

MORPHOMETRIC AND BIOCHEMICAL CHARACTERISTICS OF VENTRICULAR HYPERTROPHY IN MALE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

We examined the morphometric and biochemical effects of ventricular hypertrophy in male rainbow trout (*Oncorhynchus mykiss*) during sexual maturation. Our investigation focused on characterizing the growth of ventricular layers, on cardiomyocyte dimensions (length, cross-sectional area and cell volume) and on the activities of enzymes involved in intermediary metabolism.

Relative ventricle mass ($100 \times \text{ventricle mass/body mass}$) increased by as much as 2.4-fold during sexual maturation [as defined by an increasing gonadosomatic index ($100 \times \text{gonad mass/body mass}$)], and this resulted in an increased proportion of epicardium relative to endocardium. Ventricular enlargement was associated with increased length (+31%) and transverse cross-sectional area (+83%) of cardiomyocytes, which resulted in an expansion of up to 2.2-fold in mean myocyte volume (from 1233 to 2751 μm^3). These results indicate that sexual maturation induces ventricular enlargement through myocyte hypertrophy. Cell length and cross-sectional area were similar in both myocardial layers, and myocytes were elliptical rather than circular in transverse cross section. Ventricular hypertrophy did not alter transverse cell shape, perhaps reflecting the maintenance of short

diffusion distances for small molecules as cells hypertrophy. Myocyte hypertrophy could not account entirely for the sevenfold range of ventricle masses from different-sized fish, indicating that myocyte hyperplasia contributes substantially to ventricular growth as trout grow.

Measurements of the maximal activities of metabolic enzymes demonstrated that ventricular hypertrophy was associated with (1) higher epicardial but not endocardial activities of citrate synthase (by 23%) and β -hydroxyacyl-CoA dehydrogenase (by 20%); (2) lower activities of hexokinase (by 50%) in both layers, and (3) no change in lactate dehydrogenase or pyruvate kinase activities, which were also similar between layers. These results suggest that the energetic needs of the hypertrophied trout ventricle may be met through increased reliance on fatty acid oxidation, particularly by the endocardium, but decreased reliance on glucose as a metabolic fuel in both layers.

Key words: *Oncorhynchus mykiss*, rainbow trout, electron microscopy, myocyte, hyperplasia, cardiac hypertrophy, energy metabolism, myocardial layers.

Introduction

The vertebrate heart displays a remarkable capacity for morphological and biochemical adaptation. One illustration of this adaptability is cardiac growth in adult animals, which functions to accommodate changes in work demand on the heart. Patterns of cardiac growth can be segregated into two categories: (1) growth that maintains a constant allometry between body size and heart size, termed normal growth, and (2) growth that increases the heart mass:body mass ratio, termed excessive growth or cardiac hypertrophy. In both cases, cardiac growth can be accomplished through enlargement of individual cardiomyocytes (myocyte hypertrophy), proliferation of cardiomyocytes (myocyte hyperplasia) or both. It is well known in mammals that postnatal cardiac growth, either normal or excessive, occurs solely through myocyte hypertrophy and is associated with well-characterized

alterations in tissue morphology and/or cellular biochemistry (e.g. energy metabolism) (Zak, 1984). By comparison, the morphological and biochemical consequences of cardiac growth in non-mammalian vertebrates are poorly understood, and how growth is accomplished (myocyte hypertrophy, hyperplasia or both) has received scant attention. Such knowledge would offer comparative insights into the relationship between development, functional capabilities and plasticity of cardiac tissue in vertebrates.

Rainbow trout (*Oncorhynchus mykiss*) represent a non-mammalian model for investigating cardiac hypertrophy. Ventricular hypertrophy has been demonstrated in trout in response to cold-temperature acclimation (Graham and Farrell, 1989; Patey and Driedzic, 1997) and sexual maturation (Franklin and Davie, 1992; Graham and Farrell, 1992). In

response to gonadal enlargement, male rainbow trout exhibit an increase of as much as 143 % in relative ventricle mass ($100 \times \text{ventricle mass/body mass}$) (Franklin and Davie, 1992). In their study of heart growth in trout, Farrell *et al.* (1988) report that ventricular growth occurs through a combination of myocyte hypertrophy and hyperplasia. This observation, which differs greatly from the currently accepted paradigm for postnatal cardiac growth in mammals, stimulated our interest in determining the relative contribution of myocyte hypertrophy and hyperplasia to sexual maturation-induced ventricular hypertrophy in male trout. Furthermore, the effects of ventricular hypertrophy, induced by sexual maturation, on the growth of the layers of the trout ventricle, which consists of a compact, well-oxygenated epicardium and a trabecular, poorly-oxygenated endocardium, have not been investigated. Transmural differences in oxygen availability, myoarchitecture and possibly energy metabolism may promote distinct, layer-specific patterns of growth as ventricles enlarge during sexual maturation.

Cardiac work in trout is supported non-preferentially by fatty acid and carbohydrate metabolism (Driedzic, 1992). On the basis of measurements of enzyme activities, there is some indication that the salmonid heart exhibits transmural gradients in energy metabolism. The endocardium appears better poised for oxidative and glycolytic carbohydrate metabolism, whereas oxidative capacity for fatty acids may be better developed in the epicardium (Poupa *et al.* 1974; Ewart and Driedzic, 1987; Farrell *et al.* 1990). Whether sexual maturation-induced ventricular hypertrophy affects energy metabolism of the trout heart is unknown; however, this may depend on the ventricular layer examined and the type of tissue growth (hypertrophy or hyperplasia) occurring. For example, myocyte hypertrophy in mammals can result in increased diffusion distances for small molecules that, unless balanced by increased surface areas of transverse tubules and mitochondria, may reduce contractile efficiency and modify energy metabolism (Hamrell and Alpert, 1986). Because trout cardiomyocytes lack a transverse tubular system (Santer, 1985), and mitochondria are located centrally rather than peripherally in the myocyte, even mild hypertrophy might increase diffusion distances sufficiently to restrict aerobic energy metabolism and to inhibit contractility. Cellular function may also be influenced by transverse cell shape, which determines effective diffusion distances for small particles (e.g. Ca^{2+} , oxygen) to intracellular sites of action. Previous investigations of ventricular growth in fishes have assumed that cardiomyocytes were cylinders with circular cross sections, although it was acknowledged that this was an oversimplification (Farrell *et al.* 1988; Rodnick and Sidell, 1997). The transverse dimensions of cardiomyocytes, or the effect of ventricular growth on myocyte transverse shape, have not been determined quantitatively in any fish species.

The goal of the present study was to examine morphometric and metabolic aspects of ventricular hypertrophy induced by sexual maturation in male rainbow trout. We posed the following questions. (1) Do the size and transverse shape of cardiomyocytes change during ventricular hypertrophy, and

does myocyte hypertrophy or hyperplasia explain ventricular enlargement? (2) Does ventricular hypertrophy result in alterations in maximal activities of key enzymes involved in energy metabolism? While addressing both questions, we looked specifically at the effects of sexual maturation-induced ventricular hypertrophy on the myocardial layers. Both electron and light microscopy were employed to measure the dimensions, and ultimately the size and shape, of ventricular myocytes over a range of relative ventricle masses. We also measured maximal activities of key enzymes involved in aerobic and anaerobic metabolism to assess the effects of ventricular hypertrophy on cardiac energy metabolism.

