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RELATIVE CONTRIBUTION OF QUANTITATIVE AND QUALITATIVE CHANGES IN MITOCHONDRIA TO METABOLIC COMPENSATION DURING SEASONAL ACCLIMATISATION OF RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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Summary

This study examined whether changes in the properties of mitochondria from red muscle of rainbow trout *Oncorhynchus mykiss* are accompanied by ultrastructural changes during cold acclimatisation. We compared measurements at five levels of organisation in red muscle of winter- (1 °C) and summer- (16 °C) acclimatised trout. We examined (1) maximal rates of pyruvate and palmitoyl carnitine oxidation by isolated mitochondria, (2) enzymatic activities [cytochrome *c* oxidase (CCO), citrate synthase (CS), carnitine palmitoyl transferase (CPT) and phosphofructokinase (PFK)] of the muscle and isolated mitochondria, (3) mitochondrial protein content in the muscle, (4) the ultrastructure of muscle fibres, and (5) the cristae surface density of the mitochondria. All variables were measured on each trout sampled. The mitochondria from winter-acclimatised trout possessed higher maximal capacities for the oxidation of pyruvate and palmitoyl carnitine than those from summer-acclimatised trout. Muscle activities of CCO, CS and CPT were greater in winter than in summer trout, whereas the levels of PFK did not differ seasonally. Similarly, the mitochondria from winter trout had elevated levels of CCO, CS and CPT compared with those isolated from summer trout. The cristae surface density of the mitochondria from winter trout (40.2±0.6 $\mu m^2 \mu m^{-3}$; mean ± S.E.M.) was significantly higher than that from summer trout (36.4±1.2 $\mu m^2 \mu m^{-3}$), whereas there was no difference in the mitochondrial volume densities of muscle fibres from winter and summer trout. Thus, the considerable compensation of muscle aerobic capacity at low temperatures in trout is not accompanied by changes in mitochondrial volume density, but rather by shifts in enzyme levels and cristae surface density.

Key words: mitochondria, enzymatic activity, muscle, ultrastructure, thermal acclimatisation, fish, oxidative capacity, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Changes in the long-term bioenergetic requirements of a tissue often modify its aerobic capacity. To increase the aerobic capacity of a tissue, quantitative modifications could raise mitochondrial abundance or qualitative shifts could enhance the oxidative capacities of individual mitochondria. Both responses have been found in muscle of fish species that stay active in the cold. In goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), striped bass (*Morone saxatilis*), chain pickerel (*Esox niger*) and threespine stickleback (*Gasterosteus aculeatus*), cold acclimation augments muscle aerobic capacity by increasing the proportion of oxidative fibres in the musculature (goldfish, carp), the activities of mitochondrial enzymes (goldfish, bass, stickleback, pickerel) and the mitochondrial volume density in the fibres (goldfish, bass, crucian carp; for reviews, see Guderley, 1990; Johnston, 1993; Sänger, 1993). Furthermore, cold acclimation modifies the apparent activation energy and phospholipid composition of mitochondria isolated from red muscle of goldfish and common carp (Van den Thillart and Modderkolk, 1978; Van den Thillart and De Bruin, 1981; Wodtke, 1981*a*,*b*), but does not increase the cristae surface density of muscle mitochondria from goldfish and striped bass (Tyler and Sidell, 1984; Egginton and Sidell, 1989). The changes in phospholipid composition that occur during cold acclimation can heighten the activity of certain mitochondrial enzymes (Hazel, 1972; Wodtke, 1981*b*). Mitochondria from red muscle of cold-acclimated sculpin (*Myoxocephalus scorpius*) have higher oxidation rates, at a given assay temperature, for pyruvate and palmitoyl carnitine than those isolated from warm-acclimated sculpin (Guderley and Johnston, 1996). However, it is not known whether functional changes in mitochondrial properties are accompanied by changes in tissue ultrastructure.

Although rainbow trout *Oncorhynchus mykiss* cope with

important seasonal variations in temperature and stay active in the cold, few quantitative modifications in the musculature occur during cold acclimation/acclimatisation. In fact, cold acclimation/acclimatisation does not consistently lead to positive compensation of the activities of mitochondrial enzymes (Guderley and Gawlicka, 1992; Thibault *et al.* 1997; Milanesi and Bird, 1972). Cold acclimatisation increases the proportion of oxidative fibres in the musculature, although the highest capacity for sustained exercise at habitat temperature is found in trout acclimatised to an intermediate temperature (Taylor *et al.* 1996). In contrast to the limited responses at other levels, mitochondria from red muscle of cold-acclimatised trout show positive compensation of maximal rates of pyruvate and palmitoyl carnitine oxidation accompanied by changes in membrane phospholipid composition (Guderley *et al.* 1997).

Since quantitative and qualitative modifications to the mitochondria have not been examined simultaneously during investigations of a given response to thermal change, the relative importance of these levels of change cannot be evaluated. In this study, we aimed to determine whether changes in muscle ultrastructure accompany the marked positive compensation of mitochondrial oxidative capacities that occurs during cold acclimatisation of trout.

Materials and methods

Fish

Rainbow trout *Oncorhynchus mykiss* (Walbaum) were obtained from a local hatchery, Ferme Piscicole Richard Boily, Ile d'Orléans, Québec, Canada. The trout were kept in an outside pond and thus were exposed to seasonal changes in temperature and photoperiod. Fish were fed *ad libitum* daily with a shrimp-based food. Trout were sampled in February (N=9) at 1 °C and with a photoperiod of 10 h:14 h L:D, and in August (N=11) at 16 °C and with a photoperiod of 15 h:9 h L:D. After transport to Université Laval, trout were maintained at these temperature and photoperiod conditions in a 500 l recirculating aquarium for a maximum of 10 days. Trout sampled in February (winter-acclimatised trout; WT) and August (summer-acclimatised trout; ST) had a mean mass of 243±14 g and 543±21 g, and a mean fork length of 27.7±0.4 cm and 35.0±0.6 cm (means ± S.E.M.), respectively. We experienced considerable difficulty preparing coupled mitochondria from smaller summer-acclimatised fish, presumably because of the smaller amount of mitochondrial protein available in the volume of red muscle. Since working on individual fish was central to our sampling approach, we chose to use larger trout from which it was possible to isolate coupled mitochondria.

