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RESPONSES OF INTERMEDIARY METABOLISM TO ACUTE HANDLING STRESS AND RECOVERY IN UNTRAINED AND TRAINED *LEUCISCUS CEPHALUS* (CYPRINIDAE, TELEOSTEI)

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Summary

1. Juvenile *Leuciscus cephalus* L. were forced to swim against a current of 25 cm s^{-1} (3–5 body lengths s^{-1}) intermittently for more than 2 months. Their metabolic responses to acute handling stress and recovery were compared to those of untrained *L. cephalus*.

2. The concentrations of glycolytic intermediates, malate and phosphocreatine were determined in whole-body homogenates of different fish before and immediately after mechanical stimulation leading to exhaustion, as well as after 5, 15, 30, 60 and 120 min of recovery.

3. The time course of recovery was described by fitting a bi-exponential equation. In untrained fish glycolytic metabolites, except pyruvate, showed maximum concentrations immediately after termination of the stress period, whereas in trained fish these maxima were delayed.

4. In trained *L. cephalus* the concentrations of all metabolites investigated returned to pre-exercise levels much faster than in untrained fish. Most characteristically, lactate was removed about four times faster from the tissues of trained than from those of untrained *L. cephalus*.

5. It is argued that *anaerobic recovery*, a well-known characteristic of exercise physiology in man and other vertebrates, is the driving force of accelerated recovery of trained *L. cephalus*.

Introduction

In fish, endurance exercise training leads to a general increase in aerobic capacity but, in contrast to mammals and man, the training effect appears to be dominated by systemic rather than by molecular or cellular adaptations. Fish exercised for several weeks in a flume show an increase in maximum sustainable swimming speed (Brett *et al.* 1958; MacLeod, 1967), stamina (Hochachka, 1961; Hammond & Hickman, 1966) and growth rate (Davison & Goldspink, 1977; Nahhas *et al.* 1982). This correlates with fibre hypertrophy (Greer-Walker & Pull,

Key words: endurance training, stress, recovery, lactate shuttle, glycolysis, malate, citrate cycle, phosphagen stores, *Leuciscus cephalus*.

1973), with a small increase in the total mass of the red swimming muscle (Broughton *et al.* 1981) and with an increase in the activities of some glycolytic enzymes in the red fibres (Johnston, 1982; Johnston & Moon, 1980*a*,*b*). In contrast to exercise programmes involving mammals (Gollnick *et al.* 1972; Holloszy & Booth, 1976; Blomstrand *et al.* 1986), endurance training of fish does not appear to lead to an increase in mitochondrial density or in the activity of the enzymes of aerobic metabolism (Johnston, 1982), a fact that has been linked to the nearly isometric nature of muscle contraction in fish myotomes (Alexander, 1969). The well-known functional and morphological division of the swimming muscles into slow red fibres, used mainly for sustained swimming, and fast white fibres, used mainly for burst swimming (Johnston, 1980), may also be responsible for the more systemic-oriented reactions of fish, since all adaptive responses to exercise programmes involve shifts in the relative and absolute contribution of one of these fibre types to total muscle mass.

If it is true (a supposition still based on the study of very few species) that, in fish, endurance training does not affect the activities of aerobic enzymes and causes only a small increase in the activities of glycolytic enzymes in red muscle fibres (Johnston, 1982), the question arises whether, and to what extent, trained and untrained fish differ in their metabolic responses. A second question is whether the metabolic responses of trained fish differ from those of trained mammals in which the biochemical adaptations to endurance training are sometimes said to be more important than systemic (cardiovascular) adaptations (Holloszy & Coyle, 1984).

