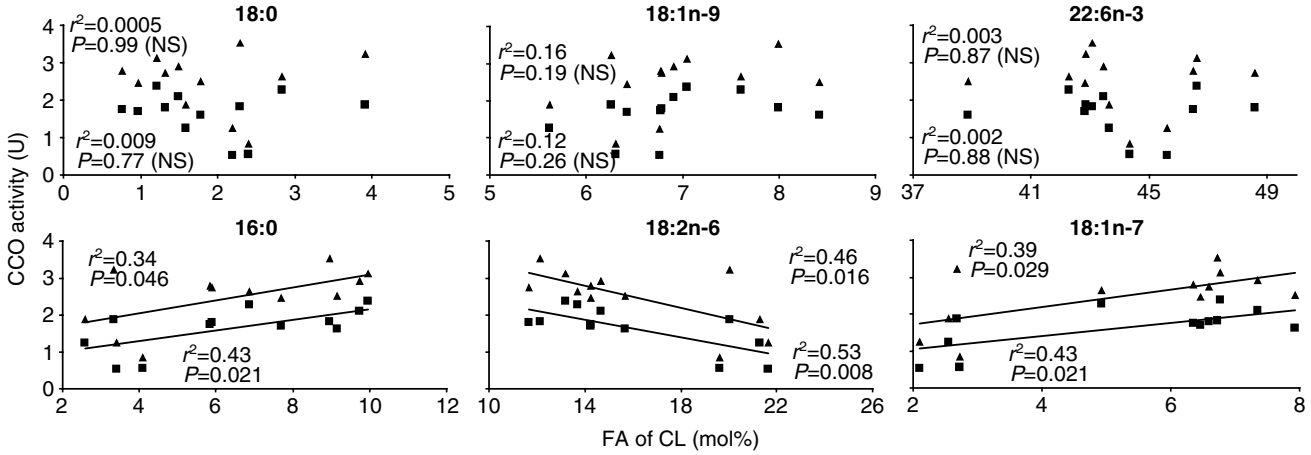

Fig. 5. Correlations between the activity of CCO and the main FA associated to CL of red muscle mitochondrial preparations. Activities plotted were measured at 5°C (squares) and 15°C (triangles). Lines represent statistically significant relationship.

thermal acclimation: the plasmalogen forms of PE and PC. The limited changes in the UI and the ratio of unsaturated to saturated FA in total phospholipids suggest that adjustments in overall membrane unsaturation are not the primary target of mitochondrial membrane restructuring. Rather, acyl chain restructuring seems to target specific minor phospholipid classes. Oxidative phosphorylation is presumably controlled by numerous reactions that each require adjustment during thermal acclimation. The modifications in respiration rates during the time course of warm acclimation may reflect adjustments of CCO activity and the affinity of ANT for ADP. Our data suggest that restructuring of membrane phospholipid classes and subclasses and their specific FA compositions plays an important role in such adjustments. Since the specific FA composition of CL has been suggested to have functional significance for many enzymes of oxidative phosphorylation, changes in the molecular species composition of CL are likely to be a central mechanism.

List of abbreviations

ANT	adenine nucleotide translocase
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAT	carboxyatractyloside
CCO	cytochrome *c* oxidase
CL	cardiolipin
diacylPC	diacylphosphatidylcholine
diacylPE	diacylphosphatidylethanolamine
DMA	dimethylacetal
FA	fatty acid
FAME	fatty acid methyl ester
GC	gas chromatography
HPLC	high performance liquid chromatography
HVA	homeoviscous adaptation
LysoPC	natural lysophosphatidylcholine
MUFA	monounsaturated fatty acid
PI	phosphatidylinositol
PlsmPC	plasmalogen choline
PlsmPE	plasmalogen ethanolamine
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Q_{10}	thermal sensitivity
RCR	respiratory control ratio
ROS	reactive oxygen species
SFA	saturated fatty acid
UI	unsaturation index

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