The Journal of Experimental Biology 206, 3455-3465

doi:10.1242/jeb.00578

Time course of the response of mitochondria from oxidative muscle during thermal acclimation of rainbow trout, *Oncorhynchus mykiss*

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Accepted 2 July 2003

Summary

The time course of changes in the properties of mitochondria from oxidative muscle of rainbow trout was examined during warm (15°C) and cold (5°C) acclimation. Mitochondrial oxidative capacities showed a biphasic response during thermal acclimation: at a given assay temperature, capacities first increased and then decreased during warm acclimation and showed the inverse pattern during cold acclimation. This was most apparent for maximal rates of state 3 oxygen consumption expressed per mg mitochondrial protein. Rates expressed per nmol ADP–ATP translocase (ANT) showed this pattern during cold acclimation. A biphasic pattern was also apparent for state 4 and oligomycin-inhibited (state 4_{ol}) rates of oxygen uptake expressed per mg protein. Changes in states 4 and 4_{ol} were smaller during cold than warm acclimation. Warm acclimation reduced the proportion of cytochrome *c* oxidase and citrate synthase needed during mitochondrial substrate oxidation. Phospholipid concentrations per mg mitochondrial protein changed little with thermal acclimation. Mitochondrial properties changed more quickly during warm than cold acclimation. While the biochemical modifications during thermal acclimation may eventually compensate for the thermal change, compensation did not occur at its onset. Rather, the initial changes of mitochondrial oxidative capacity in response to temperature change accentuated the functional impact of the thermal change, and prolonged exposure to the new temperature was required to attain a degree of thermal compensation.

Key words: mitochondria, thermal acclimation, oxidative muscle, enzyme activity, thermal compensation, rainbow trout, *Oncorhynchus mykiss.*

Introduction

Due to its marked effects on biochemical activities, temperature is of crucial importance to ectotherms, particularly for fish whose branchial respiration forces body temperature to follow that of the habitat. During an annual cycle, fish from temperate zones cope with a wide thermal range. To offset these thermal shifts, many species modify their biochemical and physiological properties (Hazel and Prosser, 1974). A common response in cold-active temperate-zone fish occurs at the level of the aerobic capacity of skeletal muscle. During cold acclimation, fish tissues can increase mitochondrial volume density (Jankowsky and Korn, 1965; Johnston and Maitland, 1980; Tyler and Sidell, 1984; Egginton and Sidell, 1989), activities of mitochondrial enzymes (Jankowsky and Korn, 1965; Hazel, 1972a,b; Shaklee et al., 1979; Johnston and Wokoma, 1986; Rodnick and Sidell, 1994; St. Pierre et al., 1998) or protein-specific oxidative capacities of their mitochondria (Guderley and Johnston, 1996; Guderley et al., 1997).

In rainbow trout (*Oncorhynchus mykiss*), cold acclimation and acclimatisation decrease the ratio of phosphatidylcholine to phosphatidylethanolamine (PC/PE) in membranes from liver mitochondria (Miranda and Hazel, 1996), increase the capacity of skeletal muscle mitochondria to oxidise pyruvate and acyl carnitines and increase polyunsaturation of mitochondrial phospholipids (Guderley et al., 1997). Cold acclimation and acclimatisation also increase the activity of some mitochondrial enzymes: β-hydroxyacyl CoA dehydrogenase (Guderley and Gawlicka, 1992), cytochrome *c* oxidase (CCO), citrate synthase (CS) and carnitine palmitoyl transferase (CPT) (St. Pierre et al., 1998). Furthermore, the cristae surface density of mitochondria (St. Pierre et al., 1998) and the total mitochondrial volume in oxidative muscle fibres increase at low acclimatisation temperature (Egginton et al., 2000). However, cold acclimation of rainbow trout does not increase the proportion of oxidative fibre volume occupied by mitochondria (St. Pierre et al., 1998; Egginton et al., 2000).

Little is known about the time course of thermal acclimation and, in particular, the time course of changes in mitochondrial capacities. During cold acclimation (from 15°C to 5°C) of goldfish (*Carassius auratus*), enzymatic activities initially decrease (between 6 h and 12 h) and subsequently stabilise between 48 h and 72 h (Lehmann, 1970). The PC/PE in plasma membranes of rainbow trout kidney decreases after 8 h of cold acclimation (from 20°C to 5°C; Hazel and Landrey, 1988a).

The proportions of saturated and monounsaturated fatty acids change most rapidly (16–48 h), while long-chain polyunsaturated fatty acids only increase after 10–21 days of thermal acclimation (Hazel and Landrey, 1988b). The beginning of warm acclimation (from 9°C to 28°C) of white sucker (*Catostomus commersoni*) leads to an induction of heat-shock protein 70 in glycolytic muscle, suggesting degradation of protein (Hardewig et al., 2000). The concomitant decrease of CS activity may reflect the lack of protection against degradation in the mitochondrial matrix.

Our study examines the time course of changes in oxidative capacities of mitochondria from oxidative muscle of trout during warm (5°C to 15°C) and cold (15°C to 5°C) acclimation. Trout were studied during the initial thermal change and during acclimation to the new temperature. Although these temperatures are well within the range naturally experienced by trout (Thibault et al., 1997), their best performance is observed after acclimatisation to intermediate temperatures (Taylor et al., 1996). By examining mitochondrial substrate oxidation at 5°C and 15°C, as well as the concentrations of mitochondrial components, we sought to evaluate the potential mechanisms by which oxidative capacities change. Thus, we measured (1) the levels of ADP–ATP translocase (ANT), the inner mitochondrial membrane carrier of nucleotides that constitutes a point of control of mitochondrial respiration (Groen et al., 1982), (2) the concentration of an integral component of the electron transport chain, cytochrome *b*, (3) activities of CS, CPT and CCO and (4) phospholipid and protein contents. We also measured these enzyme activities in oxidative muscle to assess the time course of changes in the aerobic capacity of muscle during thermal acclimation.

Materials and methods

Animals

Rainbow trout *(Oncorhynchus mykiss* Walbaum*)* were obtained from a fish hatchery (Ferme Piscicole Richard Boily, Île d'Orléans, Québec, Canada). Thermal acclimation occurred at the LARSA (*Laboratoire Régional des Sciences Aquatiques*) at Laval University in 15 80-litre tanks during warm acclimation and in two 1000-litre tanks during cold acclimation, with a constant photoperiod of 12 h:12 h L:D. Fish were fed commercial food (Corey Aquafeeds, New Brunswick, Canada) at maintenance rations, calculated for their size and tank temperatures, once a day. Fish for warm acclimation and cold acclimation had a mean initial body mass of 355±12 g (*N*=73) and 330.5±8.1 g (*N*=61), respectively.