Fish of a similar mass and fork length to those used in the winter (189±15 g and 25.1±0.5 cm; means ± S.E.M.) were sampled in August (small summer-acclimatised trout; SST) at a temperature of 16 °C and a photoperiod of 15 h:9 h L:D to verify whether these trout possessed the same enzyme activities as those used for mitochondrial studies in August and to compare the enzyme activities of similarly sized winter and summer trout.

Sampling strategy

Fish were killed by a blow to the head and transection of the spinal cord before measuring their body mass and fork length. For WT and ST, red muscle was sampled at two sites on one side of the fish. Red muscle sampled at the level of the adipose fin (dorsal and ventral halves of the muscle in relation to the lateral line) was prepared for electron microscopy, and the red muscle remaining along the lateral line (dorsal and ventral halves) of the fish was used for mitochondrial isolation.

Fish were then frozen at −80 °C for subsequent determinations of enzyme activities and protein concentrations of the red muscle. Red muscle for enzymatic studies was sampled along the lateral line (dorsal half in relation to the lateral line) and at the level of the adipose fin (ventral half in relation to the lateral line). We sampled muscle at the level of the adipose fin to verify that the muscle sampled for electron microscopy was representative of the muscle sampled for mitochondrial and enzymatic studies. Protein concentrations were assessed in the red muscle homogenates used to measure enzyme activities.

SST were killed and frozen at −80 °C for subsequent measurements of enzyme activities and protein concentrations of the red muscle. The red muscle was sampled along the lateral line (dorsal half in relation to the lateral line) only. Protein concentrations were assessed in the red muscle homogenates used to measure enzyme activities.

Isolation and assay of mitochondria

Mitochondria were isolated and assayed according to Guderley *et al.* (1997) with the following modifications. Assay temperatures were maintained at 1, 8, 15 and 22 °C for WT and at 8, 15 and 22 °C for ST. Approximately 0.3 mg of mitochondrial protein was added to 1.0 ml of assay medium. Pyruvate and palmitoyl carnitine were used at saturating concentrations, namely 2.36 mmol l^{-1} and 94.3 μmol l^{-1}, respectively. For each experiment, we added malate (0.37 mmol l^{-1}) to spark the Krebs cycle. Maximal state 3 oxygen consumption rates were obtained by adding ADP to a final concentration of 0.47 mmol l^{-1}. The respiratory control ratio (RCR) was calculated from the ratio of the state 3 rate to the state 4 rate determined after all the ADP had been phosphorylated. A sample of the mitochondrial suspension was frozen at −80 °C for later assay of enzyme activities.

Assay of enzyme activities

Samples of red muscle from WT, ST and SST, as well as suspensions of mitochondria from WT and ST, were frozen for 1 month and thawed only once. According to Thibault *et al.* (1997), this freezing/thawing procedure does not affect the activity of cytochrome *c* oxidase (CCO), carnitine palmitoyl transferase (CPT) and phosphofructokinase (PFK), whereas the activity of citrate synthase (CS) is increased by approximately 30 %.

Samples of red muscle were homogenised on ice according to Thibault *et al.* (1997), except that 0.1 % Triton X-100 was added. The homogenates were centrifuged at 300 ***g*** for 10 min to clarify the supernatants. The use of Triton X-100 in the homogenisation buffer and the absence of high-speed centrifugation of the muscle homogenates allowed us to obtain maximal CCO activities (Tyler and Nathanailides, 1995). Mitochondrial suspensions were diluted in 10 volumes (v/v) of a buffer containing (in mmol l^{-1}): 50 imidazole-HCl, 5 EDTA, 1 reduced glutathione, and 0.1 % Triton X-100; pH 7.5, adjusted at room temperature (20 °C). Enzyme activities were measured according to Thibault *et al.* (1997). Assays were carried out at 1, 8, 15 and 22 °C for WT, at 8, 15 and 22 °C for ST and at 15 °C for SST. For WT and ST, enzyme activities in the samples of red muscle corresponding to those used for electron microscopy were measured at 8 °C. The enzyme activities in muscle and in mitochondrial suspensions were expressed in units g^{-1} fresh muscle and in units mg^{-1} mitochondrial protein, respectively (where 1 unit corresponds to 1 μmole of substrate transformed to product per minute).

Protein concentrations

All the protein concentrations were determined using the bicinchoninic acid method (Smith *et al.* 1985). Protein concentrations of mitochondrial suspensions were determined after removal of bovine serum albumin as described by Guderley *et al.* (1997), using 2 % Triton X-100 to solubilise the membranes. Total protein concentrations of the homogenates of red muscle were assessed after facilitation of protein solubilisation with urea and acetic acid (Somero and Childress, 1990). Mitochondrial protein concentrations in red muscle were calculated by dividing the activities of CCO and CS measured in the red muscle by the activity present in the mitochondrial suspensions:

$$[(\text{units g}^{-1}\text{ fresh muscle})/(\text{units mg}^{-1}\text{ mitochondrial protein}) = \text{mg mitochondrial protein g}^{-1}\text{ fresh muscle}].$$

Preparation of muscle for electron microscopy

Samples of red muscle were first fixed in 2 % glutaraldehyde, 0.1 mol l^{-1} cacodylate and 0.05 % $CaCl_2$ (m/v), pH 7.2, and then in 2 % osmium tetroxide (OsO_4), 0.1 mol l^{-1} cacodylate and 0.05 % $CaCl_2$, pH 7.2. After fixation, they were dehydrated in a graded ethanol series (30–100 %), placed in a solution of propylene oxide and finally impregnated with solutions containing a decreasing ratio of propylene oxide:epon (1:1–1:3 and finally only epon). Propylene oxide was used as infiltrating chemical. For each group of fish, three blocks of muscle were randomly chosen among the seven blocks prepared for each fish and cut at a slightly oblique angle in relation to the longitudinal axis of the muscle fibres, as suggested by James and Meek (1979).

