The Journal of Experimental Biology 200, 931–939 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0670

THERMALLY INDUCED CHANGES IN INTRACELLULAR pH AND MODULATORS OF PHOSPHOFRUCTOKINASE IN TROUT WHITE MUSCLE

ERIC A. LEHOUX* AND HELGA E. GUDERLEY†

Département de Biologie, Faculté des Sciences et de Génie, Université Laval, Ste-Foy, Québec, Canada G1K 7P4

Accepted 29 December 1996

Summary

The intracellular pH (pHi) and the concentrations of lactate and selected modulators of phosphofructokinase (PFK; EC 2.7.1.11) were measured in white epaxial muscle of 15 °C-acclimated rainbow trout (*Oncorhynchus mykiss*) maintained at 8, 15 or 22 °C for 48 h and sampled at rest and after 10 min of exhaustive exercise. The lactate accumulation resulting from exercise was 13 % smaller at 22 °C than at 8 and 15 °C. The estimated duration of burst performance was shorter at cold than at warm temperatures, whereas the average rate of lactate accumulation during burst performance was higher at 8 °C than at 15 and 22 °C. pHi rose when temperature decreased, but less than predicted by the imidazole alphastat hypothesis of Reeves. The effects of temperature on the pre-exercise concentrations of PFK modulators [adenylates, fructose 6-phosphate (F6P) and fructose 1,6-bisphosphate (FBP)] were generally negligible. In exhausted trout, adenylate concentrations were almost unaffected by temperature. In contrast, post-exercise FBP and F6P concentrations were significantly higher at low than at high temperatures. We interpret the response of F6P to temperature as an indication that the covariation of pHi and temperature is insufficient to prevent a cold-enhancement of PFK inhibition. Since F6P is a potent activator of PFK, we conclude that, in trout white muscle, thermally induced changes in F6P concentration probably help buffer the effects of temperature change on PFK activity.

Key words: glycolysis, exercise, temperature, intracellular pH, lactate, glycolytic flux, adenylates, fructose phosphate, *Oncorhynchus mykiss*, fish muscle, rainbow trout.

Introduction

Fish living in temperate zones can experience large fluctuations in their body temperature. Because of the high thermal dependence of biochemical reaction rates, fluctuations in body temperature pose a problem for the maintenance of metabolic flux and regulation. To solve this problem, fish can modify their biochemical systems in response to temperature change. For example, it is well established that, during cold acclimation, fish undergo, in the course of several weeks, a series of compensatory biochemical modifications (for reviews, see Sänger, 1993; Johnston, 1993). Because of the importance of locomotion, the effects of cold acclimation on muscle metabolic organization have been extensively studied (for reviews, see Egginton and Sidell, 1989; Guderley, 1990; Johnston, 1993). In contrast, the effects of acute thermal change on fish muscle metabolism have received little attention. This is surprising since short-term responses to rapid thermal change are probably as important for fitness and survival as acclimatory responses to thermal change.

The contractile activity of fish white muscle is primarily fuelled by anaerobic glycolysis (Dobson *et al.* 1987). As in all metabolic pathways, the flux of carbon through glycolysis depends on the concentration and catalytic activity of the enzymes constituting the pathway. Since both quantitative and qualitative changes in enzyme expression are fairly slow, short-term responses to thermal change must involve changes in levels of micromolecules or in the phosphorylation state of regulatory enzymes. In the present study, we are concerned with changes in intracellular pH (pHi) and in the concentration of modulators regulating the activity of a flux-controlling enzyme. These changes have the potential to provide a significant and essentially instantaneous response to thermally induced modifications in enzymatic activity.

The focus of this study, phosphofructokinase (PFK; EC 2.7.1.11), is the main flux-controlling enzyme of glycolysis in muscles (Voet and Voet, 1995). Increases in the activity of this enzyme following cold acclimation in rainbow trout *Oncorhynchus mykiss* indicate a need for long-term thermal compensation of PFK activity in white muscle (Guderley and Gawlicka, 1992). Muscle PFK is an excellent candidate for the study of responses to acute thermal change. Key ionizable

*Present address: Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University, Stillwater, OK 74078, USA.
†Author for corrspondence (e-mail: helga.guderley@bio.ulaval.ca).

groups (the imidazole moiety of histidine residues; His) which control the activity of muscle PFK make this enzyme unusually sensitive to a decrease in temperature (Bock *et al.* 1975; Tellam and Frieden, 1981; Hand and Somero, 1983). A decrease in temperature enhances the sensitivity of PFK to proton inhibition by increasing the pK of the key histidine groups (pK_{His}) of the enzyme. At constant pH, this increased sensitivity to proton inhibition results in a greater proportion of inactive protonated forms of the enzyme (Bock *et al.* 1975). In fish muscle, the inverse relationship between pHi and temperature helps compensate for the effect of temperature on the pK_{His} of PFK and other enzymes (for a review, see Heisler, 1986).

The purpose of the present study is to answer the following questions. Is the covariation of pHi and temperature ($\Delta pHi/\Delta T$) sufficient to offset fully the effects of a decrease in temperature on PFK activity in fish white muscle? If not, can changes in the concentrations of PFK modulators help buffer the effects of short-term temperature changes on PFK activity? To answer these and other questions, we compared the pHi and the concentration of lactate and PFK modulators in the white epaxial muscle of rested and exhausted, 15 °C-acclimated rainbow trout maintained for 48 h at 8, 15 and 22 °C. The modulators we selected for our metabolic analysis [ATP, ADP, AMP, fructose 6-phosphate (F6P) and fructose 1,6-bisphosphate (FBP)] are considered to be of major physiological importance in the regulation of muscle PFK (Spriet, 1990) and, as they all exert their effect by shifting the pK_{His} of PFK (Bock and Frieden, 1976; see also Su and Storey, 1992), they are particularly likely to respond to the cold-enhanced proton inhibition of this enzyme.

Materials and methods

Chemicals

Enzymes and biochemicals of the highest quality available were purchased from Boehringer Mannheim (Laval, Canada). Buffers, glutamate, glucose and nitrilotriacetic acid were purchased from Sigma Chemical Co. (St Louis, USA). The pH reference buffer solution was purchased from Canlab (Montréal, Canada). All other chemicals were purchased from Fisher Scientific (Québec, Canada) or Anachemia (Montréal, Canada).

