Throughout the life cycle of the rainbow trout (Oncorhynchus mykiss), the heart exhibits periods of enhanced growth. Two such instances are cardiac enlargement associated with sexual maturity in males and heart growth at seasonally low environmental temperatures. Heart growth includes a parallel increase in the number of mitochondria. These natural models of heart growth have been exploited to study protein synthesis directed by the mitochondrial genome.

Methods were developed to assess protein synthesis in mitochondria isolated from the heart of rainbow trout. Protein synthesis was assessed by tracking the incorporation of L-[2,6-3H]phenylalanine into trichloracetic-acid-precipitable protein. Amino acid incorporation into mitochondrial protein was linear with respect to time and was inhibited by chloramphenicol. Radiolabel was selectively enhanced in molecular mass fractions over the same size range as polypeptides known to be encoded by the mitochondrial genome. Protein synthesis was measured in mitochondria isolated from sexually mature animals and from animals subjected to different thermal regimes.

The relative ventricular mass of sexually mature male rainbow trout was significantly greater than that of sexually mature females (0.104±0.004 versus 0.087±0.002; mean ± S.E.M.). Mitochondria isolated from the heart of males synthesized protein at a faster rate than mitochondria isolated from the heart of females (0.22±0.02 versus 0.11±0.02 pmol phenylalanine mg⁻¹ protein min⁻¹). That is, ‘male’ mitochondria are inherently predisposed to synthesize protein at faster rates. We speculate that the difference may result from higher levels of mitochondrial RNA in males than in females.

Mitochondria isolated from the heart of sexually immature rainbow trout acclimated to 13 °C synthesized protein at the same rate at 25 °C (0.456±0.075 pmol phenylalanine mg⁻¹ protein min⁻¹) and 15 °C (0.455±0.027 pmol phenylalanine mg⁻¹ protein min⁻¹). However, the rate of protein synthesis was severely impaired at 5 °C (0.125±0.02 pmol phenylalanine mg⁻¹ protein min⁻¹). Since the rate of state 3 respiration by isolated mitochondria decreased in a linear fashion over the temperature range 25 to 5 °C, the rate of mitochondrial protein synthesis is not directly coupled to the rate of respiration. Thermal acclimation to 5 °C did not result in positive thermal compensation in either the rate of protein synthesis or the rate of oxygen consumption by isolated mitochondria.

In a further series of experiments, total protein synthesis and oxygen consumption were measured in isolated myocytes. The rate of oxygen consumption by myocytes remained constant over the temperature range 25 to 5 °C. There was no difference in the rate of total cell protein synthesis between 25 °C (1.73±0.29 pmol phenylalanine 10⁶ cells⁻¹ h⁻¹) and 15 °C (2.12±0.19 pmol phenylalanine 10⁶ cells⁻¹ h⁻¹), but at 5 °C protein synthesis was substantially impaired to approximately one-sixth of the level observed at 15 °C. As such, rates of total cell protein synthesis were not directly coupled to rates of respiration and were curtailed at low temperature.

In vitro studies show that mitochondria isolated from the heart of sexually mature male rainbow trout are inherently different from mitochondria isolated from the heart of females such that the former are able to synthesize protein at a faster rate. The rate of mitochondrial protein synthesis does not correlate with the greater than twofold changes in rates of oxygen consumption induced by acute changes in assay temperature, suggesting that protein synthesis is not directly coupled to rates of ATP or GTP synthesis.

Key words: cardiac hypertrophy, mitochondria, myocyte, Oncorhynchus mykiss, protein synthesis, rainbow trout, respiration, temperature acclimation, heart.
Introduction

The heart of some fish species exhibits enhanced rates of protein turnover under a variety of conditions and, as such, provides a useful model system in which to study the control of protein synthesis. In the current experiments, protein synthesis is monitored in rainbow trout (Oncorhynchus mykiss) during cardiac development associated with sexual maturity and as a function of acute and chronic thermal transitions. The major part of this study relates to the expression of proteins synthesized by the mitochondrial genome. Mitochondrial protein accounts for approximately one-third of the total protein in heart. Most of the protein in mitochondria is synthesized via the nuclear-cytoplasmic system and incorporated into mitochondria. But 13 peptide subunits, essential for four different inner membrane proteins, are encoded by mitochondrial DNA and synthesized on ribosomes in the mitochondria (Poyton and McEwen, 1996).

Relative ventricle mass (RVM, ventricle mass as a percentage of body mass) increases in male but not in female rainbow trout in association with seasonal gonadal development (Franklin and Davie, 1992; Bailey et al., 1997). Growth associated with sexual maturity is due to myocyte hypertrophy and results in increases in activity of mitochondrial marker enzymes in the epicardial layer (Clark and Rodnick, 1998), suggesting that mitochondrial volume density may be enhanced. Tissue growth is a function of the balance between rates of biosynthesis and degradation. The increase in heart mass during sexual maturity, which in some cases may double over the course of a few weeks, suggests that there is an increase in rates of protein synthesis, although decreases in rates of protein degradation cannot be ruled out. In this study, we test whether rates of protein synthesis expressed by the mitochondrial genome differ between males and females.