We have tried to provide answers to these questions by comparing the metabolic responses of trained and untrained juvenile fish. We hypothesized that the most sensitive assay for physiological or biochemical reorganizations due to training would be to impose a severe stress on the animals and to follow the changes in concentration of some of the key metabolites during the period of stress and during recovery. We chose juvenile fish because fast changes in energy metabolism can be interpreted only if it is possible to immobilize the object of study within a few seconds (J. Dalla Via, M. Huber, W. Wieser & R. Lackner, in preparation): this can be done, as in mammalian exercise physiology, by quick-freezing small biopsy samples, or by freeze-clamping the whole organism. The latter is possible with fish weighing up to a few grams.

Materials and methods

Animals: training and sampling

One-year-old chub, *Leuciscus cephalus*, were used in this investigation. Groups of fish were kept in a training flume for at least 2 months prior to experiments. The flume was constructed of PVC plates and was subdivided into four compartments so as to allow the simultaneous treatment of up to four groups of fish at different velocities. Two pumps (746 and 1119 W) were used to recirculate the water and to maintain the flow. A filter consisting of Perlon wool was installed in a by-pass of

the circulation system. Grids of stainless steel and PVC were used to rectify and to regulate the water current. 'Trained' fish were forced to swim against a current of 25 cm s^{-1} which was equivalent to approximately 3–5 body lengths s^{-1}. The control or 'untrained' fish were kept at 3 cm s^{-1}.

The temperature in all experiments was 20°C. Fish were fed four times a day with commercial carp food (Alma, FRG) by means of an automatic feeder, 4–5 % of the fish mass being the daily ration. During each feeding period of 20 min the flow in the flume was automatically reduced to the control level of 3 cm s^{-1}. A daily regime of 15 h light and 9 h dark was maintained throughout the experiments, but the flow regime was not altered during the night.

Trained and untrained fish grew equally well in the flume. They were introduced into the flume on 1 July 1987. On 12 July, trained fish weighed 1·905 ± 1·446 g ($N = 91$) and untrained fish 1·376 ± 0·854 g ($N = 90$). On 28 July, fresh body masses were 2·345 ± 1·64 ($N = 88$) and 1·701 ± 0·942 ($N = 90$), respectively.

The second phase of the investigation started in late August. Before experiments, individual fish were transferred to separate vessels and left undisturbed in constant light for 1 day. Acute stress was induced by chasing the fish mechanically until they gave the appearance of total exhaustion, the test being that they would not respond to touch or prodding. To reach this state took 5–7 min of chasing, no difference being apparent between trained and untrained fish. Fish were caught with forceps behind the opercula and freeze-clamped immediately. Other specimens were allowed to recover in individual, shielded vessels so that they would not be disturbed by movements in the temperature-controlled chamber in which these experiments were carried out. It should be emphasized that skill and experience are required to catch the fish during rest or recovery without introducing a measurable additional stress (see J. Dalla Via, M. Huber, W. Wieser & R. Lackner, in preparation). The sampling protocol consisted of the following stages: before stress (pre-exercise); at exhaustion; and at 5 min, 15 min, 30 min, 1 and 2 h of recovery.

Extraction and determination of metabolites

Freeze-clamped fish were stored in liquid nitrogen or at −70°C. Usually on the following day they were weighed and pulverized with frozen 10 % perchloric acid in a mortar mill (Retsch, FRG) under liquid nitrogen. The frozen powder was thawed in a refrigerator and centrifuged at 26000 *g* for 20 min. The supernatant was neutralized with 5 mol l^{-1} K_2CO_3 and recentrifuged. All metabolites were determined enzymatically following the standard procedures described by Bergmeyer (1984).