Animals

Immature rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were purchased from local hatcheries (La Pisciculture Sans Limites and La Pisciculture des Alleghanys). The fish were kept in 500 l tanks of recirculated, biologically filtered water under a 15 h:9 h light:dark photoperiod. The animals were fed *ad libitum* with commercial trout pellet food. The trout were acclimated to a water temperature of 15.0±1.2 °C for a minimum of 2 months. They were fasted for 48 h before being transferred to the cages or the swimming arena. Experiments were performed in winter and early spring. All protocols involving the use of rainbow trout were approved by the local animal care committee.

Rest protocol and muscle sampling

Trout (N=37) of mean body mass 42.3±0.5 g (mean ± S.E.M.), mean fork length 15.2±0.1 cm and mean condition factor (CF) 12.0±0.2 mg cm^{-3} were divided into three groups of comparable body mass, fork length and CF. The CF was calculated by dividing the body mass of a fish by the cube of its fork length.

Trout were confined in specially designed cages made of an inner chamber (19 cm length × 4.5 cm diameter) constructed of semi-flexible 8 mm plastic mesh, tightly fitted into an outer chamber (23 cm length × 10.5 cm height × 6 cm width) constructed of black Plexiglas. The open end of the inner chamber was closed with a section of 2 mm plastic mesh held in place with a rubber band. To keep the trout in the dark, each cage was covered with a piece of black fabric. A wire handle, passing through the fabric, was attached to the top of the inner cage. Circulation and oxygenation of the water were ensured by a small air stone and a series of 5 mm diameter holes drilled in the bottom of the outer chamber. The cages were partially submerged (approximately 65 % of the cage) in a thermostatted tank maintained at one of the experimental temperatures: 8.0, 15.0 and 22.0 °C (±1.2 °C).

After a 48 h rest period, each trout was rapidly killed by discreetly grabbing the handle of the cage and hitting the flexible inner cage (containing the trout) against a concrete block. The time elapsed between the grabbing of the handle and the moment the trout was killed was only a fraction of a second. The fish was taken out of the cage and white epaxial muscle immediately sampled between the dorsal and anal fins, approximately 2 mm above the lateral line. To minimize neural stimulation of the muscle, the longitudinal cut through the fish was performed first. Dissection was carried out directly on the concrete block which was cooled in an ice/water bath. Muscle samples (approximately 2 g) were wrapped in aluminium foil and freeze-clamped in liquid N_2 (Wollonberger *et al.* 1960) precisely 20 s after the trout was killed. An additional sample of white epaxial muscle was excised from under the dorsal fin, freeze-clamped as above and kept in liquid N_2 for determination of water content. All fish were sampled in the evening.

Exercise protocol

Trout (N=33) of mean body mass 45.9±0.6 g, mean fork length 16.3±0.1 cm and mean CF 10.6±0.1 mg cm^{-3} were divided into three groups of comparable body mass, fork length and CF.

The trout were transferred from a 15 °C holding tank to a half-filled 450 l oval arena (1.80 m length × 0.50 m width × 0.55 m height) maintained at one of the experimental temperatures: 8.0, 15.0 and 22.0 °C (±0.5 °C). Dissolved oxygen levels were maintained at saturation. After a 48 h acclimation period, each trout was individually chased with a 13 V electric prod (Pearson *et al.* 1990). At the beginning of the chase, the trout immediately started swimming in burst mode and maintained this mode of swimming until they could no longer do so. The end of the burst swimming period was

marked by a relatively sudden and drastic decrease in swimming velocity. Using this change in velocity as a criterion, we estimated the duration of the burst swimming performance with a manually operated chronometer. Although burst swimming was never maintained for more than 2 min, the trout were always chased for 10 min. Some trout remained capable of very short bursts of swimming after the initial burst swimming period had ended. Because of their short duration (typically <1–2 s) and low frequency of occurrence (typically 0–3 bursts per trout), the duration of these bursts was not recorded. No differences in swimming behaviour or velocities were discerned between the different temperatures. At the end of the chase, the trout were no longer capable of burst swimming but they could still swim very slowly. The trout were rapidly captured by hand and killed by a blow to the head. Muscle was sampled as in the rest protocol.

Muscle extraction and pHi determinations

These procedures were carried out within 24 h of the sampling. Muscle samples stored in liquid N_2 were rapidly transferred to a porcelain mortar half-filled with liquid N_2 and kept in a liquid N_2 bath. The aluminium foil was discarded and the muscle was ground to a fine powder using a pre-cooled pestle. Skin fragments were removed during grinding, which typically took approximately 1 min.

The mean pHi of the muscle sample was estimated by direct measurement of the homogenate pH after metabolic inhibition, as described by Pörtner *et al.* (1990). Briefly, a sample (approximately 150 mg) of the muscle powder in liquid N_2 was taken from the mortar, rapidly strained under the liquid N_2 atmosphere of the bath and transferred to a 500 µl Eppendorf microcentrifuge tube containing 200 µl of inhibitor solution (150 mmol l^{-1} potassium fluoride; 6 mmol l^{-1} nitrilotriacetic acid, pH approximately 0.15 below the value to be measured). The transferred powder was rapidly covered with the inhibitor solution and mixed with a needle. The microcentrifuge tube was then filled with inhibitor solution (final volume approximately 700 µl). The inner lid of the cap of the microcentrifuge tube was nicked, to allow complete filling of the tube by the extrusion of air bubbles during closing. The mixture was agitated in a Vortex mixer (maximal setting) for 3 s, and centrifuged for 15 s (excluding acceleration time) at 500 ***g*** (4 °C) in a Sorval RC-5B centrifuge using an SS-34 rotor. A sample of the supernatant was immediately taken for the measurement of pH at one of the experimental temperatures (8.0, 15.0 and 22.0 °C, ±0.1 °C) in a thermostatted (Haake D8-G circulating water bath) microcapillary pH electrode (Radiometer G297/G2) coupled with a pH meter (Radiometer PHM 84). The instrument was calibrated at the appropriate temperature using a pH 7.00±0.01 (25 °C) reference buffer solution.