The impact of temperature on heart protein turnover is poorly understood. Protein synthesis was assessed in intact, perfused hearts isolated from rainbow trout. Acute changes in assay temperature between 5 and 15°C had little impact on rates of total protein synthesis, but adaptation to low temperature resulted in decreased rates of protein synthesis by both isolated mitochondria and isolated cells. In a second experiment, sexually immature fish were held at 5°C for 7–12 days and at 5°C for more than 1 month prior to the start of experiments. Studies involving isolated myocytes were performed from June to September 1998 with animals weighing 250–1100 g and obtained from Randall Fish Farms in Gagetown, New Brunswick, Canada. We report that mitochondria isolated from the hearts of sexually mature males have higher rates of protein synthesis than mitochondria from the hearts of females. Acute decreases in temperature result in substantial decreases in rates of protein synthesis by both isolated mitochondria and isolated cells. Acclimation to low temperature does not result in compensation of protein synthetic rate nor does the rate of protein synthesis correlate with the rate of oxygen consumption.

Materials and methods

Animals

Rainbow trout Oncorhynchus mykiss (Walbaum) were transported to Mount Allison University and held in tanks with running well water at 13°C unless stated otherwise. All fish were fed commercial trout pellets up to the day of experimentation. Photoperiod was adjusted to approximate the natural light:dark cycles. The sexual maturation study was conducted from 22 September to 30 October 1997 with fish obtained in early September from the Florenceville Fish Hatchery in Florenceville, New Brunswick, Canada. Studies to assess the impact of temperature on mitochondrial protein synthesis were carried out between February and April 1997 with rainbow trout from Carleton-Victoria Fish Hatchery, New Brunswick, and between January and March 1998 with animals from the Florenceville Fish Hatchery. Animals used in the temperature studies were maintained at Mount Allison for more than 1 month prior to the start of experiments. Studies involving isolated myocytes were performed from June to September 1998 with animals weighing 250–1100 g and obtained from Randall Fish Farms in Gagetown, New Brunswick, Canada.

Experimental protocols

Three experiments were conducted involving measurements of protein synthesis and respiration. In one experiment, measurements were made on mitochondria isolated from the hearts of sexually maturing rainbow trout maintained at 13°C. In a second experiment, sexually immature fish were held at 13°C for many months, at 5°C for 7–12 days and at 5°C for longer than 4 weeks. In animals maintained at 13°C, measurements of rates of mitochondrial protein synthesis and respiration were made at assay temperatures of 5, 15 and 25°C. Assays at each temperature were conducted with different subpopulations of fish. In fish held at 5°C, measurements were made at 5°C only. Evidence to support the contention that mitochondrial protein synthesis was being monitored was...
obtained via separation of proteins into selected mass ranges. In a third experiment, rates of total protein synthesis and oxygen consumption were monitored in heart cells isolated from fish maintained at 13 °C. Studies were conducted at assay temperatures of 5, 15 and 25 °C. At 5 °C, extracellular pH values of 7.6 and 8.0 were applied. In these experiments, each preparation yielded enough cells to obtain measurements under all conditions.

**Surgical procedures**

Animals were killed by a blow to the head, and the heart was quickly excised and placed in either cold isolation medium for mitochondrial extraction or perfusion medium for isolated myocyte studies. For all experiments involving isolated mitochondria, non-ventricular tissue was trimmed from hearts and wet ventricular mass was recorded. In experiments involving myocytes, hearts were mounted for perfusion by inserting a stainless-steel cannula into the ventricle through the atrial opening and securing it in place using surgical silk.

**Isolation of mitochondria**

Mitochondria were isolated using a modification of the method of Suarez and Hochachka (1980). Ventricles were minced and homogenized in 9 volumes of ice-cold isolation medium using a cold ground-glass homogenizer. In the temperature experiments, two or more ventricles were occasionally pooled to yield a single mitochondrial preparation. Isolation medium consisted of 5 mmol l⁻¹ KH₂PO₄, 5 mmol l⁻¹ K₂HPO₄, 2 mmol l⁻¹ EGTA and 250 mmol l⁻¹ sucrose, pH 7.4 at 4 °C. The crude homogenate was centrifuged at 600 g and the resulting supernatant was centrifuged at 9000 g. The resulting mitochondrial pellet was twice gently resuspended in 2 ml of isolation medium and centrifuged at 9000 g. All centrifugations were for 10 min at 4 °C. The final pellet was gently resuspended in 0.3–0.6 ml of isolation medium. A 35 μl sample of the resuspended mitochondria was frozen for later measurement of protein using a cold ground-glass homogenizer. In the temperature experiments, two or more ventricles were occasionally pooled to yield a single mitochondrial preparation. Isolation medium consisted of 5 mmol l⁻¹ KH₂PO₄, 5 mmol l⁻¹ K₂HPO₄, 2 mmol l⁻¹ EGTA and 250 mmol l⁻¹ sucrose, pH 7.4 at 4 °C. The crude homogenate was centrifuged at 600 g and the resulting supernatant was centrifuged at 9000 g. The resulting mitochondrial pellet was twice gently resuspended in 2 ml of isolation medium and centrifuged at 9000 g. All centrifugations were for 10 min at 4 °C. The final pellet was gently resuspended in 0.3–0.6 ml of isolation medium. A 35 μl sample of the resuspended mitochondria was frozen for later measurement of protein content using the BioRad D₄ assay. Isolation medium containing bovine serum albumin (BSA, fraction V, Sigma) was added to the remaining suspension to give a final concentration of 1 mg ml⁻¹. Mitochondria were kept on ice until used.