Stereology: muscle fibres and mitochondrial ultrastructure

Ultrathin sections (approximately 90 nm) of fibres were placed on nickel grids (150 mesh) and double-stained with 2 % uranyl acetate/50 % ethanol and lead citrate (for the preparation technique, see Reynolds, 1963). They were then viewed with a transmission electron microscope (JEOL 1200 EX). Micrographs from nine randomly chosen fibres per fish for each group were taken at 1500× and printed at a final magnification of 3800×. Volume densities of total mitochondria *Vv*(mt,f), subsarcolemmal mitochondria *Vv*(ms,f), intermyofibrillar mitochondria *Vv*(mi,f), lipids *Vv*(lip,f) and myofibrils *Vv*(myo,f) were calculated using the point-counting method (Weibel, 1979). The space between the squares was between 1 and 1.5 times the average size of the profile of interest, namely 1.32 μm.

Micrographs from 18 randomly selected intermyofibrillar mitochondria per fish were taken at 30 000× and printed at a final magnification of 75 000×. The surface density of the cristae [*Sv*(cr,mi); the ratio of the surface area of the mitochondrial inner membrane to the volume of the mitochondrion] was determined using line-intercept measurements (Weibel, 1979). We traced the outline of the cristae before undertaking counting. No corrections were made for the effects of thickness or compression. Since we did not use a goniometer stage to evaluate the surface density of the cristae, the absolute values presented here are probably underestimates. However, comparisons among groups should not be affected.

Calculations and statistical analyses

To evaluate the oxygen content in the assay medium of mitochondria at each experimental temperature, we used solubility coefficients for oxygen determined by couloximetry (Johnston *et al.* 1994). The Fulton's condition index *I* of the fish was calculated using the following formula:

$$I = 1000m/L^3,$$

where *m* is mass (g) and *L* is fork length (cm).

Statistical comparisons were carried out using SigmaStat. Seasonal comparisons between WT and ST were made using the Student's *t*-test except when the SST group was included in the analysis. Comparisons among the three groups of fish (WT, ST and SST) evaluating the effect of size on the results (fish condition and size as well as CCO, CS and PFK activities of the red muscle at 15 °C) were made using one-way analysis of variance (ANOVA) followed by Tukey's *a posteriori* test. Comparisons between sampling sites of red muscle (lateral line and adipose fin) for WT and ST used the Student's paired *t*-test. Comparisons between respiratory control ratios (RCR values) of mitochondria for pyruvate and palmitoyl carnitine for a given season and assay temperature used the Student's paired *t*-test, whereas comparisons between assay temperatures within a season used one-way ANOVA followed by Tukey's *a posteriori* test. The significance level was P=0.05. Values are presented as means ± S.E.M.

Results

Fish condition and size

ST had a significantly higher condition factor (12.7±0.3)

than WT (11.3±0.4). However, the condition factor did not differ between ST and SST (12.7±0.3 for ST and 11.7±0.3 for SST) nor between WT and SST (P>0.05). ST (543±21 g and 35.0±0.6 cm) were larger than WT (243±14 g and 27.7±0.4 cm; P≤0.05). SST (189±15 g and 25.1±0.5 cm) were similar in size to WT (P>0.05).

Seasonal variation in maximal rates of substrate oxidation by isolated mitochondria

The RCRs for pyruvate at 15 °C and for palmitoyl carnitine at 8 and 15 °C were significantly higher in WT than ST (Table 1). For both WT and ST, the RCRs did not differ between the assay temperatures (P>0.05). For a given season and assay temperature, RCRs were generally higher for palmitoyl carnitine than for pyruvate (Table 1).

Maximal state 3 rates of oxidation of pyruvate and palmitoyl carnitine at 8, 15 and 22 °C by isolated mitochondria were higher for WT than for ST (Fig. 1). There was no difference between WT and ST in the relative importance of pyruvate and palmitoyl carnitine oxidation (P>0.05). Maximal rates of oxidation of pyruvate and palmitoyl carnitine at habitat temperatures indicated considerable, although incomplete, compensation of mitochondrial capacities with cold acclimatisation. We assumed a constant Q_{10} between 15 and 22 °C to evaluate rates at habitat temperature for ST. For pyruvate oxidation, mean winter rates were 46.3±2.6 nmol O min^{-1} mg^{-1} mitochondrial protein at 1 °C and summer rates were 106.5 nmol O min^{-1} mg^{-1} mitochondrial protein at 16 °C. For palmitoyl carnitine, mean winter rates were 54.6±4.5 nmol O min^{-1} mg^{-1} mitochondrial protein at 1 °C and summer rates were 109.1 nmol O min^{-1} mg^{-1} mitochondrial protein at 16 °C.

Protein concentrations

The protein concentration of the samples of red muscle taken along the lateral line and at the level of the adipose fin did not differ between WT and ST (Table 2). For ST, but not WT, the red muscle sampled at the adipose fin had a higher protein concentration than that sampled along the lateral line (Table 2). The concentration of mitochondrial protein in red muscle was higher in WT than in ST (Table 2).

Table 1. *Effects of thermal acclimatisation and assay temperature on respiratory control ratios of mitochondria isolated from red muscle of the rainbow trout* Oncorhynchus mykiss

Fish	Assay temperature (°C)	Respiratory control ratio	
		Pyruvate	Palmitoyl carnitine
WT	1	7.2±0.6 (9)	7.0±0.5 (8)
ST		ND	ND
WT	8	6.8±1.0‡(9)	8.1±0.6* (8)
ST		5.5±0.4 (9)	5.0±0.2 (9)
WT	15	6.5±0.5*,‡ (9)	8.2±0.6* (8)
ST		4.4±0.1‡ (8)	4.9±0.1 (8)
WT	22	5.5±0.4‡ (7)	6.4±0.6 (7)
ST		4.7±0.4‡ (3)	5.1±0.5 (5)

Values are means ± S.E.M. The number of individuals is indicated in parentheses. When a Student's paired t-test was used, the number of individuals was considered to be the lower N value for the pair.