Using a liquid-N_2-cooled polypropylene funnel, approximately 1 g of the remaining partially dried muscle powder was transferred to the bottom of a pre-weighed centrifugation tube containing 1500 µl of ice-cold 7.5 % (v/v) perchloric acid solution. The powder was immediately homogenized for 30 s using a Polytron homogenizer (intermediate setting) coupled with a 7 mm generator. The tube was reweighed and the dilution was brought to 1:5 (m/v) with the perchloric acid solution. The mixture was then briefly agitated in a Vortex mixer and centrifuged for 15 min at 26 000 ***g*** (4 °C). A known volume of supernatant was transferred to a pre-cooled centrifugation tube and neutralized to pH 7 by slow addition of 5 mol l^{-1} potassium carbonate. Neutralization was performed in an ice bath with a 1 min cooling pause half-way through the procedure. The suspension was centrifuged for 5 min as above, and the supernatant was immediately frozen and stored in liquid N_2. All metabolite assays were performed within a week.

Metabolite assays

The muscle concentrations of ATP, ADP, AMP, F6P, FBP and lactate were measured enzymatically as described in Bergmeyer (1983). In the ADP/AMP assay, the pH of the triethanolamine hydrochloride buffer was adjusted to 7.6 (25 °C) with potassium carbonate and the NADH concentration was decreased to 120 µmol l^{-1}. Because some metabolites are unstable in thawed neutralized extracts, the assays were always performed in the same order. All enzymatic assays were routinely controlled for accuracy using standard solutions prepared with commercially available purified metabolites.

Water content determination

Muscle water content was determined by measuring the difference between the wet and dry masses of tissue samples. To minimize condensation during wet mass measurements, the freeze-clamped samples were rapidly weighed following a short incubation at –20 °C. The dry mass of the samples was measured after 72 h at 60 °C.

Data analysis

All data are reported as mean values ± S.E.M. Metabolite concentrations and pHi values as well as the mass, fork length and CF of the trout were compared using two-factor analysis of variance (ANOVA; factors: temperature and exercise status). Data which did not conform to the assumptions of normality and homogeneity of variance were transformed to meet these assumptions before applying the tests. Untransformed data are shown. When no interactions were found between the factors, one-way ANOVAs were carried out, followed by a Student–Newman–Keuls (SNK) multiple-comparisons test (SuperANOVA version 1.1, Abacus Concepts, Berkeley, USA). When the data could not be transformed to meet the above assumptions, they were compared using a Kruskal–Wallis test (Statview SE, Abacus Concepts, Berkeley, USA) followed by a Noether *a posteriori* test (Scherrer, 1984). The duration of burst swimming and the glycolytic flux data were compared using one-way ANOVAs, followed by an SNK multiple-comparisons test (SuperANOVA). A Welch's ANOVA (JMP, version 2.0; SAS

Table 1. *Pre- and post-exercise white muscle water content of 15 °C-acclimated rainbow trout maintained at different temperatures for 48 h*

Status	8 °C	15 °C	22 °C
Pre-exercise	3.69±0.04 (14)	3.75±0.05 (12)	3.77±0.05 (10)
Post-exercise	3.97±0.05 (11)	3.95±0.04 (12)	3.78±0.04 (10)

Values are means ± S.E.M. (*N*) in g water g^{-1} dry muscle.
Temperature and exercise interacted to determine water content (P<0.01; two-factor ANOVA).

Institute) followed by a Scheffé's multiple-comparisons test was used to analyse the F6P data. Linear regressions (Statview) were used to examine the covariation of pHi and temperature, and 95 % confidence intervals were used to compare the slopes. The 0.05 significance level was used throughout.

Results

Water content

The mean white muscle water content of all trout was 3.82±0.05 g water g^{-1} dry muscle (Table 1), and the largest difference between two experimental groups was 7.6 %. In the remainder of this paper, we express the concentrations of metabolites per gram fresh muscle since we are interested in the propensity of a solute (metabolite) to participate in a biochemical reaction. This propensity depends on the chemical potential of a solute which, in turn, is dependent on water content.

Lactate and pHi

The accumulation of lactate resulting from exercise (Fig. 1) was 13 % smaller at 22 °C than at 8 and 15 °C (P<0.05; ANOVA followed by SNK). This smaller lactate accumulation was not accompanied by a smaller drop in pHi (Fig. 2):

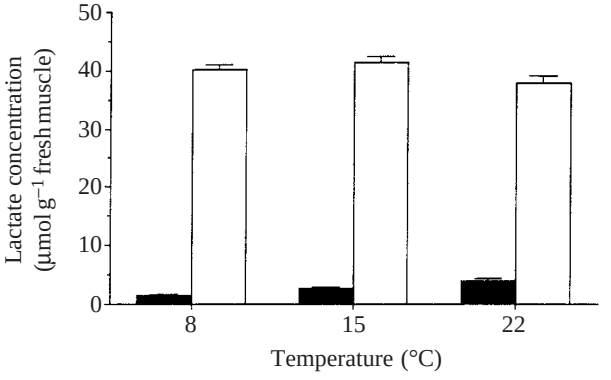

Fig. 1. Pre-exercise (filled bars) and post-exercise (open bars) white muscle lactate concentrations of 15 °C-acclimated rainbow trout maintained at different temperatures for 48 h. Columns and error bars represent means + S.E.M. Rested trout, *N*=14 (8 °C), *N*=12 (15 °C), *N*=11 (22 °C). Exhausted trout, *N*=11 (8 °C), *N*=12 (15 °C), *N*=10 (22 °C). Temperature and exercise interacted to determine lactate concentration (two-factor ANOVA).

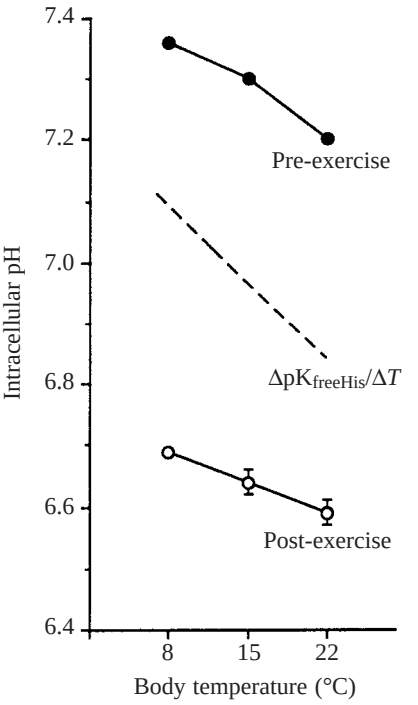

Fig. 2. Relationship between body temperature (*T*) and pre- and post-exercise white muscle intracellular pH of 15 °C-acclimated rainbow trout. Muscle was sampled in trout maintained at the indicated temperatures for 48 h. Symbols and error bars represent means ± S.E.M. Rested trout, *N*=13 (8 °C), *N*=11 (15 °C), *N*=10 (22 °C). Exhausted trout, *N*=10–12 (see Fig. 1). All values are significantly different from one another (ANOVA followed by SNK *a posteriori* test). Standard errors equal to 0.01 are covered by the symbols. The dashed line shows $\Delta pK_{freeHis}/\Delta T$ (Reeves, 1972).