**Mitochondrial respiration**

Rates of oxygen consumption were measured using a Clark-type oxygen electrode and a Yellow Springs Instrument (model 16582) oxygen meter interfaced with a strip-chart recorder. The medium in which oxygen consumption was measured consisted of 12.5 mmol l⁻¹ KH₂PO₄, 12.5 mmol l⁻¹ K₂HPO₄, 10 mmol l⁻¹ Tris, 100 mmol l⁻¹ KCl and 2.7 mg ml⁻¹ BSA. This medium was gassed with compressed air for 30 min, the pH was set to 7.4 at 5, 15 or 25 °C, and BSA was then added. Suspended mitochondria (50–100 μl) were added to the medium in a water-jacketed chamber equipped with a stirring bar and motor. Once a stable chart reading had been obtained, pyruvate (final concentration 5.0 mmol l⁻¹) was added followed by malate (2.2 mmol l⁻¹). State 3 oxygen consumption was recorded following the addition of ADP (0.2 mmol l⁻¹), and state 4 respiration was recorded upon ADP depletion (Estabrook, 1967). The total volume in the chamber was 2.0 ml. Respiratory control ratios (RCRs) and the ratios of ATP synthesised to oxygen consumed (P:O ratio) were calculated.

**Mitochondrial protein synthesis**

Protein synthesis rates were measured by the incorporation of L-[2,6,³H]phenylalanine (Amersham Life Sciences) into trichloroacetic acid (TCA)-precipitable protein over time. The protein synthesis medium (Table 1) was modified from that of McKee et al. (1990) and included the following amino acids: Asp, Thr, Ser, Asn, Gln, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, His, Lys, Trp, Arg and Pro. The medium had an osmotic pressure of 356 mosmol l⁻¹, which is within the range for rainbow trout tissues. The protein synthesis assay is based on that of Kwast and Hand (1993). In this assay, 75 μl of [³H]phenylalanine (2775 kBq) was added to 325 μl of protein synthesis medium in a glass test tube from which 20 μl was taken to calculate the specific activity based on the radioactivity divided by the concentration of labelled plus unlabelled phenylalanine. Protein synthesis was initiated by adding 100 μl of suspended mitochondria, which corresponded to a final concentration of 1.3–4.1 mg mitochondrial protein ml⁻¹. Synthesis assays were accompanied by control assays containing the sodium salt of chloramphenicol succinate (Sigma) to inhibit mitochondrial protein synthesis (Kwast and Hand, 1993). A final concentration of 10 or 20 mg ml⁻¹ was used, there being no difference between these amounts with respect to the efficacy of inhibition. Test tubes containing the

<table>
<thead>
<tr>
<th>Table 1. Contents of protein synthesis medium</th>
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<tbody>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgSO₄</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>Mops</td>
</tr>
<tr>
<td>Glutamate</td>
</tr>
<tr>
<td>Malate</td>
</tr>
<tr>
<td>Amino acids (except phenylalanine)</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>ADP</td>
</tr>
<tr>
<td>Cycloheximide (mg ml⁻¹)</td>
</tr>
<tr>
<td>Bovine serum albumin (mg ml⁻¹)</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>EGTA</td>
</tr>
<tr>
<td>Mitochondrial protein (mg ml⁻¹)</td>
</tr>
<tr>
<td>pH</td>
</tr>
</tbody>
</table>

Sucrose and EGTA are components of the isolation medium and are added with the mitochondria. All concentrations are in mmol l⁻¹ unless otherwise stated. *2 mmol l⁻¹ was used in experiments with sexually maturing fish and 4 mmol l⁻¹ in the temperature experiments. The higher level of MgSO₄ yielded marginally higher rates of protein synthesis.
reaction medium were sealed and agitated in a water bath at the desired temperature (5, 15 or 25°C) for 3 h. Duplicate samples (20μl) were taken every 30 or 60 min and delivered onto glass microfibre filters (Whatman 934-AH) to dry for 5 min. Dry filters were transferred into ice-cold 10% TCA containing 5 mmol l⁻¹ unlabelled phenylalanine, washed in two separate lots of 5% TCA at room temperature (=19–24°C) and finally washed in 95% ethanol at room temperature. All washes were for 15 min. Filters were dried and placed in scintillation vials containing 10 ml of CytoScint liquid scintillation cocktail (ICN Biomedicals). Radioactivity (disint min⁻¹) was converted (using the specific activity value) to pmoles of phenylalanine incorporated per milligram of mitochondrial protein for experiments in the presence and absence of chloramphenicol.

Values obtained in control assays with chloramphenicol were subtracted from the experimental values at each time point. Incorporation (expressed as pmol phenylalanine mg⁻¹ mitochondrial protein) was plotted against time. The slope of the curve yielded the rate of protein synthesis.