Warm acclimation (cold to warm) began in February 2001 at 5°C. Fifteen fish were used to assess the oxidative capacities of these winter-acclimatised trout (field temperature, ~4°C). Then, water temperature was raised by 4°C over 2 days, and 12 specimens were studied during the subsequent 3 days. The water temperature was then warmed by a further 4°C over 2 days, and 12 specimens were studied during the subsequent 3 days. During the third and final thermal change, the water temperature was increased by 2°C over 1 day to reach 15°C. Another 12 fish were studied during the first 3 days at 15°C. Four weeks after the beginning of the temperature increments, eight trout were examined. Finally, 8 weeks after the beginning of the thermal change, when we assumed the trout were completely warm acclimated, 14 trout were used to assess final oxidative capacities.

Cold acclimation (15°C to 5°C) began in August 2001. Fifteen fish were used to assess the oxidative capacities of summer-acclimatised trout (field temperature, ~15°C). Water temperature was decreased by 4°C over 2 days, and 12 trout were studied during the next 3 days. Another decrease of 4°C corresponded to the second step of acclimation, and 10 more trout were examined during the subsequent 3 days. Due to some problems with the cooling system, the decrease to the final temperature (5°C) occurred 6 weeks after the initial thermal change. Because cold acclimation is typically slower than warm acclimation (Cossins et al., 1977), we assessed the final capacities 10 weeks after the beginning of the thermal change. Twelve trout were studied at each of the last two steps.

Tissue sampling

Fish were stunned by a blow to the head and rapidly killed by transection of the spinal cord behind the head. After measuring body mass and length, oxidative muscle on both sides was sampled. The intact caudal section, including the tail, was frozen at –80°C for later analysis of enzyme activities.

Isolation of mitochondria and respirometry

Mitochondria were isolated according to Guderley et al. (1997). The mitochondrial pellet was re-suspended in a volume of reaction buffer corresponding to one-tenth of the mass of muscle used (i.e. 300 ml of buffer for 3 g of muscle).

Oxygen consumption was measured at 5°C and 15°C according to Guderley et al. (1997). For each assay, malate was added to a final concentration of 0.38 mmol l^{-1} to spark the Krebs cycle, and pyruvate or palmitoyl carnitine was added to a final concentration of 2.38 mmol l^{-1} or 47.6 mmol l^{-1}, respectively. Oxidative phosphorylation (state 3) began with the addition of ADP to a final concentration of 0.93 mmol l^{-1}. After measurement of state 4 rates, 1 mg ml^{-1} oligomycin was added (state 4_{ol}) to evaluate oxygen consumption in the absence of oxidative phosphorylation (Estabrook, 1967).

Cytochrome b and ANT concentrations

Cytochrome *b* contents were evaluated by difference spectra read after reduction by 2 mmol l^{-1} succinate with electron flow blocked between cytochrome *b* and cytochrome c_1 by 2.28 mmol l^{-1} antimycin (Sherratt et al., 1988). Difference spectra against the oxidised sample were obtained with a double-beam spectrophotometer (Varian-Cary 210).

The concentration of ANT was measured in mitochondrial suspensions by titration with its noncompetitive irreversible inhibitor, carboxyatractyloside (CAT). Using the polarographic method, oxygen consumption with saturating ADP levels (3.72 mmol l^{-1}) was inhibited by adding small

Table 1. *Characteristics of experimental trout*

Acclimation step	Body mass (g)	Fork length (cm)	Condition factor[1]	Hepatosomatic index[2]	Number of individuals
Warm acclimation					
Initial	373.4±27.1	31.9±0.8	1.12±0.04	1.41±0.10^{a}	15
Week 1	333.8±31.4	31.7±0.9	1.05±0.03	1.22±0.14^{a,b}	12
Week 2	322.7±30.5	32.4±0.8	1.03±0.04	1.11±0.07^{a,b,c}	12
Week 3	322.5±22.7	30.8±0.7	1.10±0.03	0.95±0.06^{b,c}	12
Week 4	390.4±38.8	33.3±1.0	1.04±0.04	0.85±0.14^{b,c}	8
Week 8	387.2±29.1	33.1±0.8	1.06±0.02	0.80±0.04^{c}	14
Cold acclimation					
Initial	306.4±13.9^{b}	30.3±0.6	1.09±0.02	0.93±0.05^{b}	15
Week 1	305.4±18.1^{b}	30.5±0.5	1.07±0.03	0.95±0.05^{b}	12
Week 2	315.3±16.5^{a,b}	30.4±0.7	1.10±0.03	1.10±0.04^{a,b}	10
Week 6	375.2±17.2^{a}	32.3±0.5	1.11±0.01	1.08±0.03^{b}	12
Week 10	353.6±18.7^{a,b}	31.7±0.5	1.10±0.03	1.30±0.06^{a}	12

[1]Condition factor = (body mass × fork length^{-3}) × 100.

[2]Hepatosomatic index = (liver mass × body mass^{-1}) × 100.

Data are means ± S.E.M. and were compared between acclimation steps within each experiment. When values in a column are followed by different letters, they are significantly different (P≤0.05; ANOVA and Tukey's *a posteriori* test).

volumes (10 ml decreasing to 0.5 ml) of a 0.1 mmol l^{-1} CAT solution. 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 half of the CAT needed for inhibition, because two CAT molecules bind to one ANT molecule (Willis and Dallman, 1989).

Protein concentrations

The protein concentration in mitochondrial suspensions was determined by the bicinchoninic acid method (Smith et al., 1985), using 2% Triton X-100 to solubilise the membranes. Mitochondrial protein concentrations in oxidative muscle were calculated using CS activity in the muscle and mitochondrial preparations (U g^{-1} and U mg^{-1} protein) (St. Pierre et al., 1998): mg mitochondrial protein g^{-1} muscle = U g^{-1} muscle/U mg^{-1} mitochondrial protein.

Enzymatic activities

Enzymes were measured in oxidative muscle dissected from the frozen tails and in aliquot parts of the mitochondrial preparations that had been frozen at –80°C. Citrate synthase (CS), cytochrome *c* oxidase (CCO) and carnitine palmitoyl transferase (CPT) were measured at 5°C and 15°C according to the extraction and assay conditions in Thibault et al. (1997), except that the extraction buffer included 0.1% Triton X-100 and did not include fructose-2,6-bisphosphate. Mitochondrial suspensions were diluted in a buffer containing 50 mmol l^{-1} imidazole-HCl, 5 mmol l^{-1} EDTA, 0.1% Triton X-100 and 1 mmol l^{-1} reduced glutathione, pH 7.5. All assays were run in duplicate. 1 unit of enzymatic activity (U) corresponds to 1 mmol of substrate transformed to product per minute.