0.61±0.02 at 22 °C *versus* 0.67±0.01 and 0.65±0.02 at 8 and 15 °C respectively (P>0.05; ANOVA). The exercise-induced covariation in pHi and [lactate] (ΔpHi/Δ[lactate] where Δ[lactate] is expressed in μmol g^{-1} fresh muscle) did not differ at the three temperatures: –0.0170±0.0004 (8 °C), –0.0170±0.0004 (15 °C) and –0.0180±0.0010 (22 °C) (P>0.05, ANOVA).

In both rested and exhausted trout, white muscle pHi decreased when temperature rose (Fig. 2; Table 2). While the $\Delta pHi/\Delta T$ appeared to be greater in rested than in exhausted trout, the difference was not statistically significant. In rested trout, $\Delta pHi/\Delta T$ was greater between 15 and 22 °C than between 8 and 15 °C. $\Delta pHi/\Delta T$ was always smaller than –0.017, the $\Delta pK/\Delta T$ of the imidazole moiety of free histidine, except between 15 and 22 °C in the rested trout.

Duration of burst swimming and glycolytic flux

The estimated duration of burst swimming was significantly shorter at 8 °C than at 15 and 22 °C (P<0.05; ANOVA followed by SNK; Fig. 3A). As a result, the estimated average glycolytic flux (rate of lactate accumulation) during burst swimming was higher in trout exercised at 8 °C (P<0.05; ANOVA followed by SNK; Fig. 3B).

Table 2. *Changes in intracellular pH with changes in temperature range (ΔpHi/ΔT) in white muscle of rested and exhausted 15 °C-acclimated rainbow trout*

Status	8–15 °C	15–22 °C	8–22 °C
Pre-exercise	–0.008±0.002* (–0.005 to –0.011)	–0.015±0.001 (–0.012 to –0.018)	–0.011±0.001* (–0.010 to –0.013)
Post-exercise	–0.007±0.002* (–0.003 to –0.011)	–0.007±0.005* (0.002 to –0.017)	–0.007±0.002* (–0.003 to –0.011)

ΔpHi/Δ*T* values were calculated using the data for which the mean values are shown in Fig. 2.
Values are means ± S.E.M. with the 95 % confidence limits indicated in parentheses.
* indicates a value differing significantly from the ΔpK/Δ*T* of the imidazole moiety of free histidine.

Adenylates, fructose 6-phosphate and fructose 1,6-bisphosphate

Temperature had no significant effect on pre-exercise muscle adenylate concentrations (Fig. 4A–C). Post-exercise adenylate concentrations were also mostly unaffected by the temperature at which exercise occurred. Exercise drastically decreased the muscle [ATP] by an average of 76 %, but had little effect on the corresponding [ADP] and [AMP].

In rested trout, [F6P] was not significantly affected by temperature, whereas in exhausted trout [F6P] was higher at 8 °C than at 15 and 22 °C (Fig. 4D) (P<0.05, ANOVA followed by SNK test). Exercise increased [F6P] but only at 8 and 15 °C (P<0.05; ANOVA followed by SNK). High pre-exercise [FBP] was found at all temperatures (Fig. 4E). In exhausted trout, [FBP] rose when temperature was decreased (P<0.05; ANOVA followed by SNK). Despite the impressive suppression of the [FBP]/[F6P] ratio by exercise and the significant effect of temperature on post-exercise [FBP] and [F6P], the [FBP]/[F6P] ratios in exhausted trout were similar at all three temperatures: 0.085±0.006, 0.106±0.014 and 0.106±0.017 at 8, 15 and 22 °C respectively (P>0.05, ANOVA).

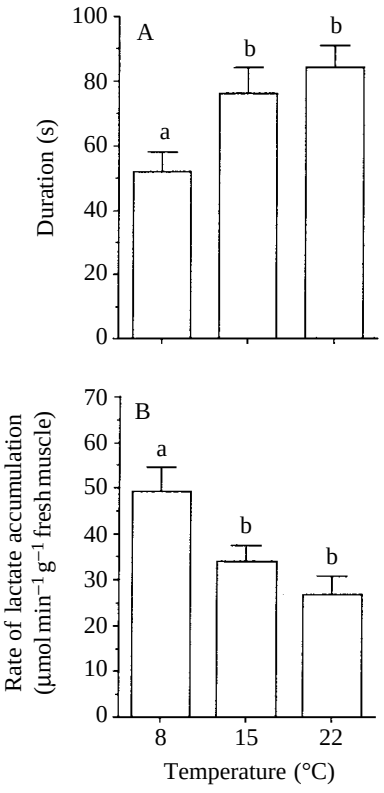

Fig. 3. Estimated durations of burst swimming (A) and white muscle glycolytic flux during burst swimming (B) for 15 °C-acclimated rainbow trout exercised at different temperatures after a 48 h acclimation period. Columns and error bars represent means + S.E.M. N=10 (8 °C), N=11 (15 °C), N=10 (22 °C). Different superscripts indicate a significant difference (ANOVA followed by SNK *a posteriori* test).