Isolation of cardiac myocytes

Heart cells were isolated and viewed via modifications of protocols used by Legate et al. (1998). The perfusion system consisted of a wash-out line and chamber and a closed recirculating system controlled to 15°C. The recirculating media were constantly gassed with 0.25% CO₂ in air. Hearts were electrically paced above threshold voltage, media were constantly gassed with 0.25% CO₂ in air. Preparations were run with controls containing cycloheximide (1 mg ml⁻¹) to inhibit protein synthesis on cytoplasmic ribosomes that otherwise contributed to oxygen consumption by isolated myocytes. Oxygen consumption was initiated by adding 50 μmol l⁻¹ phenylalanine, 0.2 mmol l⁻¹ NaH₂PO₄, 0.05 mmol l⁻¹ CaCl₂, 2.5 mmol l⁻¹ KCl, 2.5 mmol l⁻¹ NaCl, 5 mmol l⁻¹ CaCl₂, 0.05 mmol l⁻¹ KCl, 10 mmol l⁻¹ Hepes, 10 mmol l⁻¹ NaHCO₃ and 5 mmol l⁻¹ glucose and was gassed with 0.5% CO₂/99.5% air for 30 min prior to use. The pH was then set to 7.6. Hearts were perfused with this medium for 15 min to clear the blood out and then partially digested by perfusion for 30 min with enzyme medium 1, which consisted of perfusion medium containing 0.1 mmol l⁻¹ CaCl₂, 2H₂O, 0.1% Type 1A collagenase (Sigma) and 0.06% hyaluronidase (Sigma). The hearts were removed from the cannula, trimmed of non-ventricular tissue and patted dry. The ventricle was bisected and covered in enzyme medium 2 (enzyme medium 1 containing 1% BSA). The endocardium was gently scraped free of the epicardium, and large pieces of endocardium were gently teased into smaller pieces. The endocardial suspension was transferred into a darkened flask with 20 ml of enzyme medium 2 and set in a shaker bath to digest for 20 min at 15°C. The suspension was filtered through 225 μm Spectra/Mesh polyethylene filters into chilled centrifuge tubes and spun at 50 g for 5 min at 5°C. The supernatant was discarded, and the pellet was gently resuspended in 6 ml of suspension medium (perfusion medium containing 0.50 mmol l⁻¹ CaCl₂·2H₂O and 4% BSA) and centrifuged twice more at 50 g for 5 min. The pellet was resuspended in suspension medium and centrifuged for 2–5 min in an Eppendorf 5414 microcentrifuge to pellet the myocytes. The myocytes were resuspended in 1.6 ml of suspension medium for oxygen consumption experiments or in 1.0 ml of suspension medium for protein synthesis experiments.

Samples of suspended cells (25–100 μl) were used and taken up in 2.5 ml of suspension medium containing 0.2 mmol l⁻¹ phenylalanine and 0.1 mmol l⁻¹ of all other amino acids at 5, 15 or 25°C and at a pH of 7.6. The cells were stored in air-tight 1 ml Yale Tuberculin glass syringes equipped with 18 gauge needles and incubated at the desired temperature in a shaking water bath. A Hamilton syringe was used to take 100 μl of the suspension from the glass syringe. The suspension was injected into the chamber of a Strathkelvin MC 100 Microcell, which contained a Strathkelvin 1302 microcathode oxygen electrode. The electrode was connected to a Strathkelvin (model 781) oxygen meter, which was interfaced to a chart recorder. Readings of Pₒ₂ were taken at time zero and every 10 min for 30 min. Eight experiments were conducted in total. Six complete data sets were obtained from single preparations at each of the three experimental temperatures. Two additional experiments yielded information at only two test temperatures. Rates of oxygen consumption are expressed as mmol O₂ consumed 10⁶ viable cells⁻¹ min⁻¹. Oxygen solubility coefficients given by Graham (1987) were used.

Protein synthesis in isolated myocytes

Myocytes were pelleted and taken up in 0.13–0.3 ml of suspension medium. The protein synthesis assay was similar to that used in the isolated mitochondria experiment. In this assay, 75 μl of [³H]phenylalanine (2775 kBq) was added to 400 μl of suspension medium containing 0.4 mmol l⁻¹ phenylalanine, 0.2 mmol l⁻¹ of all other amino acids, with or without cycloheximide, pH 7.6, in a glass test tube, and 20 μl was removed to calculate the specific activity. Protein synthesis was initiated by adding 50 μl of myocytes. Assays were run with controls containing cycloheximide (1 mg ml⁻¹) to inhibit protein synthesis on cytoplasmic ribosomes that would account for almost all the cellular protein synthesis. Assays were also run at pH 8.0 and 5°C. Preparations were incubated in a shaking water bath at 15 and 25°C for 3 h and at 5°C for 5 h. Tubes were loosely covered to allow air exchange. Duplicate samples (20 μl) were taken every 60 min and delivered onto glass microfibre filters to dry. Dry filters were treated as in the mitochondrial protein synthesis procedure. Incorporation and rates of protein synthesis were
Mitochondrial protein synthesis in rainbow trout heart

Mitochondrial respiration and protein synthesis in hearts of sexually mature rainbow trout

Electrophoresis of mitochondrial-encoded proteins

Samples from three protein synthesis experiments were subjected to SDS–PAGE. Frozen experimental and control (+ chloramphenicol) samples were thawed, and 150 or 165 μl samples were centrifuged at 12000g for 5 min. Pellets were resuspended in 1 ml of 25 mmol l⁻¹ Mops containing 5 mmol l⁻¹ phenylalanine. Samples were pelleted a second time, resuspended in 45 μl of buffer containing 100 mmol l⁻¹ Tris–HCl (pH 6.8), 4.8 % SDS, 20 % glycerol, 0.2 % Bromophenol Blue and 10–15 mmol l⁻¹ dithiothreitol (DTT), boiled for 5 min, and vortexed before application of 5 or 10 μl to a 15 % acrylamide gel. Sigma (M-4038) wide-range molecular mass markers were run in parallel as standards. Following electrophoresis, gels were stained in Coomassie Blue and, after destaining, entire bands were cut from the gels to capture the following relative molecular mass ranges: 97×10³ to 205×10³, 45×10³ to 66×10³ and 24×10³ to 36×10³. These bands were selected to encompass the anticipated separation of subunits I, II and III of cytochrome oxidase, which are encoded by the mitochondrial genome. The gel sections were placed in 200 or 300 μl of 30 % H₂O₂ (v/v) and heated at 60–65 °C for 2–3 h until the gel melted. CytoScint (10 ml) was added, and the resultant suspension was shaken vigorously. Thereafter, 1 or 2 ml was removed, taken up to 10 ml with further additions of CytoScint, and counted.