Phospholipid measurements

We followed Mills et al. (1984) in extracting total lipids from mitochondrial suspensions using chloroform:methanol (2:1) and 1 mol l^{-1} sulphuric acid. The phospholipid content was evaluated by measuring the phosphorus concentration. Phosphorus reacts with 8.5% ammonium molybdate and is reduced by 0.2% stannous chloride, forming a blue complex that was measured at 680 nm. The mass of phospholipid was calculated by multiplying the mass of phosphorus by 25 (Porter et al., 1996).

Statistical analysis

We used JMP IN 3.2.1 (SAS Institute Inc., Cary, NC, USA) to perform the statistical analyses. Analysis of variance (ANOVA) followed by Tukey multiple comparison *a posteriori* tests was used, with a level of significance of α=0.05. A logarithmic transformation of phospholipid data was performed to obtain homogeneity of variances, but untransformed data are shown.

Results

Overall characteristics of the trout

The externally measurable status of the trout changed little during the acclimation experiments: body mass, fork length and condition factor did not change (Table 1). Because the gonads were almost impossible to see, condition factor and hepatosomatic index were calculated using the body mass. The hepatosomatic index decreased during warm acclimation and increased during cold acclimation (P<0.05).

Muscle aerobic capacities: enzymatic activities

Thermal acclimation led to considerable compensation of

Table 2. *Activity of citrate synthase, carnitine palmitoyl transferase and cytochrome* c *oxidase in oxidative muscle, expressed in U g^{-1} wet muscle*

	Citrate synthase		Carnitine palmitoyl transferase		Cytochrome *c* oxidase	
Step	5°C	15°C	5°C	15°C	5°C	15°C
Warm acclimation						
Initial	17.1±0.6[a]	28.4±0.7[a]	0.37±0.02[a]	0.74±0.03[a]	54.3±3.0[a,b]	65.5±3.0
Week 1	16.9±0.8[a]	27.7±1.2[a]	0.25±0.03[b,c]	0.58±0.04[b]	64.4±3.2[a]	74.6±2.9
Week 2	16.8±0.6[a,b]	27.1±1.0[a]	0.30±0.02[a,b]	0.56±0.03[b]	63.4±4.2[a,b]	74.8±5.0
Week 3	17.8±0.7[a]	29.7±0.8[a]	0.31±0.02[a,b]	0.58±0.03[b]	61.9±2.5[a,b]	72.6±2.2
Week 4	14.8±0.8[a,b]	25.2±1.1[a,b]	0.23±0.02[b,c]	0.47±0.03[b,c]	57.2±4.3[a,b]	66.7±4.4
Week 8	14.2±0.6[b]	22.6±0.9[b]	0.20±0.01[c]	0.42±0.02[c]	50.4±2.5[b]	64.1±3.5
Cold acclimation						
Initial	16.6±0.4[b]	26.9±0.6	0.188±0.006[c]	0.45±0.01[c]	56.7±3.1	70.0±3.6
Week 1	17.7±0.5[a,b]	28.0±0.7	0.25±0.01[b]	0.50±0.02[c]	57.0±2.0	73.2±2.6
Week 2	17.4±0.5[a,b]	27.3±0.5	0.29±0.03[b]	0.53±0.02[b,c]	63.8±4.7	79.8±6.2
Week 6	18.1±0.5[a,b]	29.3±1.0	0.32±0.02[b]	0.59±0.02[b]	61.9±3.5	73.4±3.9
Week 10	19.2±0.7[a]	29.8±1.1	0.40±0.02[a]	0.76±0.03[a]	58.5±2.6	66.8±2.9

Data are means ± S.E.M. and are compared between acclimation steps within each experiment at each assay temperature. When values in a column are followed by different letters, they are significantly different ($P \leq 0.05$; ANOVA and a Tukey's *a posteriori* test).

CS and CPT activity. The specific activity of CS and CPT in oxidative muscle decreased by the end of warm acclimation at both assay temperatures ($P<0.05$), while CCO activities at 5°C initially increased and then returned to initial values (Table 2). By the end of cold acclimation, the activities of CS at 5°C and of CPT at both assay temperatures were higher than the initial activity ($P<0.05$; Table 2). CCO activity in homogenates was unchanged by cold acclimation.

Changes in maximal mitochondrial capacities during thermal acclimation

During warm and cold acclimation, the respiratory control ratios (RCR; state 3/state 4) of isolated mitochondria oxidising pyruvate ranged from 4.5 to 11.2, while those with palmitoyl carnitine ranged from 3.5 to 9.5. For both substrates at both assay temperatures, RCRs decreased with warm acclimation and increased with cold acclimation. During warm acclimation, for a given assay temperature, maximal (state 3) rates of pyruvate oxidation (expressed over the different denominators; i.e. mg protein, ANT, cytochrome *b* and phospholipids) were equivalent to those of palmitoyl carnitine, except at the end of the experiment when pyruvate was more readily oxidised (week 8; Figs 1, 2). During cold acclimation, mitochondria typically oxidised pyruvate at higher rates than palmitoyl carnitine (Figs 1, 2). The Q_{10} of pyruvate oxidation was approximately 2, whereas that for palmitoyl carnitine was approximately 2.5. Neither changed markedly with thermal acclimation.

During warm acclimation, maximal oxidative capacities changed in a biphasic fashion at both assay temperatures. Pyruvate and palmitoyl carnitine oxidation per mg mitochondrial protein increased until week 2 ($P<0.05$) and then decreased markedly until week 8 ($P<0.05$; Figs 1, 2). When expressed per nmol ANT, state 3 rates again decreased by the end of warm acclimation, but no initial increase was apparent. With nmol cytochrome *b* as the denominator, the decrease was less pronounced and only significant in the case of palmitoyl carnitine oxidation. When oxidation rates were expressed per mg phospholipids, rates did not change during warm acclimation (Figs 1, 2). During warm acclimation, the fraction of CCO and CS activity needed for maximal rates of pyruvate and palmitoyl carnitine oxidation decreased by week 8 (shown in Fig. 3 for pyruvate oxidation).

During cold acclimation, maximal rates of pyruvate oxidation changed in a biphasic pattern with rates at both assay temperatures, first decreasing ($P<0.05$) and then increasing ($P<0.05$; Fig. 1). This biphasic pattern was apparent when rates were expressed per mg mitochondrial protein and per nmol ANT. Six weeks of cold acclimation increased rates of pyruvate oxidation per nmol cytochrome *b* and palmitoyl carnitine oxidation per mg protein, nmol ANT and nmol cytochrome *b* ($P<0.05$; Figs 1, 2). Rates expressed relative to mg phospholipids decreased by week 1 and subsequently rose slightly (Figs 1, 2). The proportion of mitochondrial CCO and CS activity used during maximal rates of pyruvate oxidation (state 3/CCO and state 3/CS) initially decreased and then rose slightly during cold acclimation (Fig. 3).