Discussion

Pre-exercise metabolite concentrations and pHi

In fish, excitement can lead to a stimulation of anaerobic glycolysis and result in marked changes in white muscle pHi and metabolic status (Wieser *et al.* 1986; Tang and Boutilier, 1991; Dobson and Hochachka, 1987). The determination of true 'resting' pHi and the true 'resting' concentrations of several metabolites in fish white muscle is therefore highly dependent on the resting environment and the sampling procedure (Wieser *et al.* 1986). In the present study, trout were housed in specially designed cages that restrained activity and provided a stable resting environment. The design of the cages minimized handling stress by facilitating rapid tissue sampling. The use of these cages resulted in low muscle [lactate] while avoiding the technical difficulties associated with the use of anaesthetics. The white muscle [lactate] determined in trout rested at 8 °C (1.51±0.07 μmol g^{-1} fresh muscle) compares well with the lowest values published for rainbow trout (1.02±0.10 μmol g^{-1} fresh muscle at 6–9 °C by Tang and Boutilier, 1991; 1.54±0.15 μmol g^{-1} fresh muscle at 10 °C by Pearson *et al.* 1990). The muscle pHi measured at 15 °C is also in excellent agreement with the value reported by Tang and Boutilier (1991).

The pre-exercise adenylate, F6P and FBP concentrations we determined also agree well with other reports for rainbow trout (Dobson *et al.* 1987; Dobson and Hochachka, 1987; Parkhouse *et al.* 1988*a*,*b*; Pearson *et al.* 1990).

Effect of exercise on metabolite concentrations and pHi

The large accumulations of lactate and large reductions in pHi that resulted from exercise are characteristic of a strenuous anaerobic effort and agree well with previous studies using exhaustive exercise protocols (Milligan and Wood, 1986; Dobson *et al.* 1987; Parkhouse *et al.* 1988*a*,*b*; Tang and Boutilier, 1991). The responses of the [ATP] and [ADP] to exercise were typical for rainbow trout, whereas the absence of a significant increase in the [AMP] at 8 °C contrasts with other reports for rainbow trout (Dobson *et al.* 1987; Dobson and Hochachka, 1987; Parkhouse *et al.* 1988*b*; Pearson *et al.* 1990). The cause of this discrepancy in the response of [AMP] is unknown.

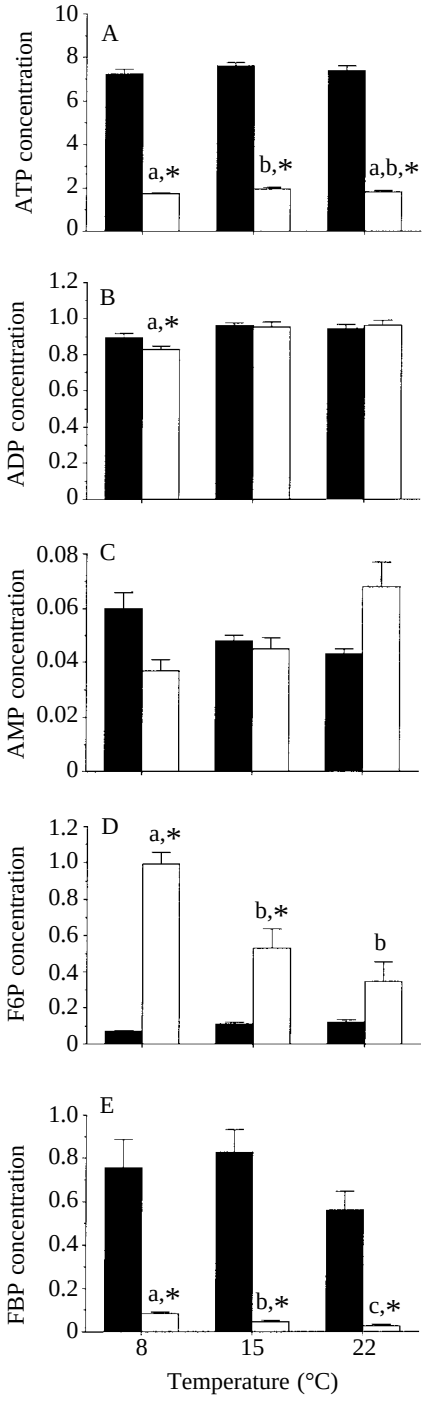

Fig. 4. Pre-exercise (filled bars) and post-exercise (open bars) ATP (A), ADP (B), AMP (C) fructose 6-phosphate (F6P) (D) and fructose 1,6-bisphosphate (FBP) (E) concentrations (μmol g^{-1} fresh muscle) in white muscle of 15 °C-acclimated rainbow trout maintained at different temperatures for 48 h. Columns and error bars represent means + S.E.M. N=10–14 as in Fig. 1, except for FBP in rested trout where N=8 at 8 °C and N=6 at 15 and 22 °C. The FBP data presented for trout rested at 8 and 22 °C were collected in a complementary experiment performed with a different group of trout. Because these data were collected separately, they were not and should not be directly compared with the FBP data presented for trout rested at 15 °C. Different superscripts indicate a significant effect of temperature and an asterisk indicates a significant difference between the corresponding pre- and post-exercise values (ANOVA followed by SNK *a posteriori* test, ATP, ADP and FBP; Kruskal–Wallis, AMP; Welch's ANOVA followed by a Scheffé's *a posteriori* test, F6P).

At 8 °C, the exercise-induced 14.6-fold increase in [F6P] compares well with the 8.5-fold increase (10 °C) reported by Pearson *et al.* (1990). More modest increases have been reported in studies in which rainbow trout were exercised at 10 °C in a swim tunnel (Dobson *et al.* 1987; Parkhouse *et al.* 1988*a*). Exercise drastically decreased [FBP] below resting values, in good agreement with existing reports on rainbow trout (Dobson *et al.* 1987; Parkhouse *et al.* 1988*a*).

Effect of temperature on the recruitment of anaerobic glycolysis

After the first few tail beats, burst swimming is mainly fuelled by anaerobic glycolysis in white muscle. Since lactate clearance from white muscle is quite slow (Milligan and Wood, 1986; Dobson and Hochachka, 1987), it is possible to estimate muscle glycolytic flux from the duration of an anaerobic effort and the resulting lactate accumulation. The method we used to estimate the duration of burst performance in trout is, by its nature, sensitive to systematic errors. Our burst swimming data were therefore collected with the best of efforts to minimize such errors. Notwithstanding, in the rest of this paper, we interpret the glycolytic flux data (which are based on the burst swimming data) in a conservative fashion.