Materials and methods

Animals

Male rainbow trout [*Oncorhynchus mykiss* (Walbaum)], 18–36 months old (1533 ± 798 g) were reared at a commercial hatchery (Clear Springs Foods, Buhl, ID, USA) and maintained in flow-through outdoor concrete raceways receiving spring water at 15 °C. Animals were anesthetized with 0.1 % tricaine methanesulfonate (MS-222, Crescent Research Chemicals, Phoenix, AZ, USA), their body mass and fork length were recorded, and a condition index was calculated ($100 \times \text{body mass/body length}^3$, where mass is in g and length is in cm). Each heart was excised rapidly, and the ventricle was isolated and weighed to determine relative ventricle mass. Further processing of ventricles was specific to each experiment. The testes were removed and weighed, and the gonadosomatic index (GSI, $100 \times \text{gonad mass/body mass}$) was recorded for each animal as a measure of sexual maturity. In our selection of animals, we chose those that ranged from sexually immature (testes were not developed) to mature (milt flowed upon palpation and gonads were evenly colored white), but did not use those showing signs of gonadal regression (gonads were noticeably gray and/or mottled).

The designation of ventricles as ‘normal’ or ‘hypertrophied’ required a working definition for ventricular hypertrophy in trout. We defined ventricular hypertrophy in trout as a relative ventricle mass that exceeded the ‘normal’ mean relative ventricle mass of the population from which the animals were drawn by 30 % or more. This definition assumes that the condition indices between control and experimental animals are similar. Admittedly, this definition is arbitrary, but it approximates the magnitude of increased relative ventricle masses reported in studies that demonstrate ventricular enlargement in trout (Farrell *et al.* 1988; Graham and Farrell, 1989, 1992; Franklin and Davie, 1992).

Ventricular gross morphometry

Myocardial layers were separated from trout ventricles ($N=44$) by blunt dissection with forceps, rinsed in ice-cold 1 % NaCl, blotted dry, and weighed to calculate the relative proportion of each layer. Samples were frozen, with an aluminum clamp cooled to the temperature of liquid nitrogen,

and stored at -70°C for enzyme analyses. Accurate separation of layers was accomplished easily, as the layers could be distinguished by both color and texture. Recovery of tissue following separation was always more than 90 % of original ventricle mass. To determine water content, portions (75–100 mg) of each layer were lyophilized to a constant mass.

Determination of cardiomyocyte dimensions and shape

Measurements of cross-sectional dimensions using electron microscopy

Ventricles were isolated from trout ($N=9$) in the manner described above. Prior to blunt separation of layers, small portions (10–20 mg) of each layer were carefully excised using micro-dissection scissors and rinsed in ice-cold sodium phosphate buffer (0.1 mol l^{-1} sodium phosphate, 7.5 % sucrose, pH 7.4). To avoid possible regional variation in cell morphometry, tissue was taken from the mid-zone of the anterior wall of the ventricle from all animals. Small ($<1\text{ mm}^3$) blocks of tissue were trimmed with a single-edge razor blade and placed into ice-cold paraformaldehyde–glutaraldehyde fixative (4 % paraformaldehyde, 1 % glutaraldehyde, 0.1 mol l^{-1} sodium phosphate buffer, 0.1 % CaCl_2 , pH 7.4). Tissues were transferred to fresh fixative within 24 h and stored at 4°C . Samples were post-fixed in sodium phosphate-buffered 1 % osmium tetroxide (pH 7.4), dehydrated through an acetone series (50, 70, 85, 95 and 100 %) and embedded in Spurr's low-viscosity resin (Ted Pella Inc., Redding, CA, USA). Semi-thin sections ($1.5\text{ }\mu\text{m}$) were generated on an RMC MT-7 ultramicrotome (Research and Manufacturing Co., Tucson, AZ, USA), stained with Toluidine Blue, and inspected to determine section orientation. In some cases, entire blocks of tissue were cut and remounted with epoxy resin to ensure sectioning of myocytes in cross section. Ultrathin sections (90–110 nm) were then generated, mounted on 200 mesh copper grids, and stained with filtered ($0.22\text{ }\mu\text{m}$ pore size) 2 % uranyl acetate and 0.1 % lead citrate. Stained sections were inspected at $4400\times$ magnification using a Zeiss EM 900 electron microscope (Zeiss Inc., Germany) operated at 70 kV. For each myocardial layer of each animal, we took micrographs of approximately 50 myocytes in near-perfect cross section (Fig. 1). To determine cross-sectional area, circumference and maximum diameter for each cell, outlines of myocytes were traced from negatives using a photographic enlarger (final magnification $13\,100\times$) and subsequently digitized using a Summagraphics digitizing tablet (model MM1201) and SigmaScan software (Jandel Scientific, Corte Madera, CA, USA). Magnification calibrations were made with micrographs of a diffraction grating replica ($2160\text{ lines mm}^{-1}$) (Ted Pella, Inc.).

Isolation of ventricular cardiomyocytes and measurements of myocyte length

We isolated ventricular myocytes (Fig. 2) by modifying the procedures of Farrell *et al.* (1988). All steps were performed at 15°C . Animals ($N=12$) were anesthetized, and the ventricle excised and placed immediately in 10 vols of wash-out medium. Wash-out medium was based on Ca^{2+} -free Cortland's

saline (see Farrell *et al.* 1986) and consisted of (in mmol l^{-1}): 125 NaCl, 3 KCl, 0.1 $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 2.3 Na_2HPO_4 , 1.0 $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5.5 D-glucose, 10 Hepes, 1 % polyvinylpyrrolidone ($M_r\ 40\times 10^3$) and 10 i.u. ml^{-1} sodium heparin. Preliminary experiments demonstrated that the