State 4 and state 4_{ol} rates during thermal acclimation

Generally, for a given assay temperature, state 4 and state 4_{ol} rates (per mg protein) of pyruvate oxidation were equivalent to those for palmitoyl carnitine during warm and cold acclimation (Fig. 4). State 4_{ol} rates were roughly 60% of state 4 rates, suggesting that ATPase activity increases state 4 rates above those due to proton leak. During warm acclimation, state 4 respiration rates for both substrates at 15°C increased

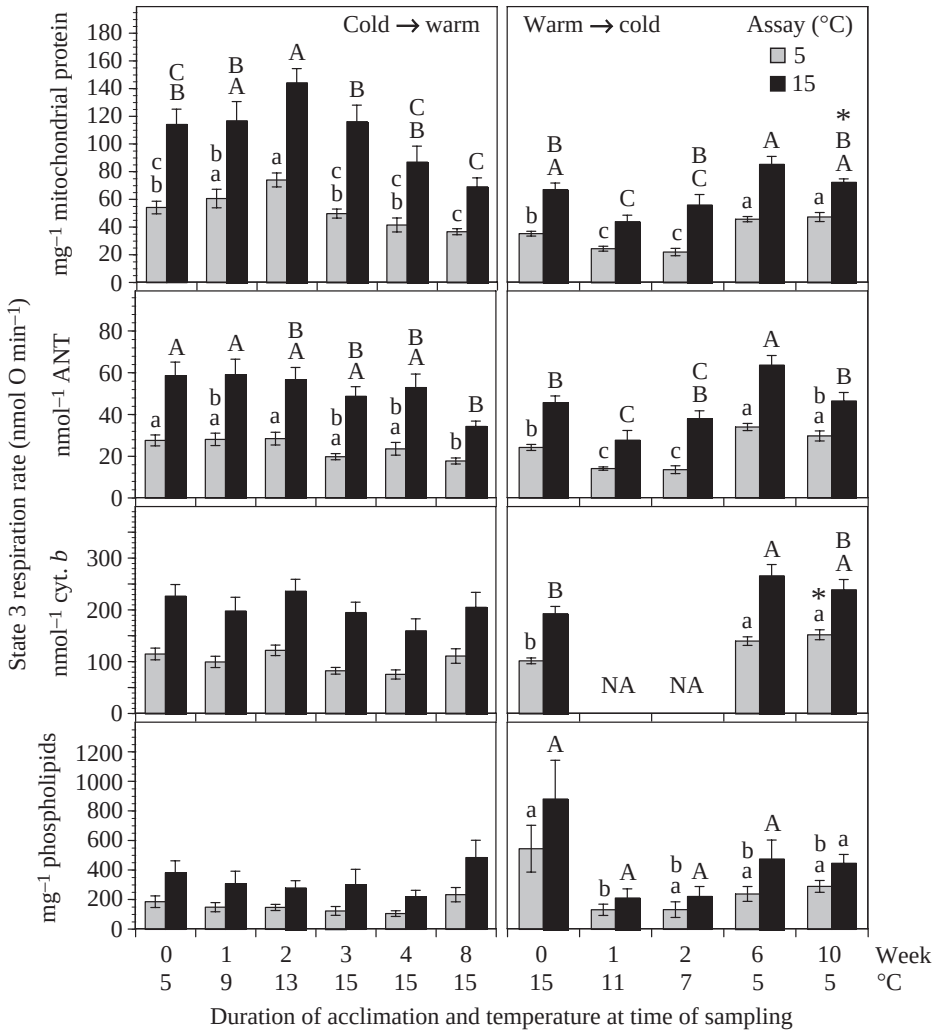

Fig. 1. Time course of changes in maximal rates of pyruvate oxidation by mitochondria from oxidative muscle during warm and cold acclimation. Rates are expressed per mg mitochondrial protein, per nmol ADP–ATP translocase (ANT), per nmol cytochrome *b* (cyt. *b*) and per mg phospholipids. Data are means ± S.E.M. When rates at a given assay temperature during each acclimation experiment differ, they are followed by different letters ($P \leq 0.05$; ANOVA and Tukey's *a posteriori* test). *Denotes a significant difference between final cold rate (week 10) and initial cold rate (initial in warm acclimation). No differences were found between final warm rate (week 8) and initial warm rate (initial in cold acclimation). The number of individuals for each experiment is shown in Table 1. NA, not available.

until week 2 ($P<0.05$) and afterwards returned to initial rates (Fig. 4). State 4_{ol} rates showed this pattern at both assay temperatures. During cold acclimation, state 4 rates tended to decrease initially and then return to or exceed initial values. This biphasic pattern was most apparent for state 4_{ol} rates (Fig. 4). Thus, states 3, 4 and 4_{ol} per mg protein showed similar time courses during thermal acclimation, with initial changes being reversed with extended acclimation.

Changes in muscle aerobic capacity during thermal acclimation

During warm acclimation, CS activity in mitochondrial suspensions (U mg^{-1} protein) increased until week 2 and afterwards returned to initial values (Table 3). CPT levels increased during weeks 1 and 2 and subsequently decreased and stabilised. CCO activities assayed at 5°C increased at week 2 and then returned to initial values. During cold acclimation, CS activity in mitochondrial suspensions increased after two weeks, returning to initial values by week 10 (Table 3). CPT activities gradually rose, while CCO activity in mitochondrial suspensions increased considerably by week 6.

Mitochondrial protein concentration in oxidative muscle was estimated from CS activity in mitochondrial and muscle extracts (see Materials and methods). During warm acclimation, mitochondrial protein concentration decreased from week 0 to week 2 ($P<0.05$) and generally remained below initial values until the end of acclimation (Fig. 5). During cold acclimation, mitochondrial protein content did not change ($P>0.05$). Using oxygen uptake per unit CS and the specific activity of CS in muscle, we calculated the maximal rates of oxygen uptake per g muscle. This calculation indicated that the capacity of muscle for mitochondrial substrate oxidation (nmol O_2 min^{-1} g^{-1} wet mass muscle) at 15°C decreased during warm acclimation. During cold acclimation, the estimated aerobic capacity at 5°C first decreased and then increased above initial values (Fig. 5). Thus, the biphasic nature of the changes in mitochondrial capacities during warm and cold acclimation was reflected in the overall aerobic capacity of muscle.

Mitochondrial concentrations of phospholipids, ANT and cytochrome b

The mitochondrial phospholipid concentration decreased from approximately 0.5 mg mg^{-1} protein to 0.27 mg mg^{-1} protein during warm acclimation and remained at 0.27 mg mg^{-1} protein during cold acclimation. Overall, phospholipids typically represented less than half the mass of protein in the mitochondrial preparations.