Data analysis

Results are presented as means ± S.E.M. Significance levels were assessed using Student’s t-test when two groups were being compared or using an analysis of variance (ANOVA) followed by the Bonferroni post-hoc test in situations involving three conditions. P<0.05 was considered significant.

Results

Evidence for the synthesis of mitochondrial-encoded proteins

Fig. 1 shows a representative example of the incorporation of phenylalanine into protein by mitochondria isolated from rainbow trout heart. Phenylalanine incorporation was linear over the 3 h of incubation, and the inclusion of chloramphenicol in the incubation medium decreased incorporation into the TCA-precipitable fraction. Net rates of protein synthesis for the experiments reported below were calculated from the differences between points on the two curves for all experiments.

Table 2 summarizes three separate experiments that track the uptake of label into specific molecular mass ranges. In the absence of chloramphenicol, [³H]phenylalanine uptake increased by 4±1.6-fold in the 45×10³ to 66×10³ relative molecular mass range that would encompass subunit 1 of cytochrome oxidase and by 6±1.8-fold in the 24×10³ to 36×10³ relative molecular mass range that would encompass subunits II and III of cytochrome oxidase. In the high relative molecular mass range, where no mitochondrial-synthesized polypeptides are anticipated, the increase in uptake between experimental and control preparations was lower at 1.7±0.3-fold. The efficacy of chloramphenicol treatment and the enhanced recovery in molecular mass fractions that include polypeptides known to be synthesized in mitochondria provide evidence that the experiments reported here are measuring protein synthesized in the mitochondria.

Mitochondrial protein synthesis in rainbow trout heart

Male and female rainbow trout both had developed gonads (Table 3). There was no significant difference in body mass between male and female fish; however, the RVM was significantly higher in males than in females. Mitochondria isolated from both males and females had high RCRs and P:O ratios, indicating that the organelles maintained functional integrity during the isolation procedure and were suitable for the protein synthesis studies (Table 4). State 4 respiration tended to be faster (P=0.08) in mitochondria isolated from male than from female rainbow trout. There was a linear incorporation of phenylalanine into protein over time in mitochondria isolated from male and female rainbow trout. There was a linear incorporation of phenylalanine into protein over time in mitochondria isolated from male and female rainbow trout.

Table 2. Radioactivity recovered from SDS gels

<table>
<thead>
<tr>
<th>Molecular mass range</th>
<th>Controls (+chloramphenicol)</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>97×10³ to 205×10³</td>
<td>47±2.7</td>
<td>82±16.7</td>
</tr>
<tr>
<td>45×10³ to 66×10³</td>
<td>53±5.9</td>
<td>197±55.3</td>
</tr>
<tr>
<td>24×10³ to 36×10³</td>
<td>64±10.2</td>
<td>350±52.9</td>
</tr>
</tbody>
</table>

Values are expressed as disits min⁻¹ μl⁻¹ of sample applied. Values are means ± S.E.M., N=3.
from both male and female fish (Fig. 2A). The rate of protein synthesis was significantly higher ($P=0.0024$) in mitochondria isolated from the hearts of male fish than in mitochondria isolated from the hearts of female fish (Fig. 2B).

**Impact of acute and chronic temperature change on rates of mitochondrial respiration and protein synthesis**

The physical characteristics of the fish used in this study are presented in Table 5. The RVM of fish acclimated to 13 °C for many weeks or even months was similar to the RVM of fish transferred to 5 °C for 7–12 days and to that of animals held at the lower temperature for more than 4 weeks.

The RCRs ranged between 5.5 and 12 and the P:O ratios ranged from 1.8 to 2.2 for all experimental groups and assay temperatures (Table 6). The mitochondria were therefore well-coupled, functionally intact and good candidates for the protein synthesis experiments. In mitochondria isolated from fish acclimated to 13 °C, the rate of state 3 respiration decreased in a linear fashion as assay temperature was decreased from 25 to 5 °C. The rate at 5 °C was approximately half that at 25 °C ($P<0.01$). The rate of state 4 respiration also decreased with assay temperature, but in this case there was a sharp drop between 25 and 15 °C and no change thereafter. At an assay temperature of 5 °C, mitochondria isolated from fish held at 5 °C for 7–12 days had significantly lower rates of state 3 respiration than animals held at 13 °C. Following acclimation to 5 °C, the rate of state 3 respiration tended to increase such that there was no significant difference between mitochondria isolated from either 13 °C- or 5 °C-acclimated fish at a test temperature of 5 °C. State 4 respiration followed the same trends; however, there were no statistically significant differences.

Incorporation of phenylalanine into the protein pool was linear with respect to time over the range 5–25 °C. Fig. 3A

---

Table 3. Body, gonad and ventricle mass in sexually mature rainbow trout

<table>
<thead>
<tr>
<th></th>
<th>Body mass (g)</th>
<th>Gonad mass (g)</th>
<th>Gonadosomatic index (%)</th>
<th>Ventricle mass (g)</th>
<th>Relative ventricle mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>514±33</td>
<td>20.6±4.1</td>
<td>3.80±0.72</td>
<td>0.45±0.032</td>
<td>0.087±0.002</td>
</tr>
<tr>
<td>Male</td>
<td>461±22</td>
<td>22.3±0.1</td>
<td>4.86±0.12</td>
<td>0.48±0.026</td>
<td>0.104±0.004*</td>
</tr>
</tbody>
</table>

* indicates a significant difference between males and females ($P<0.05$).