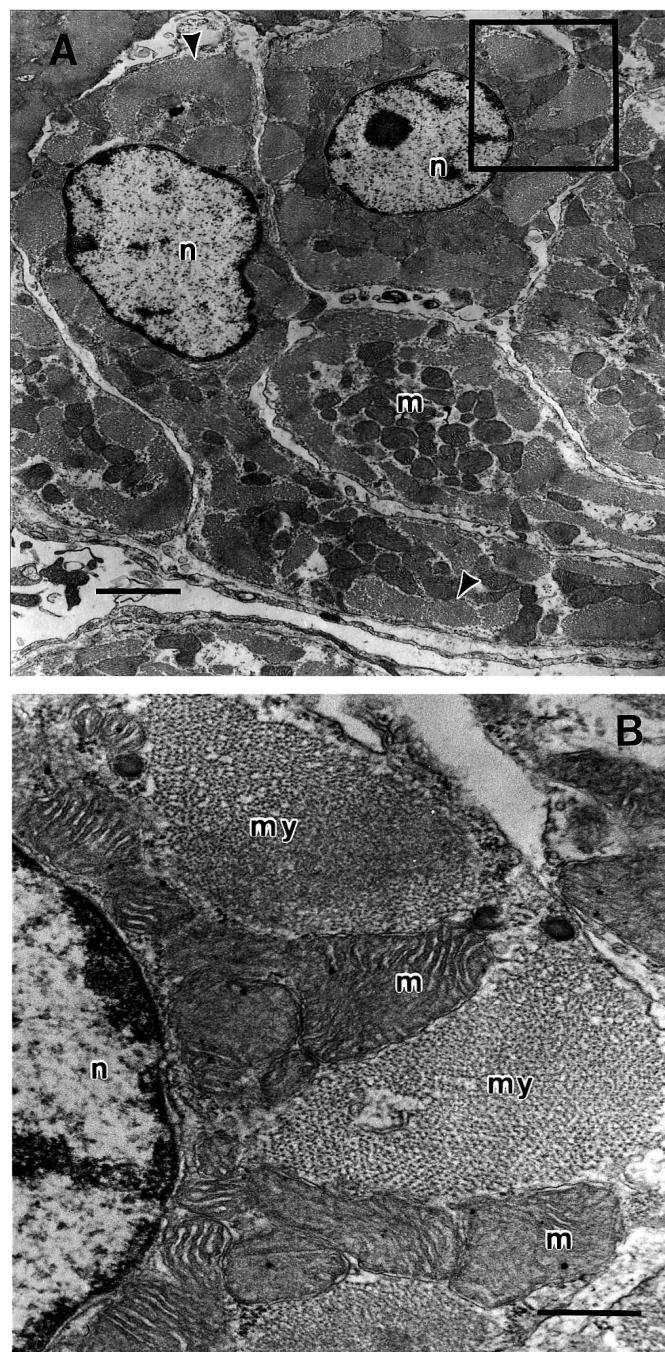


Fig. 1. Electron micrographs of ventricular epicardium of rainbow trout. (A) Transverse sections of several cardiomyocytes with prominent nuclei (n), centrally positioned mitochondria (m) and peripheral myofibrils (arrowheads) in near-perfect cross section. Scale bar, $2\text{ }\mu\text{m}$. The box indicates the area enlarged in B, which depicts myofibrils (my) in perfect cross section, subsarcolemmal mitochondria (m) and a nucleus (n). Scale bar, $0.5\text{ }\mu\text{m}$.

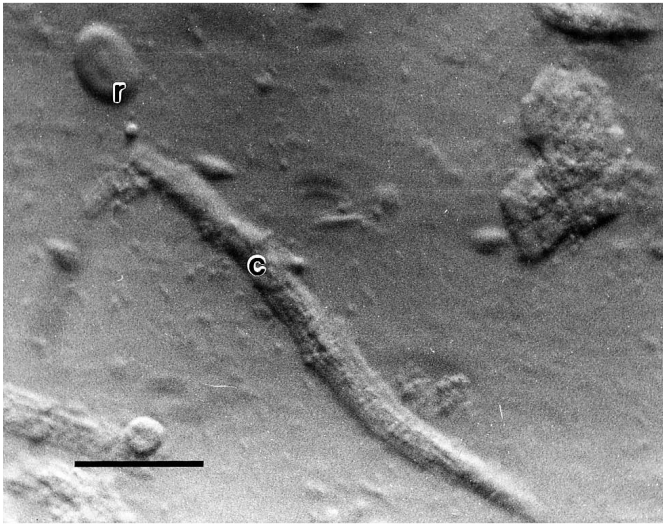


Fig. 2. Light micrograph of an isolated cardiomyocyte (c) from rainbow trout ventricular endocardium. r, red blood cell. Scale bar, 10 μm .

inclusion of heparin in the wash-out medium reduced red blood cell contamination and the occurrence of myocyte aggregates. Epi- and endocardial layers were separated and rinsed again in fresh wash-out medium. Samples of each layer (100–150 mg) were placed into sterile plastic Petri dishes with 7 ml of enzyme medium and minced finely (pieces $<1\text{ mm}^3$) with micro-dissecting scissors. Enzyme medium consisted of wash-out medium containing 0.1% collagenase (Type 1A, Sigma Chemical Co., St Louis, MO, USA) and 1% fatty-acid-free bovine serum albumin (Fraction V, Sigma). Minced tissue was transferred to stoppered 25 ml plastic Erlenmeyer flasks using a wide-bore plastic transfer pipette and incubated for 70 min in a reciprocating water bath (50 cycles min^{-1}) (Haake SWB20, Paramus, NJ, USA) while gassing with 100% O_2 . Digested material was filtered through 200 μm nylon mesh (Spectra/Mesh, Spectrum, Houston, TX, USA) into 10 ml round-bottom plastic centrifuge tubes. Cells were pelleted gently at 50 g for 5 min. The supernatant was discarded, and the pellet resuspended in 500 μl of resuspension medium (wash-out medium without heparin). The lengths of approximately 100 cells per layer per animal were recorded at 400 \times magnification from wet mounts using phase-contrast microscopy (Reichert Microstar IV, Leica, Denver, CO, USA) and a calibrated eyepiece micrometer.

Calculation of myocyte volume, mass and relative transverse cell shape

Because measurements of myocyte length and cross-sectional area were not performed on identical animals, myocyte volumes were determined indirectly. We derived linear regression equations to describe relationships between ventricle size (i.e. relative ventricle mass and ventricle mass) and myocyte dimensions (i.e. length and cross-sectional area). We employed these equations to estimate average myocyte cross-sectional area and length for the range of

relative ventricle masses and ventricle masses observed in this study. We then calculated mean myocyte volume as the product of cross-sectional area and myocyte length. We converted myocyte volume to myocyte mass to determine whether myocyte mass increased during sexual maturation and, if so, what proportion of ventricular growth was attributable to myocyte hypertrophy. For this analysis, we assumed (1) that myocyte density is 1.06 mg mm^{-3} (Mendez and Keys, 1960) and (2) that cardiomyocytes comprise approximately 85% of myocardial volume in trout, as has been demonstrated in mammals (Anversa *et al.* 1978). A comparison of these results with a hypothetical relationship describing ventricular growth as being due entirely to myocyte hypertrophy enabled us to estimate the relative contribution of myocyte hypertrophy and hyperplasia to ventricular growth.

To describe transverse cell shape, we measured circumference, maximum diameter and cross-sectional area from micrographs of myocytes in cross section. We used the circumference (C) and cross-sectional area (A) to derive a shape factor (κ) for each cell using the following equation:

$$\kappa = A^{0.5}/C.$$

To determine whether the transverse shape of cardiomyocytes was altered during ventricular hypertrophy, we compared the relationship between maximum cell diameter and κ across the observed range of relative ventricle masses. We then compared this relationship with a hypothetical distribution of maximum diameter and κ wherein myocytes were cylinders with circular cross sections. This assumption is made routinely in studies of animal cardiomyocytes (Rakusan, 1984; Farrell *et al.* 1988; Rodnick and Sidell, 1997).

Measurements of maximal enzyme activities and cellular biochemistry

General assay procedures

Ventricles from 11–14 animals were used to measure maximal activities of aerobically and anaerobically poised metabolic enzymes. These enzymes provided indices of sustainable glucose utilization (hexokinase), anaerobic metabolism (pyruvate kinase, lactate dehydrogenase), maximal aerobic metabolism (citrate synthase) and β -oxidation of fatty acids (β -hydroxyacyl-CoA dehydrogenase). Frozen samples (50–100 mg) of ventricular layers were homogenized in 9 vols of ice-cold extraction medium using a motorized Duall-22 ground-glass homogenizer. The extraction medium and final dilution were dependent on the enzyme being assayed. All enzyme activities were measured spectrophotometrically at $15 \pm 0.5^\circ\text{C}$ under saturating substrate concentrations using a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer with a thermostatically controlled recirculating water bath and water-jacketed cuvette holder. Activities are expressed as units per gram wet ventricle mass, where 1 unit denotes the conversion of 1 μmol of substrate to product per minute. All assays were linear over the reaction period. Chemicals were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA).