In our exercise protocol, the trout were forced to swim for several minutes after the end of their intense anaerobic effort. Some lactate was probably produced during this period of essentially aerobic swimming. However, the contribution of this lactate to the total lactate accumulation is unlikely to have been significant since, in rainbow trout, the end of the burst swimming period is associated with a virtually complete depletion of white muscle glycogen (Milligan and Wood, 1986). As a result, extension of the swimming period beyond the initial period of burst performance can only have led only to a slight overestimation of the glycolytic flux. We are therefore confident that, overall, the errors in our estimates of glycolytic flux are small enough for our results to show clearly that the glycolytic flux was not reduced when temperature was decreased from 22 to 8 °C. This result is compatible with the

report that in young 8 °C-acclimated rainbow trout, vigorously chased for 60 s, glycolytic flux was similar at 4, 12 and 20 °C (Wieser *et al.* 1985).

Although glycolysis was recruited more intensely and for a shorter period at 8 than at 15 °C, the accumulation of muscle lactate resulting from exercise was equal at these temperatures. This indicates that, at the end of the exercise, trout had recruited anaerobic glycolysis to a similar extent and had reached a similar state of exhaustion. Therefore, differences between pHi and PFK modulator concentrations measured in trout exhausted at 8 and 15 °C cannot be attributed to a difference in the degree of exhaustion. The smaller accumulation of lactate at 22 °C is only partly explained by the higher pre-exercise lactate concentration at that temperature. A temperature of 22 °C may be somewhat high for optimal locomotor performance of rainbow trout (Taylor *et al.* 1996).

The gradual rise in pre-exercise [lactate] that accompanied a rise in temperature probably reflects an increased metabolic rate and/or excitation of trout. The tendency of pre-exercise [F6P] to rise with increasing temperature supports this interpretation.

Effect of temperature on pHi

The similarity of the exercise-induced ΔpHi/Δ[lactate] at 8, 15 and 22 °C indicates a low thermal dependence of the *in vivo* buffering capacity of rainbow trout white muscle. This is in agreement with *in vitro* studies on muscle homogenates from rainbow trout and other fish species (Abe and Okuma, 1991; Castellini and Somero, 1981) and the *in situ* study of Aickin and Thomas (1977) on mouse soleus fibres.

In both rested and exhausted trout, the average white muscle $\Delta pHi/\Delta T$ between 8 and 22 °C was smaller than the $\Delta pH_{freeHis}/\Delta T$. A small $\Delta pHi/\Delta T$ is consistent with the predominance of inorganic phosphate among the non-bicarbonate buffers in rainbow trout white muscle (Abe *et al.* 1985; Okuma and Abe, 1992). The pK of inorganic phosphate is much less dependent on temperature than is that of histidine and, between 10 and 20 °C, its thermal dependence is opposite to that of $pK_{freeHis}$ (Heisler, 1986; Abe and Okuma, 1991).

According to the imidazole alphastat hypothesis of Reeves (1972), a $\Delta pHi/\Delta T$ equal to the $\Delta pK_{freeHis}/\Delta T$ should maintain the ionization level of an enzyme such as PFK constant. Since we have already established that, in trout white muscle, the $\Delta pHi/\Delta T$ was smaller than the $\Delta pK_{freeHis}/\Delta T$, the question we now need to address is as follows: was the $\Delta pHi/\Delta T$ in trout muscle too small to compensate fully for the $\Delta pK_{His}/\Delta T$ of PFK? As emphasized by Cameron (1989), such a question is extremely difficult to answer because of the variations in the $\Delta pK_{His}/\Delta T$, which depend on protein environment as well as on the pH and temperature range. Nevertheless, in the present study, the information provided by the F6P data allows us to address this question. Since F6P is a substrate of PFK, the increase, with a decrease in temperature, of exercise-induced accumulation of F6P, clearly indicates a cold-enhancement of PFK inhibition. This enhancement of inhibition tells us that, in trout white muscle, the $\Delta pHi/\Delta T$ was not sufficiently large to compensate fully for the effects of a decrease in temperature on PFK activity. Although the $\Delta pK_{His}/\Delta T$ of PFK is not the only physical mechanism through which a decrease in temperature affects PFK activity (see Somero, 1995), it is certainly by far the most important. The results we report therefore suggest that, in trout white muscle, the $\Delta pHi/\Delta T$ was not large enough to compensate fully for the $\Delta pK_{His}/\Delta T$ of PFK and thus to prevent a cold-enhancement of the proton inhibition of PFK. The fact that an enhancement of the proton inhibition of PFK does not have to result in a decreased flux through the PFK reaction is discussed below.

Effects of temperature on PFK modulators

In resting muscles, PFK is severely inhibited by elevated [ATP] (Rawn, 1989). This may help explain why, in rested trout, temperature generally had a negligible effect on the concentrations of PFK modulators. The reason is that a severe inhibition of PFK may alleviate the need for PFK modulation in response to thermal change. In exhausted trout, [ATP] was essentially unaffected by temperature. This suggests that ATP, which plays a central role in the regulation of PFK (Dobson *et al.* 1986), was not involved in buffering the effects of thermal change on PFK activity. The observed lack of thermally induced changes in [ADP] and [AMP] is more difficult to interpret since, in muscles, much of the measured ADP and AMP is not free to react with PFK (Veech *et al.* 1979). Keeping this in mind, it is interesting to note that, in red muscle of exercising rainbow trout, [free ADP] does not vary with temperature (P. U. Blier, E. A. Lehoux and H. E. Guderley, in preparation) in spite of the marked thermal dependence of the $K_{0.5}$ of mitochondria for ADP (Blier and Guderley, 1993). Since adenylates are ubiquitous in metabolism (Atkinson, 1977), it seems possible that large thermally induced changes in adenylate concentrations are not tolerated because they would disrupt metabolic regulation. Clearly, there is a need for further investigation of the response, or lack thereof, of free adenylate concentrations to short-term thermal change.

In rat and frog fast-twitch muscles, [FBP] transiently increases shortly after the initiation of muscular activity (Aragón *et al.* 1980; Minatogawa and Hue, 1984; Bassols *et al.* 1986; Gerhard *et al.* 1990). A similar, but less well-defined, response was observed in a study on rainbow trout exercised in a swim tunnel (Parkhouse, 1988*a*). In that study, the white muscle [FBP] was significantly increased by 30 min of swimming below the critical swimming velocity (a speed at which white fibres are only slightly recruited) and drastically decreased by an additional period of exhaustive swimming. Such a response strongly suggests that if temperature-dependent changes in [FBP] help buffer the effects of thermal change on PFK activity in trout muscle, they are likely to do so shortly after the initiation of exercise, when [FBP] is high and [F6P] is low.