Values are means ± S.E.M., $N=11$ for females and $N=10$ for males.

Gonadosomatic index is gonad mass as a percentage of body mass.

---

Table 4. Respiratory characteristics of mitochondria isolated from sexually mature rainbow trout

<table>
<thead>
<tr>
<th></th>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>96±9</td>
<td>12±2</td>
<td>9.63±2.52</td>
<td>2.43±0.086</td>
</tr>
<tr>
<td>Male</td>
<td>124±12</td>
<td>18±3</td>
<td>8.41±1.53</td>
<td>2.20±0.13</td>
</tr>
</tbody>
</table>

Rates of state 3 (ADP-activated) and state 4 (following ADP depletion) respiration are expressed as nmol O$_2$ mg$^{-1}$ protein min$^{-1}$.

Values are means ± S.E.M., $N=8$ for females and $N=9$ for males.

Assay temperature was 15 °C.

RCR, respiratory control ratio; P:O, ratio of ATP synthesised to oxygen consumed.
Mitochondrial protein synthesis in rainbow trout heart shows incorporation for experiments conducted at 5 and 25 °C for mitochondria isolated from fish acclimated to 13 °C. In mitochondria isolated from fish acclimated to 13 °C, the rate of protein synthesis was the same at assay temperatures of 15 and 25 °C, but decreased by 3.6-fold (\( P < 0.001 \)) at an assay temperature of 5 °C (Fig. 3B). At an assay temperature of 5 °C, mitochondria exhibited this same relatively low rate of protein synthesis regardless of the previous thermal history of the animal.

Impact of acute temperature change on total cell respiration and protein synthesis

The oxygen content in sealed incubates of isolated cells decreased in a linear fashion. Oxygen level typically decreased by approximately one-third over the 30 min test period (data not shown). The rate of oxygen consumption did not change over the assay temperature range of 5–25 °C (Fig. 4). The rate of incorporation of phenylalanine into the total protein pool was linear at assay temperatures of 15 and 25 °C (Fig. 5A), and there was no difference in the rate of protein synthesis at these two assay temperatures (Fig. 5B). At an assay temperature of 5 °C, the rates of incorporation were very low and highly variable at individual time points. Rates of protein synthesis at 5 °C were calculated from the isotope uptake at 5 h and should be considered to be approximations only. Nevertheless, it is clear that the rate of protein synthesis was reduced at 5 °C to a value only approximately one-sixth of that observed at the higher assay temperatures. There was no difference in the rate of protein synthesis between extracellular pH values of 7.6 and 8.0.

![Graph A](image1.png)

**Fig. 3.** Protein synthesis by mitochondria isolated from the heart of temperature-acclimated rainbow trout. (A) Incorporation of phenylalanine into mitochondrial protein versus time at assay temperatures of 25 °C (circles; \( y = 4.36 + 0.462x, r^2 = 0.992, P < 0.0001 \)) and 5 °C (squares; \( y = 0.90 + 0.125x, r^2 = 0.977, P < 0.0001 \)). (B) Rates of mitochondrial protein synthesis at assay temperatures of 25, 15 and 5 °C. 5 °C‡, animals acclimated to 5 °C for more than 4 weeks. 5 °C‡‡, animals acclimated to 5 °C for 7–12 days. Sample sizes are shown above columns. Values are means ± S.E.M.

![Graph B](image2.png)

**Table 5.** Body and ventricle mass of temperature-acclimated rainbow trout

<table>
<thead>
<tr>
<th>Acclimation temperature (°C)</th>
<th>Body mass (g)</th>
<th>Ventricle mass (g)</th>
<th>Relative ventricle mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 (N=29)</td>
<td>528±25</td>
<td>0.49±0.04</td>
<td>0.092±0.006</td>
</tr>
<tr>
<td>5 (7–12 days)* (N=5)</td>
<td>612±70</td>
<td>0.60±0.05</td>
<td>0.101±0.012</td>
</tr>
<tr>
<td>5 (&gt;4 weeks)** (N=6)</td>
<td>427±35</td>
<td>0.39±0.038</td>
<td>0.092±0.008</td>
</tr>
</tbody>
</table>

*Length of time at 5 °C was 8±0.9 days.
**Five fish were held at 5 °C for 30±0.6 days (range 28–31 days); one fish was held at 5 °C for 368 days.
Values are means ± S.E.M.
Discussion

Methodology for the study of mitochondrial protein synthesis

Mitochondria from a wide range of organisms synthesize 13 polypeptides that are incorporated into four protein complexes: coenzyme Q cytochrome c reductase, cytochrome c oxidase, F1F0-ATPase and NADH dehydrogenase (Poyton and McEwen, 1996). Protein synthesis by mitochondria isolated from rat heart (McKee et al., 1990) and Artemia embryos (Kwast and Hand, 1993) has been well documented and served as the models for our studies. As with rat heart and Artemia, mitochondria isolated from rainbow trout heart incorporate radiolabelled amino acid into protein in a linear fashion with respect to time, and incorporation is impaired by chloramphenicol, an inhibitor of the peptidyl transferase activity of ribosomes. The incubation medium included cycloheximide, a potent inhibitor of protein synthesis on cytoplasmic ribosomes to rule out any possibility of contamination from this process. Polypeptides synthesized by mitochondria encompass a wide range of sizes, with the largest being subunit 5 of NADH dehydrogenase, with a relative molecular mass of approximately 65·103 (Chomyn et al., 1986).