Hexokinase (HK; EC 2.7.1.1)

Extraction medium consisted of (in mmol l⁻¹): 40 Hepes, 1 EDTA, 2 MgCl₂ and 2 dithiothreitol; pH 7.4 at 15 °C. The reaction mixture consisted of (in mmol l⁻¹): 40 Hepes, 0.8 EDTA, 7.5 MgCl₂, 1.5 KCl, 2.5 ATP, 10 phosphocreatine, 0.4 NADP⁺, 1.0 D-glucose (omitted in control), 0.9 units ml⁻¹ creatine phosphokinase and 0.7 units ml⁻¹ glucose-6-phosphate dehydrogenase; pH 7.5 at 15 °C (procedures modified from Sidell *et al.* 1987). The final dilution of homogenate in the reaction tube was 1:1000. The reaction was initiated by the addition of glucose and followed at 340 nm for 7 min.

Pyruvate kinase (PK; EC 2.7.1.40)

The extraction buffer was identical to that for hexokinase. The reaction mixture contained (in mmol l⁻¹): 40 Hepes, 150 KCl, 10 MgCl₂, 5 ADP, 1 KCN, 0.15 NADH, 2.5 phosphoenolpyruvate (omitted in control) and 10 units ml⁻¹ lactate dehydrogenase; pH 6.9 at 15 °C (procedures modified from Hansen and Sidell, 1983). The final dilution of homogenate in the reaction tube was 1:2000. The reaction was followed spectrophotometrically at 340 nm for 6 min.

Lactate dehydrogenase (LDH; EC 1.1.1.27)

Extraction buffer consisted of (in mmol l⁻¹): 50 Hepes, 1 EDTA and 2 dithiothreitol; pH 7.5 at 15 °C. Following homogenization, samples were centrifuged for 5 min at 15 000 *g* and the supernatant was assayed. The reaction medium contained (in mmol l⁻¹): 50 Hepes, 1 KCN, 0.17 NADH and 1 pyruvate (omitted for controls); pH 7.5 at 15 °C (procedures modified from Hansen and Sidell, 1983). The final dilution of homogenate in the reaction tube was 1:2000. The reaction was followed at 340 nm for 5 min.

Citrate synthase (CS; EC 4.1.3.7)

Extraction buffer consisted of (in mmol l⁻¹): 20 Hepes and 1 EGTA; pH 7.5 at 15 °C. The assay reaction mixture consisted of (in mmol l⁻¹): 20 Hepes, 1 EGTA, 220 sucrose, 40 KCl, 0.1 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 0.05 acetyl CoA, and 1 oxaloacetate (omitted in control); pH 8.0 at 15 °C (Rodnick and Sidell, 1994). Homogenates of frozen tissue were taken through a freeze-thaw cycle to maximize enzyme activity, and the final dilution of homogenate in the reaction tube was 1:8000. The reaction was followed at 412 nm for 7 min.

β-Hydroxyacyl-CoA dehydrogenase (HOAD; EC 1.1.1.35)

Extraction buffer was identical to that for citrate synthase. The reaction mixture consisted of (in mmol l⁻¹): 20 Hepes, 1 EGTA, 1 KCN, 0.15 NADH and 0.1 acetoacetyl CoA (omitted in control); pH 7.5 at 15 °C (procedures modified from Hansen and Sidell, 1983). The final dilution of homogenate in the reaction tube was 1:800. The reaction was followed at 340 nm for 6 min.

Protein content

Total tissue protein for each myocardial layer was assayed by the bicinchoninic acid method using bovine serum albumin as the standard (Smith *et al.* 1985).

Data analysis

We calculated and tested correlation coefficients describing the relationships between ventricle mass and degree of sexual maturity (i.e. gonadosomatic index), changes in the proportion of epicardium with relative ventricle mass, and for enzyme activities and relative ventricle mass (Zar, 1996). Differences in enzyme activities between epicardial and endocardial layers were examined using Student's *t*-test, corrected for family-wise error. Regression analysis and analysis of variance (ANOVA) were used to evaluate relationships between myocyte dimensions and relative ventricle mass. Variance homogeneity was evaluated for cross-sectional area and length data using a modified Brown-Forsythe test (Keppel, 1991). Statistical significance was established at *P* < 0.05. Data are expressed as means ± S.D.

Given the wide range of animal body masses used in these experiments, we evaluated possible effects of body and ventricle size on the individual descriptive variables. For each descriptive variable, we first plotted best-fit linear regressions for body mass *versus* ventricle mass and extracted residuals. The residuals represent ventricular hypertrophy divorced from simple allometric growth. We then performed either linear regression or correlation analyses using the residuals for the abscissa and various descriptive variables (i.e. proportion of epicardium, myocyte dimensions, enzyme activities) for the ordinate (Neter *et al.* 1983). Statistical significance for this procedure was established at *P* < 0.05.

Results*Effects of body mass and ventricle mass on descriptive variables*

The results of our analyses indicated that body mass did not correlate with the descriptive variables measured in our study. Our analyses suggest that ventricle mass was correlated with percentage epicardium and myocyte cross-sectional area, but demonstrated no correlation with other variables. We interpret this relationship as resulting from the increase in ventricle mass with ventricular hypertrophy, rather than simple growth of the animals. Therefore, we used relative ventricle mass as a measure of ventricular hypertrophy in our final analyses of ventricular morphometry and energy metabolism.

Ventricular gross morphometry

Sexual maturation was associated with considerable ventricular hypertrophy in male rainbow trout, and there was a significant correlation between gonadosomatic index and relative ventricle mass (Fig. 3). Relative ventricle mass increased by as much as 2.4-fold (from 0.075 to 0.235) as animals matured (*P* < 0.001). Condition indices were similar among all animals (1.27 ± 0.11 g cm⁻³). Growth of the ventricle during sexual maturation resulted from an increase in the absolute mass of both ventricular layers, but the epicardium accounted for a greater proportion of ventricle mass than did the endocardium (*P* < 0.001) (Fig. 4). The percentage contribution by mass of epicardium ranged from 47 to 75 %

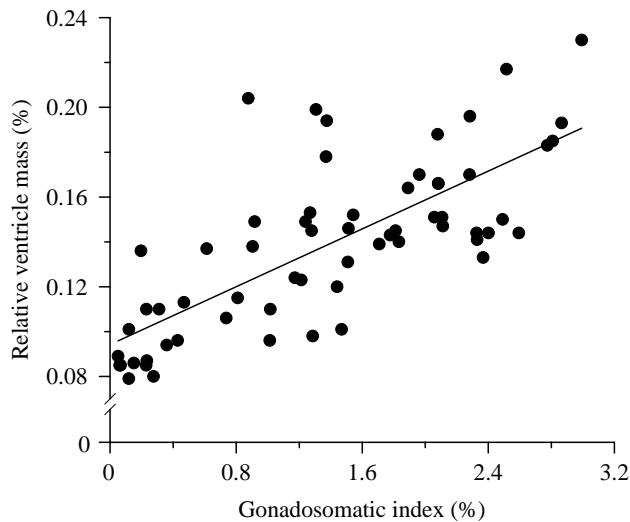


Fig. 3. Relationship between gonadosomatic index ($100 \times \text{gonad mass/body mass}$) and relative ventricle mass ($100 \times \text{ventricle mass/body mass}$) in male rainbow trout during sexual maturation ($N=44$, body mass 1918 ± 852 g; mean \pm S.D.). The correlation between relative ventricle mass and gonadosomatic index (solid line) is significant ($r=0.75$, $P<0.001$) and describes the following linear relationship: $y=0.032x+0.094$.