During warm acclimation, ANT levels were approximately 2.5 nmol mg^{-1} mitochondrial protein and did not change significantly. During cold acclimation, ANT concentrations were somewhat lower (1.5–1.8 nmol mg^{-1} protein), increased

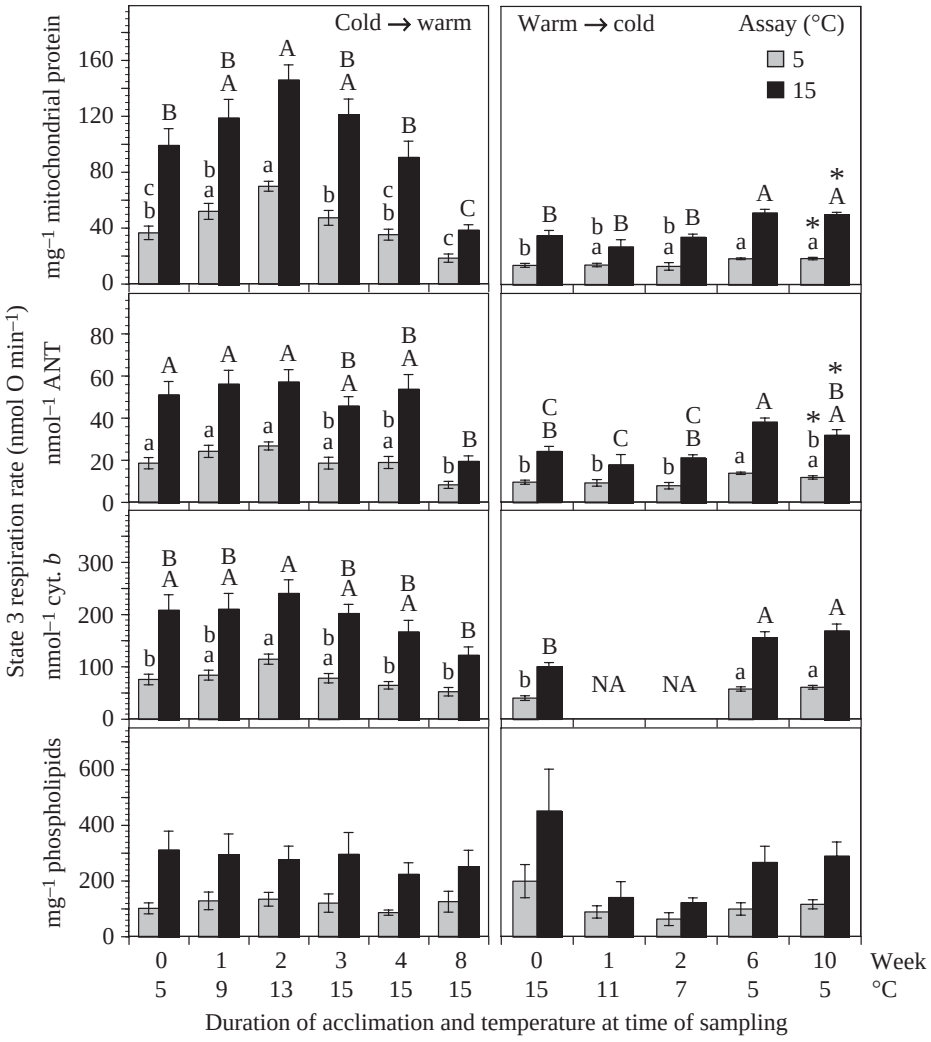

Fig. 2. Time course of changes in maximal rates of palmitoyl carnitine oxidation by mitochondria from oxidative muscle during warm and cold acclimation. Rates are expressed per mg mitochondrial protein, per nmol ADP–ATP translocase (ANT), per nmol cytochrome *b* (cyt. *b*) and per mg phospholipids. Data are means ± S.E.M. When rates at a given assay temperature during each acclimation experiment differ, they are followed by different letters ($P \leq 0.05$; ANOVA and Tukey's *a posteriori* test). *Denotes a significant difference between final cold rate (week 10) and initial cold rate (initial in warm acclimation). No differences were found between final warm rate (week 8) and initial warm rate (initial in cold acclimation). The number of individuals for each experiment is shown in Table 1.

Table 3. *Activity of citrate synthase, carnitine palmitoyl transferase and cytochrome* c *oxidase in mitochondrial suspensions, expressed in U mg^{-1} mitochondrial protein*

	Citrate synthase		Carnitine palmitoyl transferase		Cytochrome *c* oxidase	
Step	5°C	15°C	5°C	15°C	5°C	15°C
Warm acclimation						
Initial	0.74±0.05^{c}	1.02±0.03^{c}	0.014±0.002^{a,b}	0.019±0.001^{c}	1.37±0.09^{a}	1.73±0.09
Week 1	1.02±0.09^{a}	1.4±0.1^{a}	0.015±0.001^{a,b}	0.032±0.003^{a,b}	1.4±0.1^{a,b}	1.8±0.1
Week 2	0.98±0.04^{a,b}	1.54±0.05^{a}	0.0192±0.0009^{a}	0.036±0.001^{a}	1.8±0.1^{a}	2.1±0.1
Week 3	0.77±0.04^{b,c}	1.31±0.06^{a,b}	0.011±0.001^{b}	0.027±0.002^{b}	1.31±0.09^{b}	1.8±0.1
Week 4	0.64±0.04^{c}	1.05±0.06^{b,c}	0.012±0.002^{b}	0.026±0.003^{a,b,c}	1.4±0.1^{a,b}	1.6±0.2
Week 8	0.62±0.03^{c}	1.04±0.05^{c}	0.0121±0.0009^{b}	0.025±0.002^{b,c}	1.5±0.1^{a,b}	1.75±0.09
Cold acclimation						
Initial	0.59±0.02^{b}	1.01±0.06	0.0094±0.0005^{b}	0.020±0.001^{b}	1.35±0.08^{d}	1.58±0.09^{d}
Week 1	0.59±0.03^{b}	1.01±0.05	0.0106±0.0005^{b}	0.022±0.001^{b}	1.6±0.2^{c,d}	1.8±0.2^{c,d}
Week 2	0.69±0.03^{a}	1.17±0.06	0.0139±0.0006^{a}	0.028±0.001^{a}	1.93±0.05^{b,c}	2.21±0.07^{b,c}
Week 6	0.73±0.03^{a}	1.20±0.04	0.0160±0.0005^{a}	0.027±0.001^{a}	2.40±0.09^{a}	2.8±0.1^{a}
Week 10	0.66±0.02^{a,b}	1.09±0.04	0.0153±0.0006^{a}	0.028±0.001^{a}	2.19±0.07^{a,b}	2.56±0.10^{a,b}

Data are means ± S.E.M. and are compared between acclimation steps within each experiment for each assay temperature. When values in a column are followed by different letters, they are significantly different ($P \leq 0.05$; ANOVA and a Tukey's *a posteriori* test).