WT, winter-acclimatised trout; ST, summer-acclimatised trout; ND, not determined.

*Significant difference between seasons; P≤0.05 (Student's t-test).

‡Significant difference between the two substrates for a given season and assay temperature; P≤0.05 (Student's paired t-test).

Enzymatic activities in oxidative muscle

The activities of CCO, CS and CPT at a given assay temperature were higher in WT than in ST (Table 3). Winter acclimatisation led to a greater increase in the levels of membrane-bound enzymes (CCO and CPT) than in those of CS, a matrix enzyme (Table 3). PFK levels did not increase during winter acclimatisation (Table 3). None of these

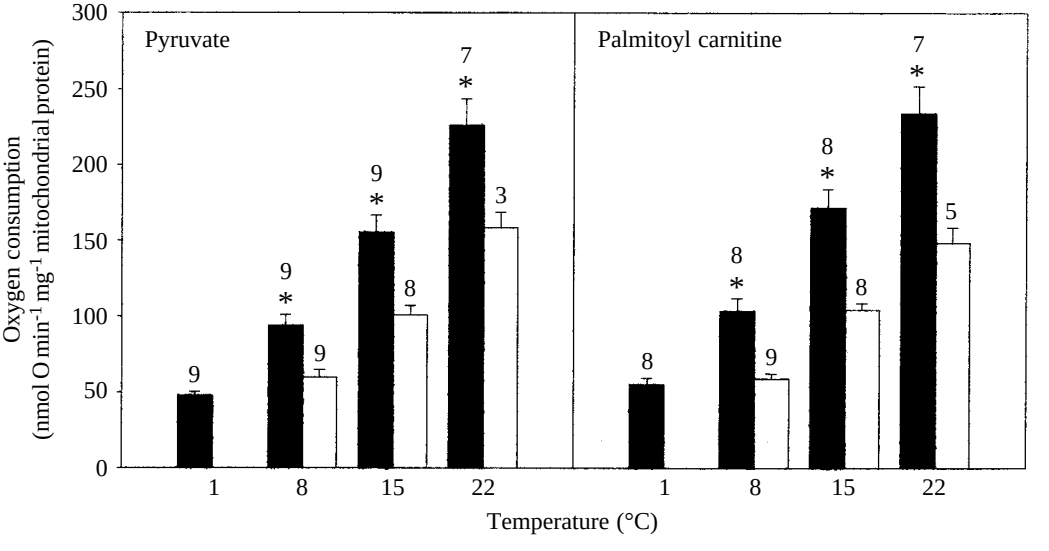

Fig. 1. Thermal sensitivity of maximal rates of pyruvate and palmitoyl carnitine oxidation by mitochondria isolated from the red muscle of winter- and summer-acclimatised rainbow trout. Oxidation rates for mitochondria from winter trout are indicated by filled columns, whereas those from summer trout are indicated by open columns. Values are means ± S.E.M. The number of individuals is indicated above each column. *Significant difference between seasons for a given assay temperature (P≤0.05; Student's t-test).

Table 2. *Influence of sampling location in red muscle on total protein content and effects of thermal acclimatisation on the total and mitochondrial protein concentrations of red muscle of the rainbow trout* Oncorhynchus mykiss

Protein (mg g^{-1} fresh tissue)	Sampling location	Fish	
		WT	ST
Total	Lateral line	126.7±4.7 (9)	118.1±4.8‡ (11)
	Adipose fin	129.9±5.9 (9)	139.3±6.7 (10)
Mitochondrial		28.2±1.9* (6)	16.1±1.7 (6)

Values are means ± S.E.M. The number of individuals is indicated in parentheses. When a Student's paired *t*-test was used, the number of individuals was considered to be the lower *N* value for the pair. Sampling locations for total proteins are described in Materials and methods.

WT, winter-acclimatised trout; ST, summer-acclimatised trout.

*Significant difference between seasons; $P \leqslant 0.05$ (Student's *t*-test).

‡Significant difference between the two sampling locations for the total protein within a season; $P \leqslant 0.05$ (Student's paired *t*-test).

conclusions was modified when allometric effects were taken into account. The levels of CS and PFK did not vary between SST and ST (Table 4). While the levels of CCO were significantly higher in SST than in ST (Table 4), this allometric difference did not modify our qualitative conclusions because the activity of CCO at 15 °C for WT is higher than that for SST ($P \leqslant 0.05$; compare Tables 3 and 4).

Enzyme activities at habitat temperatures revealed considerable compensation of aerobic capacity with cold acclimatisation. We assumed a constant Q_{10} for the enzymatic activities between 15 and 22 °C to calculate activities at habitat temperature (16 °C) for ST. The mean maximal capacities of CCO (37.0±3.5 units g^{-1} fresh tissue at 1 °C and 25.8 units g^{-1} fresh tissue at 16 °C) and CPT (0.24±0.02 units g^{-1} fresh tissue at 1 °C and 0.28 units g^{-1} fresh tissue at 16 °C) show greater compensation during cold acclimatisation than those of CS (16.0±1.3 units g^{-1} fresh tissue at 1 °C and 21.7 units g^{-1} fresh tissue at 16 °C). At first sight, the maximal capacities of CCO seem to have undergone an overcompensation during cold acclimatisation. However, allometric effects reduce this

Table 3. *Effects of thermal acclimatisation and assay temperature on enzymatic activities in red muscle of the rainbow trout* Oncorhynchus mykiss