(mean $61.0 \pm 8.4\%$) of ventricular mass, and we observed that greater absolute mass of the ventricle was also associated with an increased proportion of epicardial mass. The water content of ventricular myocardium averaged $81.5 \pm 1.9\%$ ($N=18$) and did not vary between layers or with ventricular enlargement. This indicated that increases in ventricle or layer mass were not simply the result of changes in myocardial fluid content.

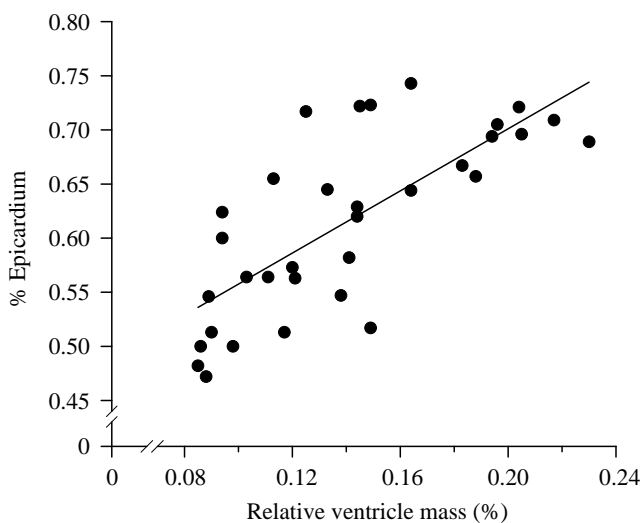


Fig. 4. Relationship between the percentage of epicardium and relative ventricle mass ($100 \times \text{ventricle mass/body mass}$) in male rainbow trout ($N=44$, body mass 1918 ± 852 g; mean \pm S.D.). The correlation is significant ($r=0.75$, $P<0.001$) and describes the following linear relationship: $y=1.44x+0.41$.

Cardiomyocyte dimensions and transverse shape

Preliminary analyses indicated that there were no differences in cellular dimensions between epi- and endocardial myocytes from the same ventricle. Cardiomyocytes of hypertrophied ventricles had significantly increased cross-sectional areas (up to 1.80-fold, $P<0.01$; Fig. 5A; Table 1), lengths (up to 1.31-fold, $P<0.01$; Fig. 5B; Table 1) and maximum diameters (up to 1.38-fold, $P<0.01$; Table 1) compared with normal ventricles. Using regression equations describing relationships between dimensional measurements and relative ventricle mass, we calculated that ventricular hypertrophy resulted in as much as a 2.2-fold increase in myocyte volume (from 1233 to $2751 \mu\text{m}^3$; Table 1) as trout matured sexually. Conversion of myocyte volumes to masses indicated that, for the range of relative

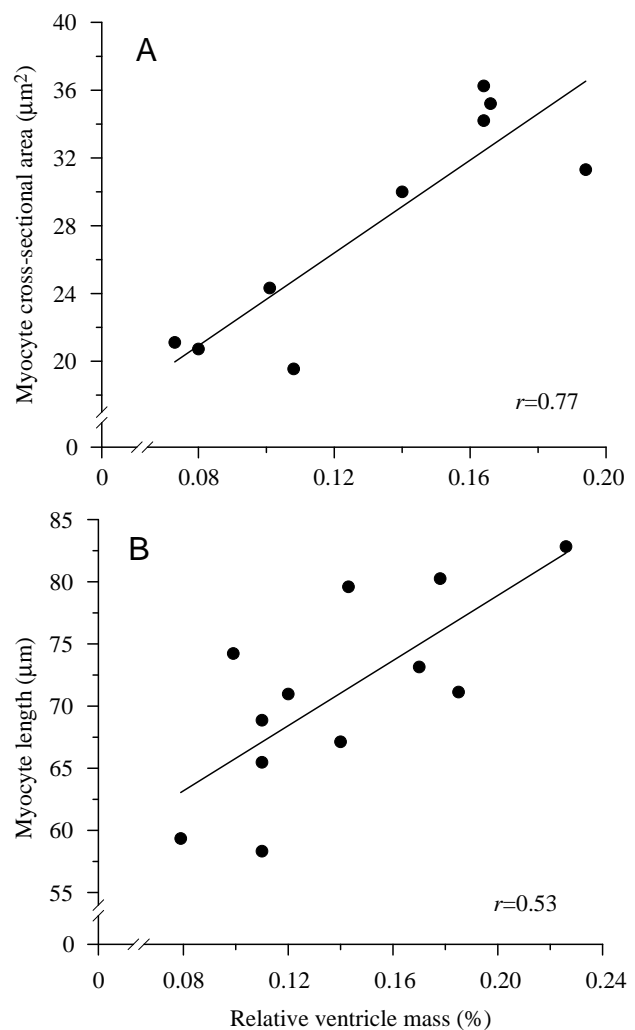


Fig. 5. Relationships between cardiomyocyte dimensions (cross-sectional area and length) and relative ventricle mass ($100 \times \text{ventricle mass/body mass}$). The absence of statistical differences between layers justified pooling measurements for each ventricle. Each point represents the mean of at least 100 cross-sectional areas (A) and 200 myocyte lengths (B). The slopes of the equations (cross-sectional area, $y=136.9x+10.0$; length, $y=130.9x+52.7$) calculated for each relationship are significant ($P<0.01$).

Table 1. Observed and calculated characteristics of ventricles and ventricular cardiomyocytes from male rainbow trout

Animal mass (g)	GSI (%)	RVM (%)	Ventricle mass (g)	Mean XSA (μm^2)	Mean cell length (μm)	Mean maximum cell diameter (μm)	Mean cell volume (μm^3)
848	0.118	0.079	0.67	(20.8)	59.3	(7.8)	1233
1025	0.275	0.080	0.82	20.8	(63.2)	7.9	1315
1556	0.390	0.099	1.54	(23.5)	74.2	(8.2)	1744
1406	0.237	0.101	1.42	24.3	(65.9)	8.0	1601
1787	0.330	0.108	1.93	19.5	(66.8)	7.8	1303
600	0.230	0.110	0.66	(25.0)	68.9	(8.5)	1722
764	0.109	0.110	0.84	(25.0)	58.3	(8.5)	1458
718	0.141	0.110	0.79	(25.0)	65.5	(8.5)	1638
642	0.330	0.120	0.77	(26.4)	71.0	(8.7)	1874
1621	1.250	0.140	2.27	30.0	(71.0)	9.2	2130
807	1.890	0.140	1.13	(29.1)	67.1	(9.2)	1953
2161	1.780	0.143	3.09	(29.6)	79.6	(9.2)	2356
2878	1.891	0.164	4.72	34.2	(74.2)	10.4	2538
1561	2.107	0.164	2.56	36.2	(74.2)	10.4	2686
1771	2.100	0.166	2.94	35.2	(74.4)	10.8	2612
2812	2.250	0.170	4.78	(33.2)	73.1	(9.9)	2427
2079	2.370	0.178	3.70	(34.3)	80.2	(10.1)	2751
859	2.810	0.185	1.59	(35.3)	71.1	(10.2)	2510
2330	3.370	0.194	4.52	31.3	(78.1)	9.0	2445

GSI (gonadosomatic index), $100 \times \text{gonad mass/body mass}$; RVM (relative ventricle mass), $100 \times \text{ventricle mass/body mass}$; XSA, cross-sectional area.