Results

General reactions to mechanical stimulation

Table 1 and Figs 1–3 illustrate the metabolic responses of juvenile *L. cephalus* to mechanical stimulation and the time course of recovery. The basic pattern of the

Table 1. *Metabolite profiles in untrained and trained* Leuciscus cephalus, *before stress, at exhaustion, and during recovery*

	Before Stress	Exhaustion	Recovery				
			After 5 min	After 15 min	After 30 min	After 1 h	After 2 h
Untrained							
Glucose	1·28 ± 0·23 (3)	1·82 ± 0·09 (5)	1·98 ± 0·40 (3)	1·95 ± 0·52 (3)	1·98 ± 0·29 (3)	2·55 ± 0·01 (2)	2·00 ± 0·28 (3)
G-1-P	0·04 ± 0·01 (3)	0·04 ± 0·01 (5)	0·06 ± 0·02 (3)	0·05 ± 0·03 (3)	0·04 ± 0·01 (3)	0·05 ± 0·01 (2)	0·04 ± 0·01 (3)
G-6-P	0·08 ± 0·01 (3)	0·41 ± 0·16 (5)	0·46 ± 0·10 (3)	0·38 ± 0·16 (3)	0·31 ± 0·11 (3)	0·33 ± 0·01 (2)	0·09 ± 0·01 (3)
F-6-P	0·03 ± 0·01 (2)	0·12 ± 0·04 (5)	0·14 ± 0·02 (3)	0·11 ± 0·03 (3)	0·11 ± 0·04 (3)	0·13 ± 0·03 (2)	0·04 ± 0·01 (3)
Gly-3-P	0·06 ± 0·01 (3)	0·31 ± 0·08 (5)	0·37 ± 0·04 (3)	0·24 ± 0·05 (3)	0·24 ± 0·05 (3)	0·25 ± 0·08 (2)	0·05 ± 0·02 (3)
PEP	0·03 ± 0·01 (3)	0·03 ± 0·01 (5)	0·04 ± 0·01 (3)	0·03 ± 0·02 (3)	0·02 ± 0·01 (3)	0·03 ± 0·01 (2)	0·03 ± 0·01 (3)
Pyruvate	0·02 ± 0·01 (3)	0·05 ± 0·01 (5)	0·05 ± 0·01 (3)	0·16 ± 0·06 (3)	0·10 ± 0·01 (3)	0·13 ± 0·01 (2)	0·03 ± 0·01 (3)
Lactate	0·37 ± 0·18 (3)	6·28 ± 1·4 (5)	5·83 ± 0·61 (3)	5·53 ± 2·2 (3)	4·32 ± 1·7 (3)	4·92 ± 0·40 (2)	0·52 ± 0·17 (3)
Phospho-creatine	10·41 ± 0·25 (3)	2·96 ± 1·9 (5)	6·67 ± 0·94 (3)	8·60 ± 2·1 (3)	10·31 ± 0·39 (3)	9·31 ± 0·14 (2)	10·45 ± 0·67 (3)
Malate	0·11 ± 0·01 (3)	0·25 ± 0·07 (5)	0·28 ± 0·02 (3)	0·32 ± 0·06 (3)	0·36 ± 0·04 (3)	0·39 ± 0·02 (2)	0·13 ± 0·02 (3)
Trained							
Glucose	2·01 ± 0·74 (5)	1·99 ± 0·68 (4)	1·61 ± 0·07 (3)	1·89 ± 0·28 (5)	2·36 ± 0·10 (2)	2·55 ± 0·24 (3)	2·22 ± 0·51 (3)
G-1-P	0·02 ± 0·01 (5)	0·06 ± 0·02 (4)	0·03 ± 0·02 (3)	0·04 ± 0·01 (5)	0·04 ± 0·01 (2)	0·04 ± 0·01 (3)	0·04 ± 0·01 (3)
G-6-P	0·11 ± 0·02 (5)	0·31 ± 0·08 (4)	0·29 ± 0·06 (3)	0·48 ± 0·24 (5)	0·27 ± 0·06 (2)	0·17 ± 0·02 (3)	0·10 ± 0·02 (3)
F-6-P	0·03 ± 0·01 (5)	0·12 ± 0·04 (4)	0·07 ± 0·03 (3)	0·14 ± 0·05 (5)	0·07 ± 0·03 (2)	0·05 ± 0·01 (3)	0·06 ± 0·02 (3)
Gly-3-P	0·08 ± 0·01 (4)	0·29 ± 0·05 (3)	0·31 ± 0·01 (3)	0·36 ± 0·05 (5)	0·22 ± 0·07 (2)	0·11 ± 0·02 (2)	0·04 ± 0·03 (3)
PEP	0·10 ± 0·02 (5)	0·06 ± 0·02 (4)	0·03 ± 0·02 (3)	0·07 ± 0·03 (5)	0·05 ± 0·01 (2)	0·06 ± 0·02 (3)	0·07 ± 0·01 (3)
Pyruvate	0·02 ± 0·01 (5)	0·04 ± 0·02 (4)	0·05 ± 0·01 (3)	0·09 ± 0·02 (5)	0·15 ± 0·01 (2)	0·07 ± 0·02 (3)	0·03 ± 0·01 (3)
Lactate	0·45 ± 0·17 (5)	3·83 ± 1·8 (4)	5·13 ± 0·99 (3)	6·93 ± 2·7 (5)	4·39 ± 1·7 (2)	1·67 ± 0·99 (3)	0·38 ± 0·32 (3)
Phospho-creatine	10·21 ± 1·6 (5)	5·55 ± 1·8 (4)	7·41 ± 1·8 (3)	10·07 ± 0·95 (5)	8·86 ± 0·73 (2)	10·18 ± 1·6 (3)	10·93 ± 0·99 (3)
Malate	0·15 ± 0·02 (5)	0·21 ± 0·04 (4)	0·24 ± 0·01 (3)	0·31 ± 0·05 (5)	0·29 ± 0·01 (2)	0·20 ± 0·08 (3)	0·12 ± 0·04 (3)