Fish	Assay temperature (°C)	CCO (units g^{-1} tissue)	CS (units g^{-1} tissue)	CPT (units g^{-1} tissue)	PFK (units g^{-1} tissue)
WT	1	37.0±3.5 (9)	16.0±1.3 (9)	0.24±0.02 (9)	0.43±0.03 (7)
ST		ND	ND	ND	ND
WT	8	61.3±5.4* (9)	21.1±1.5* (9)	0.35±0.03* (9)	1.52±0.14 (9)
ST		18.2±1.7 (11)	15.5±0.6 (11)	0.16±0.02 (7)	1.94±0.29 (10)
WT	15	86.6±7.0‡ (9)	29.1±1.9‡ (9)	0.55±0.04‡ (9)	3.75±0.33 (9)
ST		24.9±2.1 (11)	21.0±0.9 (11)	0.27±0.03 (11)	3.91±0.43 (11)
WT	22	105.3±8.4* (9)	38.0±2.6* (9)	0.77±0.06* (9)	6.40±0.47 (9)
ST		31.3±2.0 (11)	26.4±1.3 (11)	0.39±0.03 (10)	5.76±0.73 (11)

Values are means ± S.E.M. The number of individuals is indicated in parentheses.

WT, winter-acclimatised trout; ST, summer-acclimatised trout; CCO, cytochrome *c* oxidase; CS, citrate synthase; CPT, carnitine palmitoyl transferase; PFK, phosphofructokinase; ND, not determined.

*Significant difference between seasons; $P \leqslant 0.05$ (Student's *t*-test).

‡Significant difference between seasons; $P \leqslant 0.05$ (one-way ANOVA and Tukey's *a posteriori* test). A one-way ANOVA was used at 15 °C because it was the only assay temperature where we had three groups of fish to compare, namely WT, ST and SST.

The results for SST are presented in Table 4.

Table 4. *Effects of size on enzymatic activities in red muscle of summer-acclimatised rainbow trout* Oncorhynchus mykiss

Fish	*N*	Mass (g)	Fork length (cm)	CCO (units g^{-1} tissue)	CS (units g^{-1} tissue)	PFK (units g^{-1} tissue)
ST	11	543±21	35.0±0.6	24.9±2.1*	21.0±0.9	3.91±0.43
SST	9	189±15	25.1±0.5	43.5±4.3	19.3±1.7	5.08±0.74

Values are means ± S.E.M. The assay temperature was 15 °C.

ST, summer-acclimatised trout; SST, small summer-acclimatised trout; CCO, cytochrome *c* oxidase; CS, citrate synthase; PFK, phosphofructokinase.

*Significant difference between groups of fish; $P \leqslant 0.05$ (one-way ANOVA and Tukey's *a posteriori* test).

Table 5. *Effects of thermal acclimatisation and assay temperature on enzymatic activities of mitochondria isolated from red muscle of the rainbow trout* Oncorhynchus mykiss

Fish	Assay temperature (°C)	CCO (units mg^{-1} mitochondrial protein)	CS (units mg^{-1} mitochondrial protein)	CPT (units mg^{-1} mitochondrial protein)
WT	1	1.68±0.19 (9)	0.64±0.04 (9)	0.0096±0.0013 (9)
ST		ND	ND	ND
WT	8	2.01±0.17* (9)	0.88±0.06* (9)	0.0140±0.0013 (9)
ST		1.58±0.09 (10)	0.69±0.02 (10)	0.0112±0.0007 (8)
WT	15	2.70±0.22* (9)	1.19±0.08 (9)	0.0227±0.0023* (6)
ST		2.04±0.12 (10)	1.13±0.02 (10)	0.0160±0.0008 (10)
WT	22	3.28±0.23* (9)	1.68±0.11 (9)	0.0292±0.0031* (9)
ST		2.53±0.15 (10)	1.51±0.03 (10)	0.0218±0.0007 (9)

Values are means ± S.E.M. The number of individuals is indicated in parentheses.

WT, winter-acclimatised trout; ST, summer-acclimatised trout; CCO, cytochrome *c* oxidase; CS, citrate synthase; CPT, carnitine palmitoyl transferase; ND, not determined.

*Significant difference between seasons; $P \leq 0.05$ (Student's *t*-test).

degree of compensation, although it remains higher than that of CS (Table 4). WT did not show any compensation of PFK activity (0.43±0.03 units g^{-1} fresh tissue at 1 °C and 4.1 units g^{-1} fresh tissue at 16 °C).

The pattern of seasonal variation of enzymatic activities was identical when we expressed the data as units g^{-1} fresh tissue or as units mg^{-1} total protein (data not shown). Thus, the changes in enzymatic activities were not due to seasonal variation in muscle protein content.

Overall, these data show that, during cold acclimatisation, the red muscle of rainbow trout increased its aerobic capacity and its capacity for lipid catabolism, but did not increase its glycolytic capacity. CCO and CPT, both membrane-bound enzymes, underwent a greater compensation of their maximal capacity during cold acclimatisation than did CS, a matrix enzyme.

Enzymatic activities of the mitochondrial suspensions

At a given assay temperature, mitochondrial suspensions from red muscle of WT generally had higher enzyme activities (expressed as units mg^{-1} mitochondrial protein) than those from ST (Table 5). The activities of CCO at 8, 15 and 22 °C and those of CPT at 15 and 22 °C were higher for WT than for ST (Table 5). The activity of CS at 8 °C was higher for WT than for ST (Table 5). These data suggest that the relative quantity of these enzymes per milligram of mitochondrial protein was higher in WT than ST. There was no seasonal variation in the Q_{10} of CCO and CPT between 8–15 °C and 15–22 °C. The Q_{10} for CS in the interval 8–15 °C was significantly higher for ST than for WT, but there was no seasonal difference in Q_{10} values between 15 and 22 °C (data not shown). Overall, these data suggest that the intrinsic properties of the mitochondria are modified during cold acclimatisation.

How representative are the muscle samples used for electron microscopy?

The muscle samples used for electron microscopy (taken at the level of the adipose fin) showed the same magnitude and direction of change in the activities of CCO, CPT and PFK, but not in those of CS, between seasons as the strips of muscle used for enzymatic and respirometric analyses (lateral line; compare Tables 3 and 6). The samples of muscle taken for electron microscopy from WT possessed higher levels of CCO and CPT than did those from ST (Table 6), whereas the levels of CS and PFK did not differ between these samples (Table 6).