Mean cell volume was calculated as $\text{XSA} \times \text{cell length}$.

Numbers in parentheses were calculated from regression equations describing relationships between RVM and myocyte dimensions (see Fig. 5A,B).

ventricle masses observed in this study, increases in myocyte mass during sexual maturation can account for a 2.3-fold increase in ventricle mass (Table 1). Analyses of transverse cell shape (i.e. plots of κ versus mean maximum myocyte diameter) demonstrated that trout cardiomyocytes were elliptical in cross section rather than circular, but cell shape did not change with increasing myocyte diameter (i.e. myocyte hypertrophy) (Fig. 6). Predictions of myocyte volume based on a cylindrical cell with either elliptical or circular transverse cross sections (Fig. 7) demonstrate that a circular cross section predicts 2.2-fold greater myocyte volumes than the observed elliptical cross sections.

Measurements of maximal enzyme activities and cellular biochemistry

Relationships between relative ventricle mass and maximal enzyme activities in ventricular layers are shown in Fig. 8. Maximal activities of HK (Fig. 8A), CS (Fig. 8D) and HOAD (Fig. 8E) were consistently higher in endocardium than in epicardium ($P < 0.01$), whereas no such relationship was observed for PK (Fig. 8B) and LDH (Fig. 8C). Maximal activities of CS and HOAD were augmented in the epicardium of hypertrophied hearts ($P < 0.01$), but no change was observed in the endocardium. Interestingly, maximal HK activity in both layers was 50 % lower in animals with a large relative ventricle mass compared with animals with a small relative ventricle mass ($P < 0.01$). Maximal activities of PK

and LDH did not vary with ventricular hypertrophy during sexual maturation.

Protein content ($N=15$) was approximately 6 % higher ($P=0.048$) in endocardium ($139.4 \pm 12.3 \text{ mg g}^{-1} \text{ tissue}$) than in epicardium ($130.1 \pm 12.9 \text{ mg g}^{-1} \text{ tissue}$) and was not related to relative ventricle mass or ventricle mass. Expressing enzyme activities per milligram protein did not alter transmural differences in enzyme activities or the relationships between enzyme activities and relative ventricle mass.

Discussion

Ventricular hypertrophy during sexual maturation

Like previous studies (Franklin and Davie, 1992; Graham and Farrell, 1992), our study demonstrates that there is a positive correlation between the degree of sexual maturation and relative ventricle mass in male rainbow trout. Notably, the slope describing the relationship between relative ventricle mass and gonadosomatic index (0.032; Fig. 3) and the increase in relative ventricle mass with sexual maturity (2.4-fold) are nearly identical to those described by Franklin and Davie (1992) (0.033 and 2.4-fold, respectively). The effects of sex or sexual status on heart mass are not unusual in animals, and have been linked to circulating levels of testosterone in rodents (Koenig *et al.* 1982) and, more recently, to various androgens in rainbow trout (Thorarensen *et al.* 1996; Davie and Thorarensen, 1997). Although salmonid hearts express

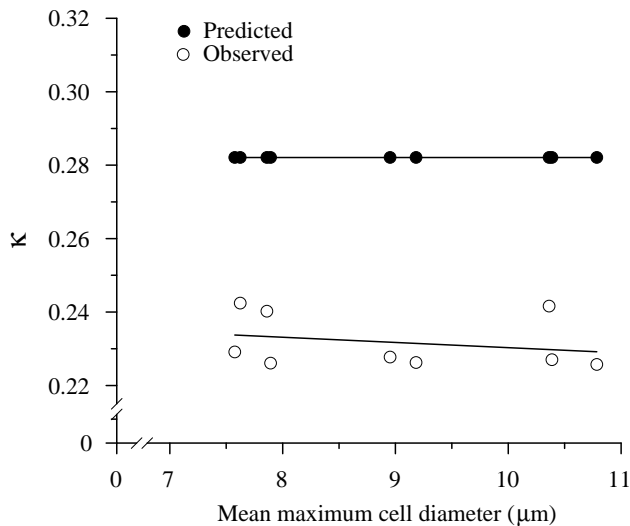


Fig. 6. Relationship between mean maximum cell diameter and myocyte shape (κ ; cross-sectional area^{0.5}/circumference). The open circles depict the relationship observed during this study (a minimum of 100 measurements per point), whereas the filled circles illustrate the relationship that would occur if myocytes had perfectly circular transverse cross sections. The difference in elevation between the lines ($P < 0.001$) indicates that trout myocytes are elliptical, rather than circular, in transverse cross section. Increasing cell diameter (i.e. cardiomyocyte hypertrophy) is not associated with a change in transverse cell shape (i.e. the slope does not differ from zero) ($P > 0.05$).

androgen receptors (Fitzpatrick *et al.* 1994), the mechanisms linking circulating androgen levels with cardiac growth have not been elucidated.

A consistent finding in this study and others (Graham and Farrell, 1992) is that increasing ventricle mass in male trout during sexual maturation results from an expansion of both myocardial layers, but growth of the epicardium exceeds that of the endocardium (Fig. 4). A greater proportion of epicardium with increasing relative ventricle mass has also been induced in trout by exogenous administration of 17- α -methyltestosterone (Davie and Thorarensen, 1997). A selective expansion of the epicardium may result from biophysical constraints imposed by the increase in ventricular diameter. According to Laplace's law, the maintenance of intraluminal pressure at larger ventricular diameters results in an increased tangential wall stress that can be counterbalanced by an increase in wall thickness. Although this compensatory response may apply to the epicardium, the numerous small pumping chambers that comprise the trabeculated endocardium probably generate a very low wall stress (Johansen, 1965) and thus may not be stimulated equivalently to grow.

Ventricular growth results from myocyte hypertrophy and hyperplasia

We demonstrate that ventricular hypertrophy in male trout results from hypertrophy of existing cardiomyocytes. The observed 2.3-fold greater mass of myocytes from hypertrophied

ventricles can account entirely for the doubling of ventricle mass that occurs with sexual maturation. This observation is consistent with the paradigm for postnatal cardiac growth and hypertrophy in mammals (Zak, 1984). However, the increase in myocyte mass observed in our study was calculated from a sevenfold range of ventricle masses from differently sized animals (Table 1). Observed changes in average myocyte mass cannot account for this range of tissue masses, indicating that hyperplasia of existing myocytes contributes substantially to increases in ventricle size that occur with normal growth of the adult trout heart. Thus, our results support and extend the observations of Farrell *et al.* (1988) that growth of the trout ventricle occurs through a combination of myocyte hypertrophy and hyperplasia. We speculate further that hypertrophy of extant myocytes is responsible for increasing ventricular mass during gonadal maturation, but that myocyte proliferation occurs between reproductive periods. It appears that trout cardiomyocytes, unlike mammalian cardiomyocytes, are not terminally differentiated, but maintain the capability for cell division even in adult animals.