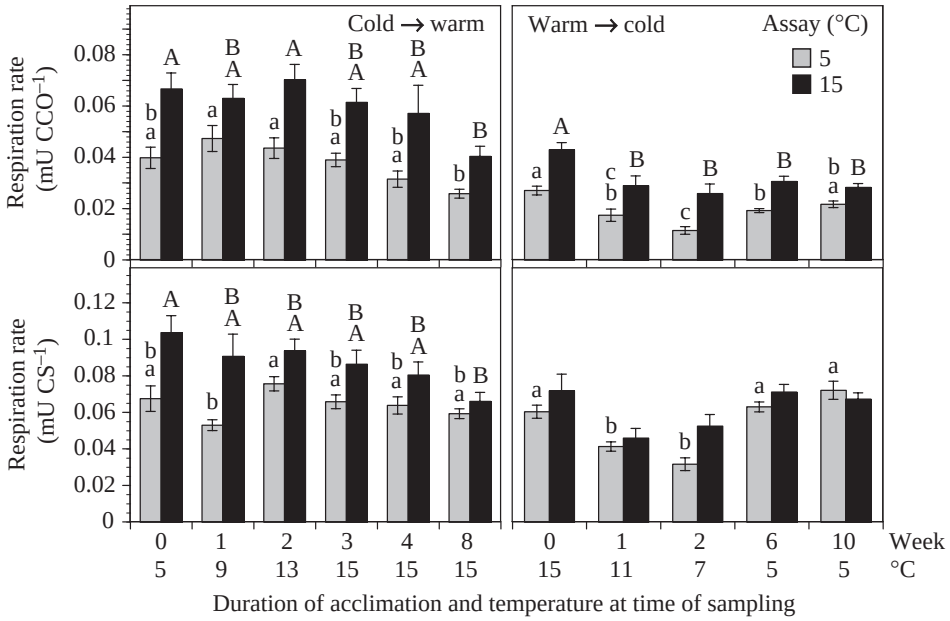

Fig. 3. Ratio of mitochondrial rates of pyruvate oxidation to maximal activities of cytochrome *c* oxidase (CCO) and citrate synthase (CS) during warm and cold acclimation. Values are expressed per mU CCO and per mU CS. Data are means ± S.E.M. When values at a given assay temperature during each acclimation experiment differ, they are followed by different letters ($P \leq 0.05$; ANOVA and Tukey's *a posteriori* test). The number of individuals in each experiment is shown in Table 1.

slightly at week 1, decreased at week 6 but did not differ at other times. During both warm and cold acclimation, ANT per mg phospholipid ranged between 5 nmol mg^{-1} phospholipid and 12 nmol mg^{-1} phospholipid but showed little significant variation.

Warm acclimation significantly increased cytochrome *b* content from 0.49 nmol mg^{-1} mitochondrial protein to 1.0 nmol mg^{-1} mitochondrial protein, but no changes were apparent during cold acclimation. Cytochrome *b* remained around 1.8 nmol mg^{-1} phospholipid during warm and cold acclimation.

Discussion

In fish muscle, mitochondria and aerobic capacity are a prime target during thermal acclimation and acclimatisation. The literature consensus is that cold acclimation increases muscle aerobic capacity and that these changes occur through increases in mitochondrial volume density or through changes in mitochondrial oxidative capacities (i.e. Jankowsky and Korn, 1965; Wodtke, 1974; Tyler and Sidell, 1984; Egginton and Sidell, 1989; Guderley and Johnston, 1996; Guderley et al., 1997; St. Pierre et al., 1998; Egginton et al., 2000). In our examination of the time course and possible mechanisms involved in this process, we show that mitochondrial oxidative capacity changes in a biphasic fashion during thermal acclimation. During warm acclimation, oxidative capacities of isolated mitochondria are first increased and then reduced. During cold acclimation, the inverse occurs, with an initial loss of capacity followed by a gradual increase. Nonetheless, an inverse symmetry in the responses during warm and cold acclimation was not found for all mitochondrial components.

As the condition factors of the trout did not change during acclimation experiments, it is unlikely that dietary limitations led to the responses of muscle mitochondria. Nonetheless, the higher hepatosomatic index in cold-acclimated and cold-acclimatised trout indicates that trout stored reserves in their liver when exposed to cold temperatures (Voss, 1985). This also occurs in goldfish and lake whitefish (*Coregonus clupeaformis*) (Van den Thillart and Smit, 1984; Blier and Guderley, 1988).

Biphasic responses during thermal acclimation were apparent for state 3, state 4 and oligomycin-inhibited rates of oxygen uptake, particularly when expressed per mg protein. During warm acclimation, these rates increased markedly at both assay temperatures until week 2. After week 3, mitochondria seemed to adjust to the warming, with rates at both assay temperatures then returning to or falling below initial values. Cold acclimation decreased state 3 rates of pyruvate oxidation at week 1 and increased these rates by week 6; states 4 and 4_{ol} showed similar tendencies. The fact that during warm and cold acclimation biphasic patterns were observed for distinct phases of mitochondrial substrate oxidation suggests a unifying causal mechanism.

The oxidative capacities of mitochondria are set by their protein content, the types of fatty acids and phospholipid head groups in their membranes, their inner membrane surface area (cristae surface density) as well as the relative levels of proteins involved in electron transport, oxidative phosphorylation and substrate breakdown. Control of oxidative phosphorylation by liver mitochondria is shared among several systems, including nucleotide translocation, substrate entry and electron transport (Groen et al., 1982). In our study, state 3 respiration rates expressed per mg mitochondrial protein and per nmol ANT changed almost in parallel with both substrates at both assay temperatures, particularly during cold acclimation. This observation is compatible with a shift in inner membrane phospholipid composition that could modulate the transport activity of ANT without altering its concentration. Effectively, cold adaptation of rats leads to a