Table 6. *Seasonal variations of enzymatic activities in samples of red muscle taken at the level of the adipose fin in rainbow trout* Oncorhynchus mykiss

Fish	CCO (units g^{-1} tissue)	CS (units g^{-1} tissue)	CPT (units g^{-1} tissue)	PFK (units g^{-1} tissue)
WT	83.4±15.7* (9)	22.8±1.9 (9)	0.42±0.04* (9)	1.58±0.15 (9)
ST	28.6±3.7 (10)	20.6±1.2 (10)	0.21±0.01 (5)	2.05±0.33 (9)

Values are means ± S.E.M. The number of individuals is indicated in parentheses. The assay temperature was 8 °C.

WT, winter-acclimatised trout; ST, summer-acclimatised trout; CCO, cytochrome *c* oxidase; CS, citrate synthase; CPT, carnitine palmitoyl transferase; PFK, phosphofructokinase.

*Significant difference between seasons; $P \leq 0.05$ (Student's *t*-test).

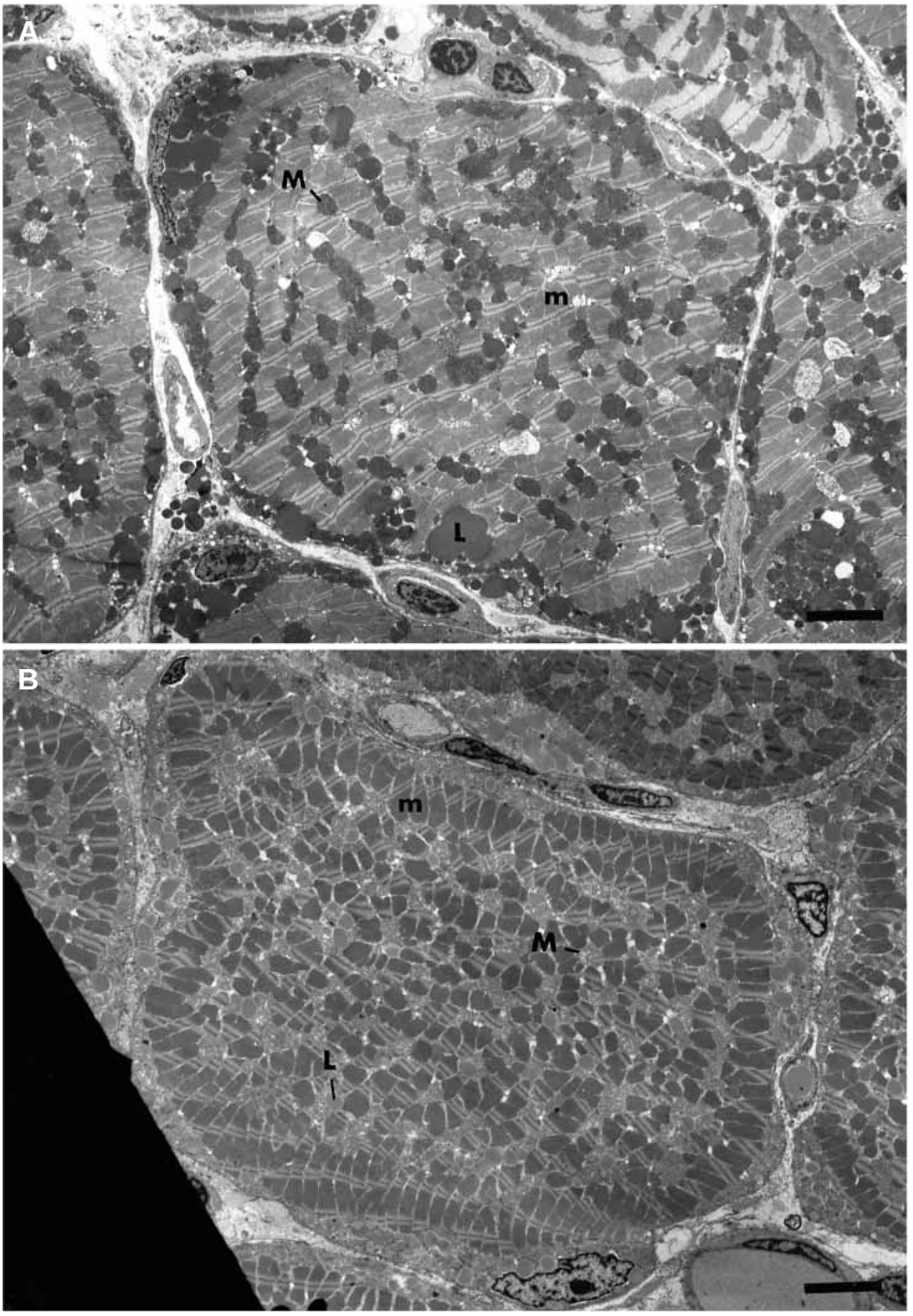

Fig. 2. Representative micrographs of red muscle fibres from rainbow trout acclimatised to winter (A) and summer (B) conditions. M, mitochondrion; m, myofibrils; L, lipids. Scale bars, 5 μm.

Together, these data suggest that the samples of muscle used for ultrastructural studies were representative of the samples taken for enzymatic and respirometric analyses, with the exception of CS levels.