How much myocyte hyperplasia must have occurred to explain the sevenfold range of ventricle mass observed in this study? Assuming that the proportion of myocyte volume to total tissue volume does not change during ventricular growth, we estimate that a 0.66 g ventricle from a sexually immature trout (gonadosomatic index 0.118, relative ventricle mass 0.079; Table 1) contains approximately 300 million myocytes, whereas a 4.78 g ventricle from a mature animal

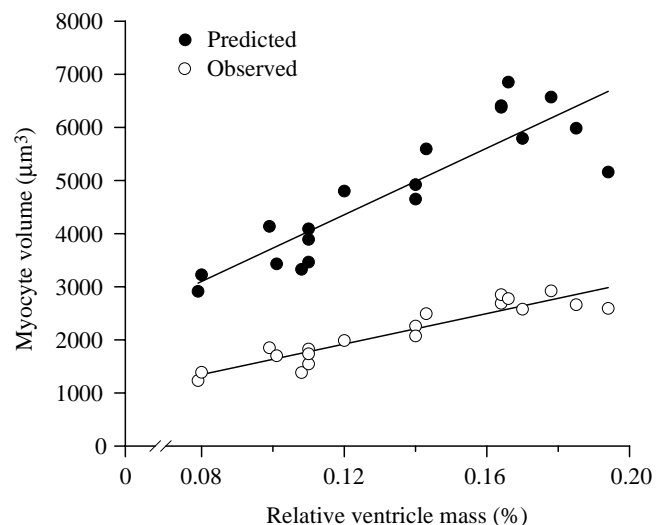


Fig. 7. Relationship between myocyte volume and relative ventricle mass (100×ventricle mass/body mass). Myocyte volume was calculated from measurements of cross-sectional area and length (Table 1). The open circles represent values derived from observed changes in myocyte lengths and cross-sectional areas with increasing relative ventricle mass ($y = 14354x + 199$) (Table 1). The filled circles portray the predicted relationship that would occur if volumes were calculated using mean maximum diameters ($y = 31395x + 589$) (Table 1) and assuming cylindrical cells with circular transverse cross sections for cell shape.

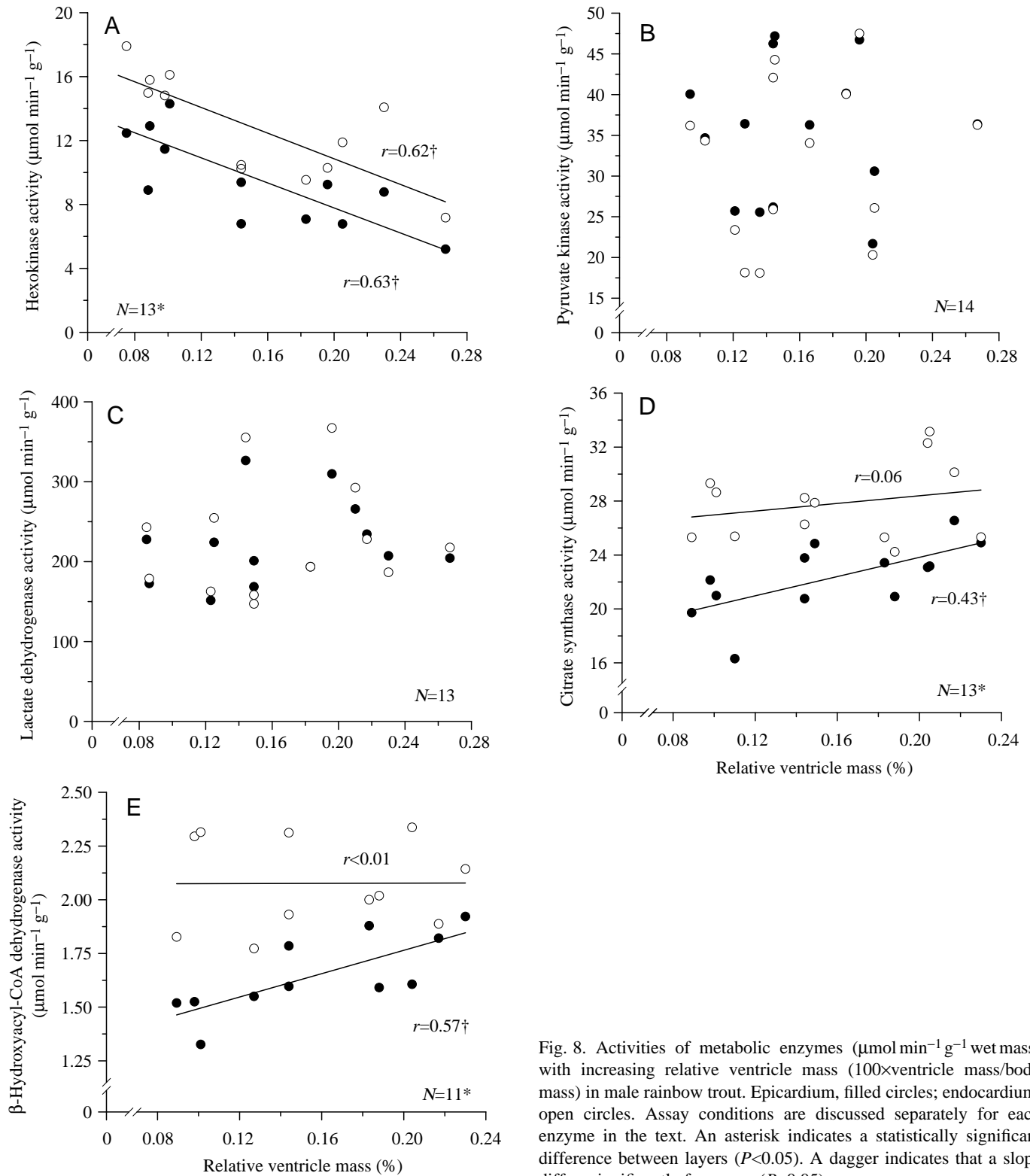


Fig. 8. Activities of metabolic enzymes ($\mu\text{mol min}^{-1} \text{g}^{-1}$ wet mass) with increasing relative ventricle mass ($100 \times \text{ventricle mass/body mass}$) in male rainbow trout. Epicardium, filled circles; endocardium, open circles. Assay conditions are discussed separately for each enzyme in the text. An asterisk indicates a statistically significant difference between layers ($P < 0.05$). A dagger indicates that a slope differs significantly from zero ($P < 0.05$).

(gonadosomatic index 2.25, relative ventricle mass 0.170; Table 1) contains approximately 1070 million enlarged myocytes. This 3.5-fold increase in myocyte numbers over the observed range of ventricle masses in the present study demonstrates that myocyte hyperplasia plays a considerable

role in growth of the trout heart. These estimates of myocyte numbers are much higher than those reported for the rat heart, where a 0.67 g ventricle was calculated to contain approximately 48 million myocytes (Anversa *et al.* 1978). Given the much smaller volume of trout myocytes

(1233–2751 μm^3 ; Table 1) compared with that of normal adult rat myocytes (9960–14 200 μm^3 ; Anversa *et al.* 1978), the larger total number of myocytes in a similar-sized trout ventricle is not unreasonable. Our observation that trout cardiomyocytes are elliptical in cross section will further influence estimations of myocyte hypertrophy and hyperplasia during cardiac growth in trout. Clearly, assuming that trout myocytes are circular in transverse cross section (Farrell *et al.* 1988; Rodnick and Sidell, 1997) will overestimate myocyte volumes in fishes (Fig. 7) and underestimate the contribution of myocyte hyperplasia to cardiac growth.