We focused our attention on subunits I, II and III of cytochrome oxidase, which have relative molecular masses of approximately 57·103, 26·103 and 30·103, respectively (Kadenback et al., 1983) and appear to be particularly well expressed in Artemia embryos (Kwast and Hand, 1993). Regions of SDS gels that contained these windows where these subunits and others of a similar size (e.g. subunit 4 of NADH dehydrogenase with cytochrome oxidase I; ATPase 6 with cytochrome oxidase II and III) were enriched with isotope compared with chloramphenicol-treated controls. Isotope enrichment in the region of the gel containing material of high relative molecular mass (>97·103) was much lower and may be attributable to larger complexes that were only partially disrupted. Taken together, the above observations are considered to be reasonable evidence that the methodology yielded measurements of bona fide mitochondrial protein synthesis.

Heart mitochondria from sexually mature males are predisposed for protein synthesis

Both the male and female rainbow trout were sexually mature, with gonadosomatic indices similar to those reported for hatchery-reared fish (Franklin and Davie, 1992). Consistent with previous findings, the RVM was significantly greater in male than in female fish (Franklin and Davie, 1992; Bailey et al., 1997). Clark and Rodnick (1998) noted that as the heart mass increases there is a preferential growth of the epicardial layer from approximately 50 % to 75 % of the ventricle mass and that, in that layer, there is a mass-specific increase in the content of two mitochondrial enzymes, citrate synthase and β-hydroxyacyl CoA dehydrogenase. These findings suggest an increase in the total mitochondrial volume density of the heart in association with sexual maturity. In the present study, whole ventricles were utilized and the preparations therefore contained mitochondria from both the endocardial and epicardial layers.

State 4 and state 3 respiration showed a tendency to be faster in mitochondria isolated from hearts of male than of female fish. This may relate to the elevated levels of enzymes noted by Clark and Rodnick (1998) and warrants further examination.

Mitochondria isolated from the hearts of male fish had rates of protein synthesis twice that of mitochondria isolated from females, suggesting that the ‘male’ mitochondria are predisposed for enhanced rates of protein synthesis. It would be of interest to ascertain whether there are differences in the capacity for protein synthesis of mitochondria isolated from the...
Mitochondrial protein synthesis in rainbow trout heart.

Studies of cardiac hypertrophy induced with thyroid hormone in rats lend insight into a potential mechanism for the enhanced rates of mitochondrial protein synthesis in rainbow trout hearts. Rats receiving injections of thyroxine for 3 days exhibited cardiac hypertrophy (Leung and McKee, 1990; Wiesner et al., 1994) yielded heart mitochondria that had higher rates of \textit{in vitro} protein synthesis (Leung and McKee, 1990) and had higher levels of heart mitochondrial 12S rRNA in concert with elevated levels of total heart RNA (Wiesner et al., 1994). Wiesner et al. (1994) contend that, in the mitochondrial compartment, mRNAs are in excess relative to assembled ribosomes and therefore one mechanism to increase the synthesis of proteins encoded on mitochondrial DNA may be to increase the availability of ribosomes. In perfused, isolated hearts from rainbow trout, there is a linear correlation between the rate of protein synthesis and total RNA content (Sephton and Driedzic, 1995) and, if the rat heart model is applicable, mitochondrial rRNA levels should mirror total tissue RNA levels. As such, it is possible that the same control mechanism that exists in rat heart mitochondria may also occur in the rainbow trout heart. That is, mitochondria from the hearts of sexually mature male rainbow trout may have higher levels of rRNA than mitochondria from female fish. Although there may be similarities at the mitochondrial level between these two models of mitochondrial biogenesis, there is no reason to suspect that the process during sexual maturity in male rainbow trout is related to thyroid hormones. There is, however, compelling evidence that 11-ketotestosterone is an important factor in enhanced cardiac growth in sexually maturing male rainbow trout (Thorarinsen et al., 1996a,b; Davie and Thorarinsen, 1997). Whether steroids can lead to cardiac mitochondrial proliferation through mechanisms similar to thyroxine has yet to be resolved.
The results reveal a clear difference between the mitochondria isolated from the hearts of sexually mature male and female rainbow trout. Given that heart growth is associated with sexual maturity in males but not in females, the implication, as articulated above, is that the difference in mitochondrial protein synthetic rates is related to maturation. However, this remains to be proved, and the possibility cannot be ruled out that male–female differences occur throughout the life cycle. Because the fish came from different sources, had a different thermal history and differed somewhat in other aspects, it is not possible to compare directly information from the sexual maturity study with that from the temperature experiment in an attempt to resolve the question of gender or maturity as a determinant of mitochondrial protein synthesis. This aspect awaits further study.

**Protein synthesis is independent of oxygen consumption and is impaired at low temperature**

Accounts of the relationship between acclimation temperature and RVM in rainbow trout are varied: some studies report an increase in RVM at low temperature (e.g. Graham and Farrell, 1990; Bailey and Driedzic, 1993), whereas others report no such change (Sephton and Driedzic, 1995). The current experiment falls into the latter category. As discussed by Sephton and Driedzic (1995), cardiac enlargement may be a function of seasonal acclimatization and not of temperature alone. The cycling of RVM on a seasonal basis for rainbow trout over a 3 year period supports this contention (Farrell et al., 1988).