In the present study, we did not measure [FBP] in exercising trout (we collected data for rested and exhausted trout only) because inter-individual differences in the swimming

performance and behaviour of fish make it extremely difficult to replicate a specific exercise status. The unavailability of FBP data in exercising trout limits our ability to evaluate the potential role of FBP in short-term responses to thermal change. Nevertheless, it is possible to infer, from the low [FBP] measured in all exhausted trout, that thermally induced changes in [FBP] do not play a major role in the regulation of muscle PFK activity late during exhaustive exercise. Further investigation is needed to determine whether changes in [FBP] can, shortly after the initiation of exercise, play an important role in a response to thermal change. Finally, the similarity of the [FBP]/[F6P] ratio in trout exhausted at different temperatures suggests that [FBP] and [F6P] are not independently regulated.

To evaluate the potential role of thermally induced changes in [F6P] in the regulation of PFK activity better, let us first examine the role played by F6P during exercise. In contracting rat skeletal muscles, the rate of F6P accumulation is positively correlated with the rate of lactate accumulation (r=0.98, calculated from the data of Minatogawa and Hue, 1984) and the glycolytic rate (r=0.95, Katz and Lee, 1988). Because lactate and proton production are tightly coupled (Hochachka and Mommsen, 1983), it follows that the accumulation of F6P during muscle stimulation must be well correlated with the acidification of the intracellular milieu. This implies that acidification results in an inhibition of PFK, which in turn leads to an accumulation of F6P. As pointed out by Trivedi and Danforth (1966), when an inhibition of PFK leads to an accumulation of F6P and a possible decrease in [FBP], the flux through the PFK reaction need not be reduced. The reason is that, as exercise proceeds and intracellular protons accumulate, a gradual rise in [F6P] can offset the proton inhibition of PFK by shifting the pK_{His} of the enzyme towards lower pH values (see Fig. 1 of Trivedi and Danforth, 1966).

In trout white muscle, we found that a decrease in temperature was associated with an increase in the exercise-induced accumulation of F6P. Since a decrease in temperature enhances the sensitivity of PFK to proton inhibition by shifting the pK_{His} of the enzyme towards higher pH values, we conclude that, in trout exercised at low temperatures, the greater accumulation of F6P helped to offset the cold-enhancement of PFK inhibition by shifting the pK_{His} of the enzyme towards lower pH values. This conclusion is consistent with the observation that a decrease in temperature was not associated with reduced glycolytic flux and with the view that the accumulation of F6P during exercise is the major factor maintaining PFK activity as contraction proceeds (Andrés *et al.* 1990). Thermally induced changes in [F6P] offer a local response to what appears to be a local problem, the unusual cold-sensitivity of PFK.

We thank Dr Pierre U. Blier for critical comments, Dr Maria Huber for technical expertise, Dr Robert G. Boutilier for helpful advice on intracellular pH determination, Dominique Gagné for the collection of the complementary pre-exercise FBP data, and Cathy Bisson and François Houle for their excellent technical assistance. This work was supported by an operating grant from the NSERC to H.E.G. E.A.L. was recipient of an FCAR scholarship.

References

ABE, H., DOBSON, G. P., HOEGER, U. AND PARKHOUSE, W. S. (1985). Role of histidine-related compounds to intracellular buffering in fish skeletal muscle. *Am. J. Physiol.* **249**, R449–R454.

ABE, H. AND OKUMA, E. (1991). Effect of temperature on the buffering capacities of histidine-related compounds and fish skeletal muscle. *Nippon Suisan Gakkaishi* **57**, 2101–2107.

AICKIN, C. C. AND THOMAS, R. C. (1977). Micro-electrode measurement of the intracellular pH and buffering power of mouse soleus muscle fibres. *J. Physiol., Lond.* **267**, 791–810.

ANDRÉS, V., CARRERAS, J. AND CUSSÓ, R. (1990). Regulation of muscle phosphofructokinase by physiological concentrations of biphosphorylated hexoses: effect of alkalinization. *Biochem. biophys. Res. Commun.* **172**, 328–334.

ARAGÓN, J. J., TORNHEIM, K. AND LOWENSTEIN, J. (1980). On a possible role of IMP in the regulation of phosphorylase activity in skeletal muscle. *FEBS Lett.* **117**, K56–K64.

ATKINSON, D. E. (1977). *Cellular Energy Metabolism and its Regulation.* New York: Academic Press.

BASSOLS, A. M., CARRERAS, J. AND CUSSÓ, R. (1986). Changes in glucose 1,6-bisphosphate content in rat skeletal muscle during contraction. *Biochem. J.* **240**, 747–751.

BERGMEYER, H. U. (1983). *Methods of Enzymatic Analysis.* 3rd edition. Weinheim: Verlag Chemie.

BLIER, P. U. AND GUDERLEY, H. E. (1993). Mitochondrial activity in rainbow trout red muscle: the effect of temperature on the ADP-dependence of ATP synthesis. *J. exp. Biol.* **176**, 145–157.

BOCK, P. E. AND FRIEDEN, C. (1976). Phosphofructokinase. II. Role of ligands in pH-dependent structural changes of the rabbit muscle enzyme. *J. biol. Chem.* **251**, 5637–5643.

BOCK, P. E., GILBERT, H. R. AND FRIEDEN, C. (1975). Analysis of the cold lability behavior of rabbit muscle phosphofructokinase. *Biochem. biophys. Res. Commun.* **66**, 564–569.

CAMERON, J. N. (1989). Acid–base homeostasis: Past and present perspectives. *Physiol. Zool.* **62**, 845–865.

CASTELLINI, M. A. AND SOMERO, G. N. (1981). Buffering capacity of vertebrate muscle: Correlations with potentials for anaerobic function. *J. comp. Physiol.* **143**, 191–198.

DOBSON, G. P. AND HOCHACHKA, P. W. (1987). Role of glycolysis in adenylate depletion and repletion during work and recovery in teleost white muscle. *J. exp. Biol.* **129**, 125–140.