Is there a functional reason why cardiac growth in trout differs from that in mammals? The lack of a transverse tubule network (Santer, 1985), the central positioning of most mitochondria in fish cardiomyocytes (Rodnick and Sidell, 1997), along with a strong dependence of contractile performance on extracellular Ca^{2+} (Hove-Madsen and Gesser, 1989), may represent important constraints on myocyte volumes in trout. Presumably, the small size and elliptical transverse shape of trout cardiomyocytes obviate the increased surface area-to-volume ratio that would result with a T-tubular network. Sole dependence on myocyte hypertrophy for normal ventricular growth in fishes could be maladaptive, resulting in myocytes with reduced surface areas for diffusion of small molecules. A reduction in the capacity for diffusion could possibly lead to decreased contractile performance, as can occur in mammals with ventricular hypertrophy and cardiac failure (Hamrell and Alpert, 1986).

Cardiac energy metabolism

We demonstrate for the first time that ventricular hypertrophy induced by sexual maturation results in altered maximal activities of metabolic enzymes in trout ventricles, suggesting that overall cardiac energy metabolism adjusts for hypertrophy of ventricular myocytes. The results for CS (Fig. 8D) and HOAD (Fig. 8E), qualitative markers of maximal aerobic capacity and fatty acid oxidative capacity, respectively (Driedzic, 1992), imply that maximal rates of aerobic energy metabolism, and fatty acid oxidation in particular, are augmented as ventricles hypertrophy during sexual maturation. The increased potential for oxidative energy metabolism results from the increased activities of CS (+23 %) and HOAD (+20 %) in the epicardium, but a maintenance of endocardial activities. This latter response, as well as the unchanging activity of endocardial HOAD during ventricular hypertrophy, may represent an important compensatory response, whereby rates of catalysis are held constant despite an increase in myocyte volume. It is noteworthy that Kiessling *et al.* (1995) observed similar increases in CS and HOAD activities in axial white muscle of maturing rainbow trout. Measurements of the activity of HK, which catalyses the phosphorylation of intracellular glucose upon entry into the cardiomyocyte, serve as qualitative indices of glucose oxidation by fish hearts (Driedzic, 1992; Rodnick and Sidell, 1997). Hypertrophied ventricles of mature male trout exhibited dramatically lower (50 %) HK activities in both layers

compared with normal ventricles (Fig. 8A), suggesting that hypertrophied ventricles have a lower capacity for glucose metabolism. Notably, this 50 % lower HK activity is found in ventricles with myocytes that have doubled in size and indicates that total HK activity per myocyte may remain constant. The reduced HK activity in enlarged ventricles may also represent a simple dilution of normal HK activity through expansion of myocyte volume, rather than active downregulation of HK expression. Our observation that the activities of PK (Fig. 8B) and LDH (Fig. 8C), both of which serve as indicators of anaerobic flux capacity (Driedzic, 1992), remain unaltered during sexual maturation supports the active maintenance of glycolytic capacity in hypertrophied ventricles of trout. Because LDH also catalyses the oxidation of lactate to pyruvate for further energy production, this may also reflect a maintenance of catalytic capacity for another important metabolic fuel.

Our observations concerning changes in the activities of metabolic enzymes with ventricular hypertrophy in male rainbow trout do not agree entirely with previous studies of cardiac energy metabolism in fishes. As in the present study, Ewart and Driedzic (1987) demonstrated that ventricles of mature Atlantic salmon and brook trout have higher activities of CS and HOAD compared with immature conspecifics. However, unlike the present study, they observed increased HK activity with maturity. Likewise, cold-temperature acclimation, which results in increased relative ventricle mass in many teleosts, is often associated with increased activities of aerobically poised enzymes, including HK, in cardiac muscle (Driedzic, 1992; Rodnick and Sidell, 1997). Comparisons with cardiac hypertrophy in mammals emphasize that trout myocardium exhibits potentially unique changes in energy metabolism as ventricles hypertrophy. Volume-overload (e.g. endurance exercise) in mammals results in cardiac hypertrophy but does not change mass-specific enzyme activities (Laughlin *et al.* 1991). Pressure-overload (e.g. hypertension), resulting in a non-failing hypertrophied heart in rats, is associated with decreased HOAD and increased HK activities, with similar alterations in the respective oxidative pathways (Allard *et al.* 1994; Christe and Rogers, 1994). However, the failing mammalian heart exhibits a reduced (by 30 %) expression of HK mRNA and an elevated (by 70 %) abundance of lipase mRNA, compared with a non-failing heart, suggesting that the oxidation of lipid fuels is enhanced and that the maximal rate of oxidation of glucose is decreased (Raynolds *et al.* 1994). Thus, our data suggest that, compared with other models of cardiac hypertrophy in mammals or fishes, ventricular hypertrophy during sexual maturation in male trout results in unique alterations in cardiac energy metabolism.

Finally, our data support the general consensus that the mixed-type ventricular myocardium of fishes exhibits transmural differences in energy metabolism. Our results are unique, however, in demonstrating that transmural differences for some oxidative enzymes (CS, HOAD) decrease with ventricular hypertrophy. The literature is equivocal with

respect to transmural CS and HOAD activities in fish hearts, but most studies have demonstrated higher endocardial than epicardial HK activity (Bass *et al.* 1973; Tota, 1983; Ewart and Driedzic, 1987; Farrell *et al.* 1990). Similarly, although Atlantic salmon (Ewart and Driedzic, 1987) and *Conger conger* (Tota, 1983) appear to have higher endocardial activities of LDH, studies in carp (Bass *et al.* 1973) and rainbow trout (Poupa *et al.* 1974; Farrell *et al.* 1990) have indicated that no transmural difference exists. The lack of transmural differences in PK and LDH activities in this study and PK in Atlantic salmon (Ewart and Driedzic, 1987) suggests that anaerobic capacity is similar between myocardial layers, regardless of the degree of sexual maturity, in salmonids. We conclude that the capacity for oxidative metabolism of both fatty acids and glucose may be higher in the endocardium of male rainbow trout than in the epicardium, but that both layers have similar capacities for anaerobic metabolism.

We have confirmed that sexual maturation in male trout increases ventricular mass out of proportion to body size. Gonadal maturation is positively correlated with a more than twofold increase in ventricle mass, which results entirely from cardiomyocyte hypertrophy. However, unlike the heart of postnatal mammals, normal growth of the trout heart must be supported by myocyte hyperplasia, which probably occurs during or after gonadal regression. Myocyte hyperplasia, together with the maintenance of the elliptical transverse shape of myocytes, may reflect a physiological requirement to maintain short diffusion distances for small molecules in trout cardiomyocytes. Ventricular hypertrophy results in an increased proportion of epicardium relative to endocardium, which may reflect the biophysical necessity of reducing wall stress as ventricular diameter increases. The dramatic decrease in endocardial and epicardial HK activities, and concomitant increase in epicardial HOAD activity in hypertrophied ventricles, represents a possible shift in fuel preference with ventricular hypertrophy.

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