Ultrastructure of fibres and mitochondria

The volume density of the mitochondria within the muscle fibres *Vv*(mt,f) and the distribution of the mitochondria between the subsarcolemmal *Vv*(ms,f) and intermyofibrillar *Vv*(mi,f) compartments were not modified during cold acclimatisation (Table 7; Fig. 2). The proportion of lipid within the muscle fibres *Vv*(lip,f) showed a tendency to be elevated in WT compared with ST, although the difference did not reach statistical significance (P=0.058). The increase in the proportion of lipid in the muscle fibres of WT contributed to the significant decrease in the volume density of the myofibrils *Vv*(myo,f) during winter (Table 7; Fig. 2). The cristae surface density of the mitochondria *Sv*(cr,mi) was higher in WT than ST (Table 7; Fig. 3). Thus, the increase in aerobic capacity of the muscle of WT is not paralleled by an increase in the volume

Table 7. *Effects of thermal acclimatisation on the ultrastructure of red muscle fibres and mitochondria of the rainbow trout* Oncorhynchus mykiss

Variable	Fish	
	WT	ST
Vv(ms,f)	11.3±0.4	11.2±0.8
Vv(mi,f)	15.4±0.5	15.7±0.5
Vv(mt,f)	26.9±0.9	27.0±1.0
Vv(myo,f)	56.3±1.5*	60.3±0.7
Vv(lip,f)	10.0±1.0	7.5±0.6
Sv(cr,mi) ($\mu m^2\,\mu m^{-3}$)	40.2±0.6*	36.4±1.2

Values are means ± S.E.M.; N=9 for WT and N=7 for ST.

WT, winter-acclimatised trout; ST, summer-acclimatised trout; *Vv*(ms,f), volume density of subsarcolemmal mitochondria; *Vv*(mi,f), volume density of intermyofibrillar mitochondria; *Vv*(mt,f), volume density of total mitochondria; *Vv*(myo,f), volume density of myofibrils; *Vv*(lip,f), volume density of lipids; *Sv*(cr,mi), surface density of mitochondrial cristae.

*Significant difference between seasons; $P \leqslant 0.05$ (Student's *t*-test).

density of the mitochondria within the muscle fibres, but is paralleled by changes in the intrinsic properties of the mitochondria.

Discussion

The oxidative capacity of mitochondria from red muscle of rainbow trout was considerably enhanced during cold acclimatisation without accompanying increases in mitochondrial volume density. Thus, changes in the mitochondrial properties *per se* seem to be sufficient to maintain an adequate aerobic capacity, even at the thermal extremes encountered by trout.

Cold acclimatisation of rainbow trout only led to partial compensation of the maximal capacities for mitochondrial oxidation of pyruvate and palmitoyl carnitine. At habitat temperatures, the mitochondria from summer trout had higher maximal oxidative capacities ($nmol\,O\,min^{-1}\,mg^{-1}$ mitochondrial protein) than those from winter trout. However, since winter trout have a higher concentration of mitochondrial protein than summer trout, the red muscles of summer and winter trout have a similar aerobic capacity at habitat temperature ($nmol\,O\,min^{-1}\,g^{-1}$ tissue). Mitochondria from red muscle of autumn-acclimatised rainbow trout possessed similar maximal capacities for the oxidation of pyruvate and palmitoyl carnitine at habitat temperatures to those of summer-acclimatised trout (Guderley *et al.* 1997). The more extensive compensation by mitochondria from autumn trout than from winter trout could be explained, in part, by the smaller decrease in habitat temperature (4 °C) for autumn trout or it may reflect a response to decreasing temperatures rather than to the autumn temperature *per se*.

The maximal oxidation rates of mitochondria are mainly set by membrane-bound systems: dicarboxylate carrier, adenylate nucleotide translocator and cytochrome *c* oxidase (Groen *et al.* 1982). Membrane lipid composition in trout tissues is rapidly modified during thermal changes (Hazel and Williams, 1990; Williams and Hazel, 1995; Miranda and Hazel, 1996; Guderley *et al.* 1997). These modifications enhance maximal oxidative capacities in the cold by altering the activity of the mitochondrial-membrane-bound systems. In fact, the lipid composition of the mitochondrial membrane of cold-acclimated goldfish *Carassius auratus* allows a higher activity of succinate dehydrogenase than that of warm-acclimated fish (Hazel, 1972). The membrane lipid composition explains the higher specific activity of CCO in cold-acclimated compared with warm-acclimated carp *Cyprinus carpio* (Wodtke, 1981*b*). The increased activity of CCO (present study) and the alterations in the membrane composition (Guderley *et al.* 1997) of muscle mitochondria from rainbow trout during cold acclimatisation could contribute to the elevated maximal oxidation rates of mitochondria from winter trout.

Changes in mitochondrial properties are a widespread, but not universal, response to thermal acclimatisation of fish. The oxidative capacity of muscle mitochondria ($nmol\,O\,min^{-1}\,mg^{-1}$ mitochondrial protein) is higher in cold- than in warm-

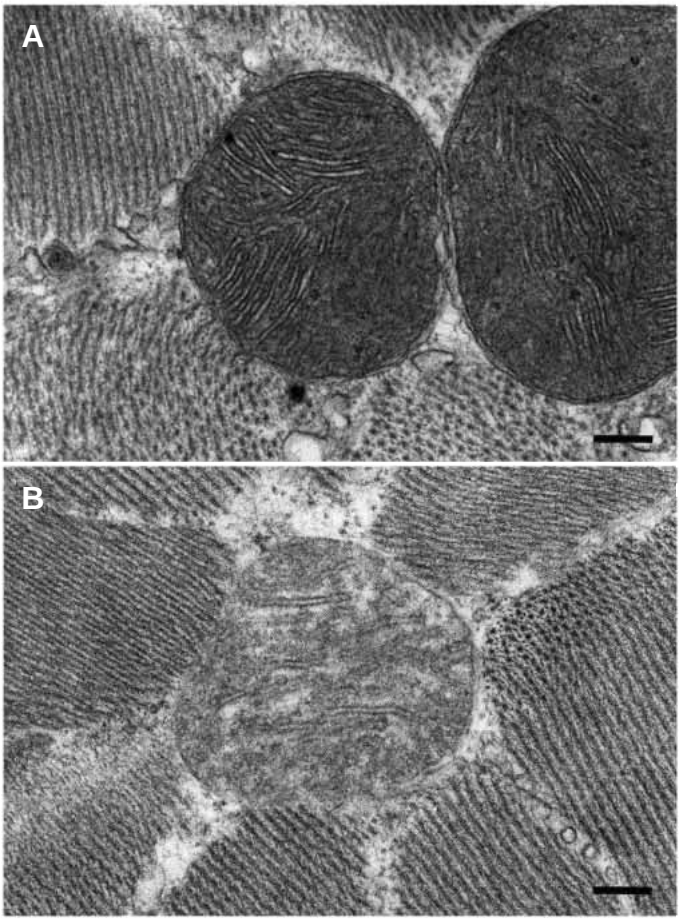

Fig. 3. Representative micrographs of intermyofibrillar mitochondria of red muscle fibres from rainbow trout acclimatised to winter (A) and summer (B) conditions. Scale bars, 200 nm.