Metabolite concentrations are given in μmol g^{-1} fresh mass as mean ± S.D., number of experiments given in parentheses.
G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; Gly-3-P, glycerol-3-phosphate; PEP, phosphoenolpyruvate.

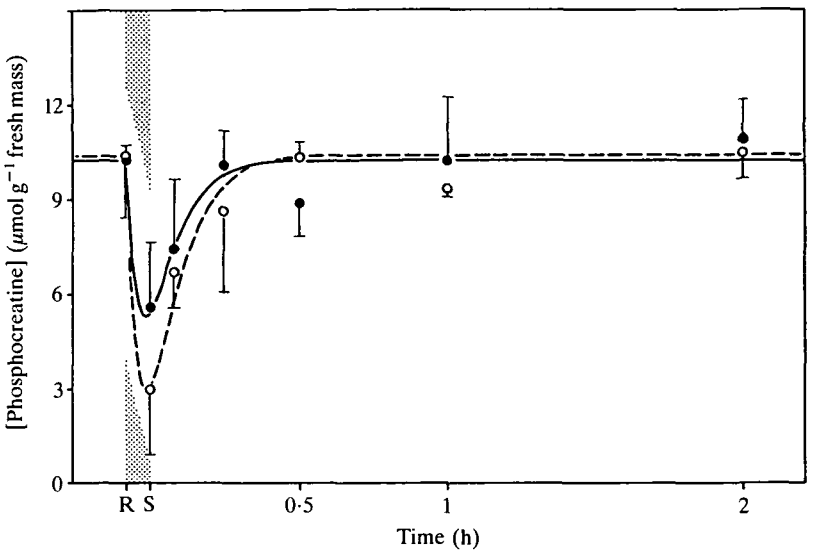

Fig. 1. Time course of changes in phosphocreatine concentration of trained (●) and untrained (○) *Leuciscus cephalus*. Standard deviations are marked as bars. R, before stress; S, after stress; the duration of stress being indicated by stippling. The curves have been obtained by fitting equation 1 to the data.

metabolic responses of untrained *L. cephalus* is identical to that of untrained *Rutilus rutilus* at the same temperature (J. Dalla Via, M. Huber, W. Wieser & R. Lackner, in preparation), except that in the former species total exhaustion results in the accumulation of only about half as much lactate as in the latter species.