DOBSON, G. P., PARKHOUSE, W. S. AND HOCHACHKA, P. W. (1987). Regulation of anaerobic ATP-generating pathways in trout fast twitch skeletal muscle. *Am. J. Physiol.* **253**, R186–R194.

DOBSON, G. P., YAMAMOTO, E. AND HOCHACHKA, P. W. (1986). Phosphofructokinase control in muscle: nature and reversal of pH-dependent ATP inhibition. *Am. J. Physiol.* **250**, R71–R76.

EGGINTON, S. AND SIDELL, B. D. (1989). Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am. J. Physiol.* **256**, R1–R9.

GERHARD, W., KRAUSSE, U. AND THUY, M. (1990). Fructose 2,6-bisphosphate and glycolytic flux in skeletal muscle of swimming frog. *FEBS Lett.* **267**, 257–260.

GUDERLEY, H. (1990). Functional significance of metabolic responses

to thermal acclimation in fish muscle. *Am. J. Physiol.* **259**, R245–R252.

GUDERLEY, H. AND GAWLICKA, A. (1992). Qualitative modification of muscle metabolic organization with thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **10**, 123–132.

HAND, S. C. AND SOMERO, G. N. (1983). Phosphofructokinase of the hibernator *Citellus beecheyi*: temperature and pH regulation of activity *via* influences on the tetramer–dimer equilibrium. *Physiol. Zool.* **56**, 380–388.

HEISLER, N. (1986). Comparative aspects of acid–base regulation. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 397–450. Amsterdam: Elsevier Biomedical Press.

HOCHACHKA, P. W. AND MOMMSEN, T. P. (1983). Protons and anaerobiosis. *Science* **223**, 1319–1322.

JOHNSTON, I. A. (1993). Phenotypic plasticity of fish muscle to temperature change. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 322–340. London: Chapman & Hall.

KATZ, A. AND LEE, A. D. (1988). G-1,6-P2 in human skeletal muscle after isometric contraction. *Am. J. Physiol.* **255**, C145–C148.

MILLIGAN, C. L. AND WOOD, C. M. (1986). Tissue intracellular acid–base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J. exp. Biol.* **123**, 123–144.

MINATOGAWA, Y. AND HUE, L. (1984). Fructose 2,6-bisphosphate in rat skeletal muscle during contraction. *Biochem. J.* **223**, 73–79.

OKUMA, E. AND ABE, H. (1992). Major buffering constituents in animal muscle. *Comp. Biochem. Physiol.* **102**A, 37–41.

PARKHOUSE, W. S., DOBSON, G. P. AND HOCHACHKA, P. W. (1988*a*). Control of glycogenolysis in rainbow trout muscle during exercise. *Can. J. Zool.* **66**, 345–351.

PARKHOUSE, W. S., DOBSON, G. P. AND HOCHACHKA, P. W. (1988*b*). Organization of energy provision in rainbow trout during exercise. *Am. J. Physiol.* **254**, R302–R309.

PEARSON, M. P., SPRIET, L. L. AND STEVENS, E. D. (1990). Effect of sprint training on the swim performance and white muscle metabolism during exercise and recovery in rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **149**, 45–60.

PÖRTNER, H. O., BOUTILIER, R. G., TANG, Y. AND TOEWS, D. P. (1990). Determination of intracellular pH and P_{CO_2} after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 255–274.

RAWN, J. D. (1989). *Biochemistry*. Burlington: Neil Patterson Publishers.

REEVES, R. B. (1972). An imidazole alphastat hypothesis for vertebrate acid–base regulation: tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* **14**, 219–236.

SÄNGER, A. M. (1993). Limits to the acclimation of fish muscle. *Rev. Fish Biol. Fish.* **3**, 1–15.

SCHERRER, B. (1984). *Biostatistique*. Chicoutimi: G. Morin Edition.

SOMERO, G. N. (1995). Proteins and temperature. *A. Rev. Physiol.* **57**, 43–68.

SPRIET, L. L. (1990). Phosphofructokinase activity and acidosis during short-term tetanic contractions. *Can. J. Physiol. Pharmac.* **69**, 298–304.

SU, Y. AND STOREY, K. B. (1992). Phosphofructokinase from white muscle of the rainbow trout, *Oncorhynchus mykiss*: purification and properties. *Biochim. biophys. Acta* **1160**, 301–308.

TANG, Y. AND BOUTILIER, R. G. (1991). White muscle intracellular acid–base and lactate status following exhaustive exercise: a comparison between freshwater- and seawater-adapted rainbow trout. *J. exp. Biol.* **156**, 153–171.

TAYLOR, S. E., EGGINTON, S. AND TAYLOR, E. W. (1996). Seasonal temperature acclimatization of rainbow trout: cardiovascular and morphometric influences on maximal sustainable exercise level. *J. exp. Biol.* **199**, 835–845.

TELLAM, R. AND FRIEDEN, C. (1981). The purification and properties of frog skeletal muscle phosphofructokinase. *Comp. Biochem. Physiol.* **69**B, 517–522.

TRIVEDI, B. AND DANFORTH, W. H. (1966). Effect of pH on the kinetics of frog muscle phosphofructokinase. *J. biol. Chem.***241**, 4110–4114.

VEECH, R. L., LAWSON, J. W. R., CORNELL, N. W. AND KREBS, H. A. (1979). Cytosolic phosphorylation potential. *J. biol. Chem.* **254**, 6538–6547.

VOET, D. AND VOET, J. G. (1995). *Biochemistry*. 2nd edition. New Jersey: John Wiley and Sons.

WIESER, W., KOCH, F., DREXEL, E. AND PLATZER, U. (1986). 'Stress' reactions in teleosts: effects of temperature and activity on anaerobic energy production in roach (*Rutilus rutilus* L.). *Comp. Biochem. Physiol.* **83**A, 41–45.

WIESER, W., PLATZER, U. AND HINTERLEITNER, S. (1985). Anaerobic and aerobic energy production of young rainbow trout (*Salmo gairdneri*) during and after bursts of activity. *J. comp. Physiol.* B **155**, 485–492.

WOLLONBERGER, A., RISTAU, O. AND SCHOFFA, G. (1960). Eine einfache Technik der extrem schnellen Abkühlung größerer Gewebestücke. *Pflügers Arch.* **270**, 399–412.