There is a linear relationship \( y = 0.0055x + 0.0708 \), \( r^2 = 0.997 \), \( P = 0.0334 \) between the rate of state 3 respiration and assay temperature in mitochondria isolated from the hearts of fish acclimated to 13 °C (Fig. 6A). This is precisely the same relationship that occurs for mitochondria isolated from red muscle of rainbow trout seasonally acclimatized to either 1 or 16 °C (St Pierre et al., 1998). A change in assay temperature between 15 and 25 °C did not influence the rate of protein synthesis by isolated mitochondria; however, a decrease to 5 °C severely inhibited the process. Fig. 6A shows that mitochondrial protein synthesis is not necessarily coupled to aerobic respiration, as has been suggested for rat heart (McKee and Grier, 1990; McKee et al., 1990): although the rates of these two processes converge at 5 and 25 °C, there is a disparity between rates at 15 °C. Therefore, flux through the citric acid cycle, with the associated generation of GTP and ATP, is not the primary determinant of the rate of mitochondrial protein synthesis. At an assay temperature of 5 °C, the rate of state 3 respiration of heart mitochondria isolated from fish acclimated to 5 °C was similar to that of mitochondria isolated from fish acclimated to 13 °C. The substantial positive thermal compensation exhibited by mitochondria isolated from red muscle (St Pierre et al., 1998) was not apparent for heart mitochondria. Whether this reflects true tissue differences or is a function of the experimental design of temperature acclimation versus seasonal acclimatization remains to be resolved. Acclimation temperature had no influence on the protein synthetic capacity of isolated mitochondria, which remained very low at an assay temperature of 5 °C. Thus, protein synthesis is impaired in isolated mitochondria or isolated hearts (Sephton and Driedzic, 1995) regardless of the thermal history of the animal. This needs to be reconciled with enhanced heart growth, in some cases, and at the very least with the maintenance of heart growth at low temperatures. Either there are potent signals for protein synthesis that the isolated preparations are lacking or protein breakdown is impaired to an even greater extent than synthesis at low temperature.

Isolated cells do not mirror the pattern of either state 3 or state 4 respiration of isolated mitochondria. Assay temperature over the range 5–25 °C does not correlate with the rate of aerobic respiration of intact cells (Fig. 6B). When perfused rainbow trout hearts are generating similar levels of power output, they show a constant rate of oxygen consumption between 5 and 15 °C (Graham and Farrell, 1990). The impact of acute temperature change on rates of oxygen consumption is therefore quite different between the levels of the mitochondria and higher levels of organization. This probably reflects the thermal sensitivity of contraction, ion pumping, biosynthesis, etc., with their varied ATP demands. As for mitochondria, a change in assay temperature between 15 and 25 °C did not influence the rate of protein synthesis by isolated cells, but a decrease in assay temperature to 5 °C severely impaired protein synthesis. This situation is in contrast to total protein synthesis by the perfused heart on which a change in acute assay temperature from 5 to 15 °C had no impact (Sephton and Driedzic, 1995). The discrepancy between total protein synthesis in isolated cells and in the intact heart is probably attributable to contractility. Work is a powerful determinant of cardiac protein synthesis in rainbow trout. Hearts performing high levels of work have higher rates of protein synthesis than hearts performing low levels of work (Houlihan et al., 1988) and, therefore, the impact of cardiac work on enhancing total protein synthesis may override any impairment due to temperature alone. Fig. 6B also shows that total protein synthesis is not directly coupled to aerobic respiration at the level of the whole cell, because overall protein synthesis is preferentially inhibited at low temperature. It is interesting to note that the interplay amongst oxygen consumption, protein synthesis and temperature in hepatocytes isolated from rainbow trout is also not straightforward (Pannevis and Houlihan, 1992). In hepatocytes, there was a linear relationship between rates of oxygen consumption and protein synthesis at low rates of protein synthesis, but higher rates of protein synthesis were achieved with little or no increase in aerobic respiration.

In conclusion, our results have shown that methodologies developed for the study of protein synthesis by mitochondria isolated from rat heart and *Artemia* embryos can be successfully applied to the rainbow trout heart. In addition, protein synthesis was successfully monitored in cells isolated from hearts, as has been reported for other cells types isolated from rainbow trout (Pannevis and Houlihan, 1992; Smith and Houlihan, 1995). Our
experiments show that mitochondria isolated from the hearts of sexually mature male rainbow trout are predisposed to synthesize protein at a greater rate than mitochondria from female trout. There was a linear relationship between the rate of state 3 oxygen consumption and assay temperature in isolated mitochondria, but changes in acclimation temperature did not alter the rate of oxygen consumption of isolated cells. A change in assay temperature from 15 to 25°C did not influence the rate of protein synthesis by either isolated mitochondria or isolated cells, but a decrease in assay temperature to 5°C severely impaired protein synthesis in both preparations. Protein synthesis is not necessarily coupled to oxygen consumption in either isolated mitochondria or isolated cells. There was no evidence for positive thermal compensation of either oxygen consumption or protein synthesis by isolated mitochondria following acclimation to low temperature. It should be noted that these conclusions are based on in vitro studies that lack elements such as myocyte contraction and circulating hormones that may influence protein synthesis. Studies are currently under way to extend the present findings to higher levels of organization.

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References