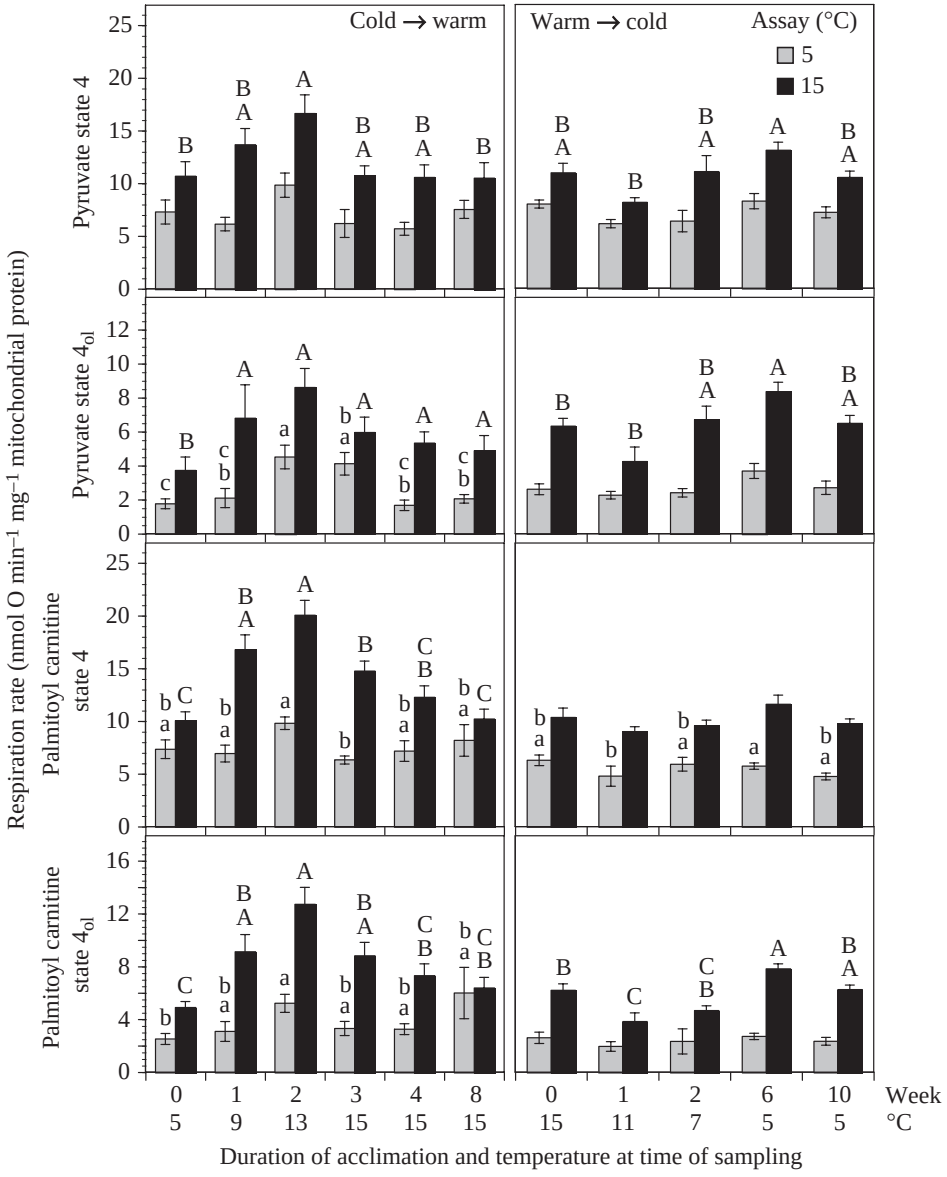

Fig. 4. Time course of changes in state 4 and oligomycin-inhibited state 4 (state 4_{ol}) rates of pyruvate and palmitoyl carnitine oxidation by mitochondria from oxidative muscle during warm and cold acclimation. Rates are expressed per mg mitochondrial protein. Data are means ± S.E.M. When rates at a given assay temperature during each acclimation experiment differ, they are followed by different letters ($P \leq 0.05$; ANOVA and Tukey's *a posteriori* test).

20% increase in V_{max} of ANT and a 20% decrease in K_m for external ADP while shifting the phospholipid composition of mitochondrial membranes, decreasing 18:2, increasing 20:4, as well as increasing PE and decreasing PC (Mak et al., 1983).

The biphasic responses of mitochondrial oxidative capacities during thermal acclimation may reflect modifications of the lipid portion of the mitochondrial membrane (Hulbert and Else, 2000). A similar mechanism may underlie the parallel changes in mitochondrial CPT and CCO activity. Interestingly, thermal transfer of rainbow trout led to biphasic changes in various lipid components of kidney plasma membranes (Hazel and Landrey, 1988a,b). A marked short-term change in phospholipid head groups (increased PC/PE with transfer to 20°C and a decrease with transfer to 5°C) was followed by a gradual return to intermediate values (Hazel and Landrey, 1988a). Transfer to 5°C led to a rapid increase in monounsaturated fatty acids in PC, with the inverse occurring during transfer to 20°C. These rapid changes were reversed during longer exposure to the new temperature. Polyunsaturated long-chain species gradually increased during cold acclimation (Hazel and Landrey, 1988b). Alternatively, the parallel changes of oxidative capacity per mg protein and per nmol ANT could be influenced by changes in the proportion of other rate-limiting elements.

Beyond the underlying biphasic response to thermal transfer, mitochondrial capacities seem to be modified by different mechanisms during warm and cold acclimation. During warm acclimation, the lack of change of state 3/cytochrome *b* coupled with a twofold increase of the levels of cytochrome *b*/mitochondrial protein suggests that cytochrome *b* levels help to set oxidative capacities. By contrast, during cold acclimation, mitochondrial oxidative capacities expressed per mg protein, per nmol ANT and per nmol cytochrome *b* showed the same time course, and cytochrome *b*/mitochondrial protein did not change. As warm and cold acclimation could stimulate different functional responses, notably on the level of chaperonin expression (Hardewig et al., 2000), differences in the underlying mechanisms may be expected.

Mitochondria from trout oxidative muscle have at least 10-fold higher capacities of CCO and CS (U mg^{-1} mitochondrial protein) than are used at maximal rates of pyruvate or palmitoyl carnitine oxidation. The capacity of these enzymes was exploited most completely in winter-acclimatised trout, where state 3 rates accounted for approximately 10% of CS activity and 6.5% of CCO activity. During both warm and cold acclimation, this percentage decreased, suggesting capacity limitations at other loci. Expressing mitochondrial oxidation rates relative to the maximal capacities of CCO and CS indicates considerable excess capacity at the level of these