acclimated sculpin *Myoxocephalus scorpius*, while the energy of activation for pyruvate oxidation is reduced (Guderley and Johnston, 1996). Cold acclimation of goldfish *C. auratus* modifies the Arrhenius plot for mitochondrial respiration and the phospholipid composition of muscle mitochondria (Van den Thillart and Modderkolk, 1978; Van den Thillart and De Bruin, 1981). In goldfish, cold acclimation increases the mitochondrial volume density without changing the cristae surface density (Tyler and Sidell, 1984). Thus, qualitative (membrane shifts) and quantitative (volume density) changes increase the mitochondrial respiration rate per gram of muscle. In contrast, long-term cold acclimatisation of the bass *Dicentrarchus labrax* diminishes the maximal oxidation rate for glutamate by heart and liver mitochondria and does not increase the unsaturation index of membrane lipids (Trigari *et al.* 1992).

Changes in tissue aerobic capacity can result from changes in mitochondrial volume density, modifications in mitochondrial capacities or a combination of both. Our studies at the tissue and mitochondrial levels indicate that the increase in mitochondrial enzyme activities (CCO, CS and CPT) in muscle of winter trout is paralleled by an increase in their levels in isolated mitochondria, but not by an increase in mitochondrial volume density. This augmentation in mitochondrial enzyme activities in winter trout resulted in CCO, CPT and CS activities at habitat temperatures similar to those of summer trout. During cold acclimation of striped bass *Morone saxatilis* and goldfish *C. auratus*, the increase in mitochondrial enzyme activities (for reviews, see Sidell, 1983; Sänger, 1993) is accompanied by a dramatic rise in mitochondrial volume density (Tyler and Sidell, 1984; Egginton and Sidell, 1989). Cold acclimation of lake whitefish *Coregonus clupeaformis* and of rainbow trout *O. mykiss* does not increase the activities of mitochondrial enzymes (Blier and Guderley, 1988; Guderley and Gawlicka, 1992), although Milanesi and Bird (1972) observed an augmentation in CCO activity following cold acclimation of rainbow trout. The activities at habitat temperature of mitochondrial enzymes in red muscle of rainbow trout increase during warm seasons and decrease during cold seasons (Thibault *et al.* 1997). The variability of the responses of trout to thermal acclimation could reflect the condition and specific strains of trout, as well as the analytical methods. Notwithstanding, our results show that, when cold acclimatisation led to an increase in muscle aerobic capacity, this increase was paralleled by modifications in the mitochondrial properties *per se* and not by increases in the mitochondrial volume density.

An augmentation of the volume density of lipids within muscle fibres has often been observed in cold-acclimated/acclimatised fishes (Egginton and Sidell, 1989; Sänger *et al.* 1990). This could accelerate the flux of oxygen in the musculature, because oxygen has a greater solubility in lipids than in water, although its coefficient of diffusion is the same for the two compartments (Egginton and Sidell, 1989). The higher proportion of lipids in the musculature of fishes in cold habitat temperatures could also indicate a higher demand for the oxidation of lipids. In fact, the red muscle of cold-acclimated rainbow trout *O. mykiss* and striped bass *M. saxatilis* oxidises palmitate more rapidly than that of their warm-acclimated counterparts (Dean, 1969; Jones and Sidell, 1982).

Although muscle aerobic capacity consistently increases with cold acclimation/acclimatisation of cold-active fishes, the ultrastructural changes vary between and within species, perhaps partly with acclimation protocols. Mitochondrial volume density in muscle increases following cold acclimation of striped bass *M. saxatilis*, crucian carp *Carassius carassius* and goldfish *C. auratus* (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Tyler and Sidell, 1984), but does not change with seasonal cold acclimatisation of rainbow trout, roach *Rutilus rutilus* and summer crucian carp (present study; Sänger *et al.* 1990; Kilarski *et al.* 1996). Mitochondrial cristae density does not change during cold acclimation of goldfish and striped bass or cold acclimatisation of crucian carp (Tyler and Sidell, 1984; Egginton and Sidell, 1989; Kilarski *et al.* 1996), although it increases with cold acclimatisation of trout (this study) and cold acclimation of summer crucian carp (Kilarski *et al.* 1996). Interestingly, in striped bass, the mitochondrial cristae surface density tends to increase during cold acclimation ($51.8\pm1.8\,\mu m^2\,\mu m^{-3}$ at 5 °C *versus* $46.9\pm1.4\,\mu m^2\,\mu m^{-3}$ at 25 °C; Egginton and Sidell, 1989), suggesting a combined response at these ultrastructural levels. Clearly, fish muscle mitochondria can undergo considerable restructuring in response to thermal change. In rainbow trout, the restructuring of mitochondria during cold acclimatisation increases maximal oxidation rates, enzyme activities and cristae surface density.

In summary, the data we present indicate for the first time that modifications in the intrinsic properties of mitochondria can suffice for compensation of muscle aerobic capacity at low temperatures. In fact, rather than increasing the volume density of mitochondria, red muscle fibres of winter-acclimatised trout possess mitochondria with different properties at both the functional and ultrastructural levels. Simultaneous monitoring at different levels of organisation led to this conclusion and could provide similar information in other systems undergoing changes in tissue aerobic capacity.

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