The effects of training

The metabolic effects of training in *L. cephalus* are best illustrated by the fates of phosphocreatine (Fig. 1), lactate and pyruvate (Fig. 2), and malate (Fig. 3). At the end of the exhausting exercise the concentration of phosphocreatine (PCr) had dropped to 28 % of its pre-exercise level in the untrained fish, but to only 54 % in the trained fish. Because of high individual variability, this difference is not statistically significant, but since it follows the trend shown by the important metabolites of glycolysis (Fig. 4) we consider it to be an additional illustration of the effects of training on metabolic responses in this species. Complete recovery of the phosphagen took about 15 min in both trained and untrained fish.

In the untrained fish lactate reached its maximum concentration at termination of the exhausting exercise, but in the trained fish it continued to be produced during the first 15 min of recovery. The same difference between the two groups held for pyruvate, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and glycerol-3-phosphate (Gly-3-P), as is clearly shown by setting the maximum concentration of each metabolite as 100 % and expressing all changes of concentration in relative terms (Fig. 4). The concentration of malate increased

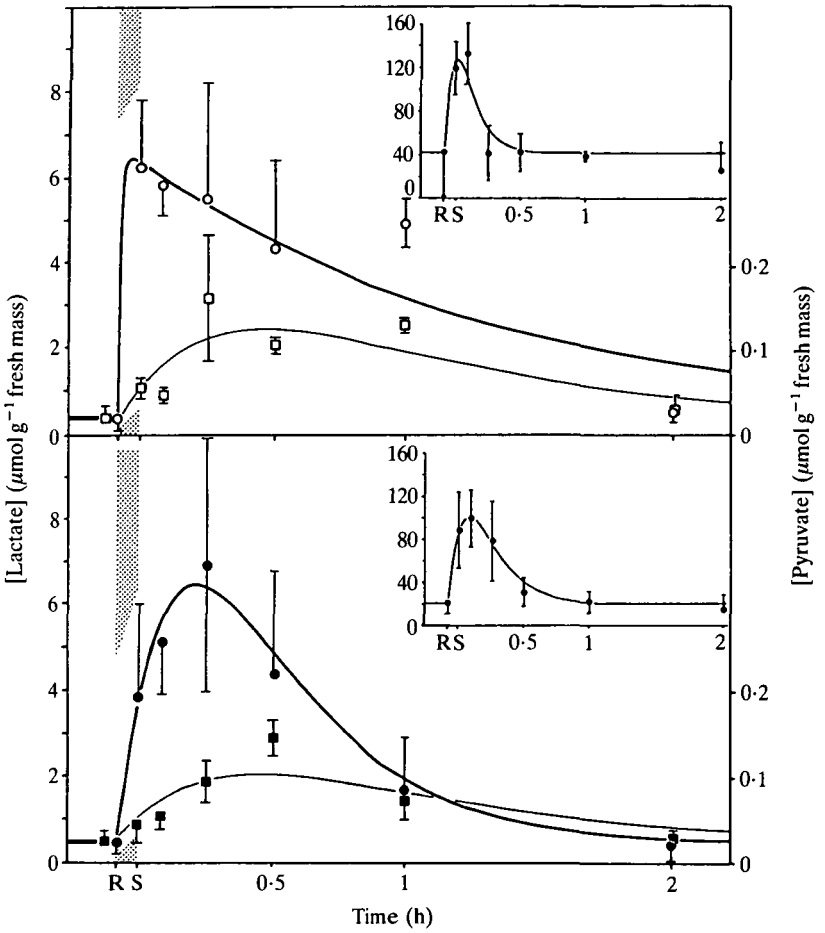

Fig. 2. Time course of changes in lactate (circles) and pyruvate (squares) concentrations in trained (filled symbols) and untrained (open symbols) fish. Inset: lactate/pyruvate ratio. For details see also Fig. 1.

during the exhausting exercise and continued to increase for about 60 min of recovery in the untrained fish, but for only 20–30 min in the trained fish.