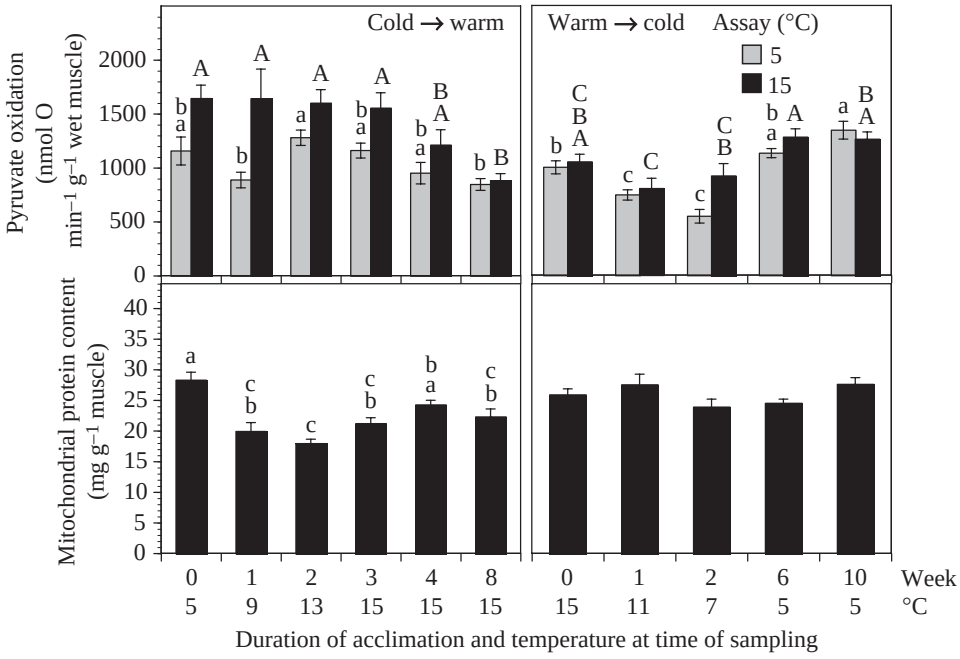

Fig. 5. Muscle aerobic capacity and mitochondrial protein content during thermal acclimation. Data are means ± S.E.M. When values at a given assay temperature during each acclimation experiment differ, they are followed by different letters ($P \leq 0.05$; ANOVA and Tukey's *a posteriori* test).

enzymes and argues against a major role limiting rates of oxidative phosphorylation.

Although CPT activities in mitochondria and oxidative muscle were 40-fold lower than the corresponding activities of CS and CCO, trout muscle mitochondria oxidised pyruvate and palmitoyl carnitine at similar rates. The explanation of this paradox may lie in the fact that CPT consists of two membrane-bound enzymes: CPT I, on the outer mitochondrial membrane, and CPT II, on the inner mitochondrial membrane. Palmitoyl carnitine is formed by CPT I, it then enters mitochondria through the carnitine carrier and is converted to palmitoyl CoA by CPT II. Therefore, the forward reaction of CPT I (palmitoyl-S-CoA + carnitine ⇌ palmitoyl carnitine + CoA-SH, as used in our activity measurements) is not needed during mitochondrial oxidation of palmitoyl carnitine. That rates of mitochondrial palmitoyl carnitine oxidation are fourfold the activity of mitochondrial CPT suggests that the catalytic rate of CPT II must be higher than that of CPT I. In mitochondria from rat heart, the formation of palmitoyl CoA by CPT II is about 10-fold faster than the formation of palmitoyl carnitine by CPT I (Palmer et al., 1977). Nonetheless, Palmer et al. (1977) found no difference between rates of mitochondrial oxidation of palmitoyl-*l*-carnitine and palmitoyl CoA. CPT activities in muscle and mitochondria from trout were similar to those in striped bass (*Morone saxatilis*) and in rat heart (Rodnick and Sidell, 1994; Palmer et al., 1977). While malonyl CoA inhibition of the forward reaction suggests that CPT I and CPT II have similar activities in striped bass muscle (Rodnick and Sidell, 1994), we know of no evaluations of the rates of the forward and reverse CPT reactions for fish muscle.

Thermal acclimation of trout led to only a partial compensation of mitochondrial oxidative capacities but to greater compensation of the capacity of muscle for substrate oxidation. Typically, mitochondrial respiration rates measured at 5°C in 5°C-acclimated individuals were slower than the same rates measured at 15°C in 15°C-acclimated fish. Furthermore, cold acclimation seemed incomplete even after 10 weeks, as mitochondrial oxidative capacities after 10 weeks of cold acclimation were weaker than rates for winter-acclimatised trout. On the other hand, after 8 weeks of warm acclimation, mitochondrial oxidative capacities resembled those for warm-acclimatised trout. The capacity of muscle for pyruvate oxidation (nmol O_2 min^{-1} g^{-1} muscle) showed marked thermal compensation during warm and cold acclimation, as did the capacity for palmitoyl carnitine oxidation during cold acclimation. These estimates combined maximal rates of mitochondrial substrate oxidation per unit CS with CS activities in muscle. CS and CPT activities in mitochondria and in oxidative muscle decreased during warm acclimation and increased during cold acclimation. However, cold acclimation led to marked changes of CCO activity per mg mitochondrial protein (present study) without changing muscle CCO activities (present study; Guderley and Gawlicka, 1992). The distinct patterns observed in mitochondrial and muscle enzyme activities could reflect distinct populations of mitochondria, one more easily extracted than the other, or could reflect differential extraction of the mitochondrial enzymes from oxidative muscle.

Phospholipid concentrations per mg mitochondrial protein changed little during thermal acclimation. The lack of significant differences in phospholipid concentrations and in respiration rates per mg phospholipid during thermal acclimation is consistent with literature reports. Studies on goldfish brain (Roots, 1968), goldfish gill mitochondria (Caldwell and Vernberg, 1970), carp (*Cyprinus carpio*) liver mitochondria (Wodtke, 1978), carp oxidative muscle mitochondria (Wodtke, 1981) and heart and liver mitochondria and microsomes from sea bass (*Dicentrarchus labrax*; Trigari et al., 1992) indicate a constant stoichiometry of phospholipid/protein with thermal acclimation. The levels of ANT and cytochrome *b* per mg phospholipid changed little during thermal acclimation. Thus, the basic membrane protein/phospholipid stoichiometry seems to change little during thermal acclimation, despite changes in fatty acid composition.

Mitochondrial oxidative capacities (states 3, 4 and 4_{ol} per mg mitochondrial protein for both substrates and assay temperatures) consistently changed more during the first three steps of warm acclimation than cold acclimation. This is consistent with literature reports that warm acclimation occurs more quickly than cold acclimation (Cossins et al., 1977) and with the underlying thermal dependence of biological processes. The more pronounced cold compensation in winter-acclimatised trout (St. Pierre et al., 1998; Guderley and St. Pierre, 1999) compared with cold-acclimated trout (Guderley and Gawlicka, 1992; present study) may reflect the additional influence of photoperiod.

In conclusion, we showed that thermal acclimation caused a biphasic response in many mitochondrial oxidative capacities, with the pattern observed during cold acclimation being an inverted image of that observed during warm acclimation. Particularly during cold acclimation, mitochondrial oxidative capacities expressed per ANT followed much the same time course as oxidative capacities expressed per mg mitochondrial protein. Fewer differences were observed when rates were expressed relative to cytochrome *b*. These responses are compatible with a major influence of the phospholipid and fatty acid composition of the inner membrane and of the concentration of proteins in the electron transport system upon mitochondrial oxidative capacities.

This study was supported by a grant from NSERC to H.G.

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