Time-course of recovery

General features of recovery have been characterized by fitting bi-exponential time functions to the concentrations of the most important metabolites (Jenkins & Watts, 1968). The functions have the form:

$$y = y_0 + A\frac{1}{k1 - k2}(e^{-t/k1} - e^{-t/k2}) , \quad (1)$$

where y is the metabolite concentration at time t, y_0 is the concentration before

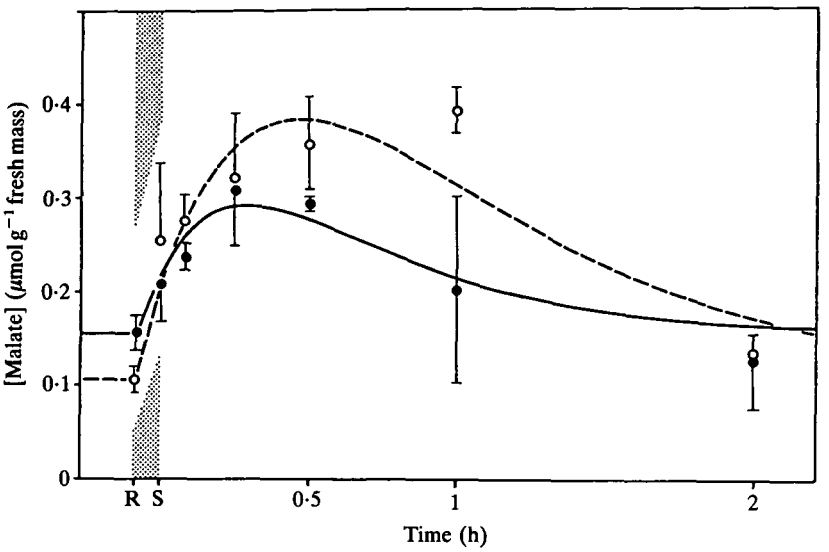

Fig. 3. Time course of changes in malate concentration in trained (●) and untrained (○) fish. The curves were obtained by fitting equation 1 to the data. The time constants are $k1 = k2 = 0{\cdot}55$ h for untrained fish, and $k1 = k2 = 0{\cdot}37$ h for trained fish.

stress, A is a scaling factor, and $k1$ and $k2$ are two time constants which describe the increase and decrease of metabolite concentrations with time (in hours).

The effect of training on the time course of recovery metabolism can be summarized as follows. (1) All glycolytic metabolites, except pyruvate, reach their concentration maximum at a later time in trained fish than in untrained fish, whereas the opposite is true for malate. (2) Metabolism returned faster to pre-exercise levels in trained than in untrained *L. cephalus*. The statistical significance of these two statements has been tested by comparing some characteristics of the fitted functions with the Wilcoxon–Mann–Whitney U-test (Table 2). The most striking difference between trained and untrained *L. cephalus* is shown by the much shorter time constant $k2$ in the former group (average of four metabolites $= 0{\cdot}3 \pm 0{\cdot}1$ compared with $1{\cdot}2 \pm 0{\cdot}3$ h; $\alpha < 0{\cdot}01$).

The driving force of accelerated recovery appears to be the continuation of lactate production during the first 15 min of recovery in the trained fish (Fig. 2). By maintaining a high rate of glycolysis into the beginning of the recovery period the trained fish appear to have been capable of reducing the time required for regaining the pre-exercise steady state of aerobic metabolism. This is clearly shown by the faster return of the citrate cycle to its normal mode of operation. In untrained fish, the concentration of malate, an indicator of citrate cycle activity, at the end of stimulation is 2·5-fold that at rest and continues to increase for another 60 min. In trained fish, however, malate reaches its peak of concentration 15–30 min after the end of stimulation. It is assumed that at this point the citrate cycle regains its normal mode of operation (Fig. 3).

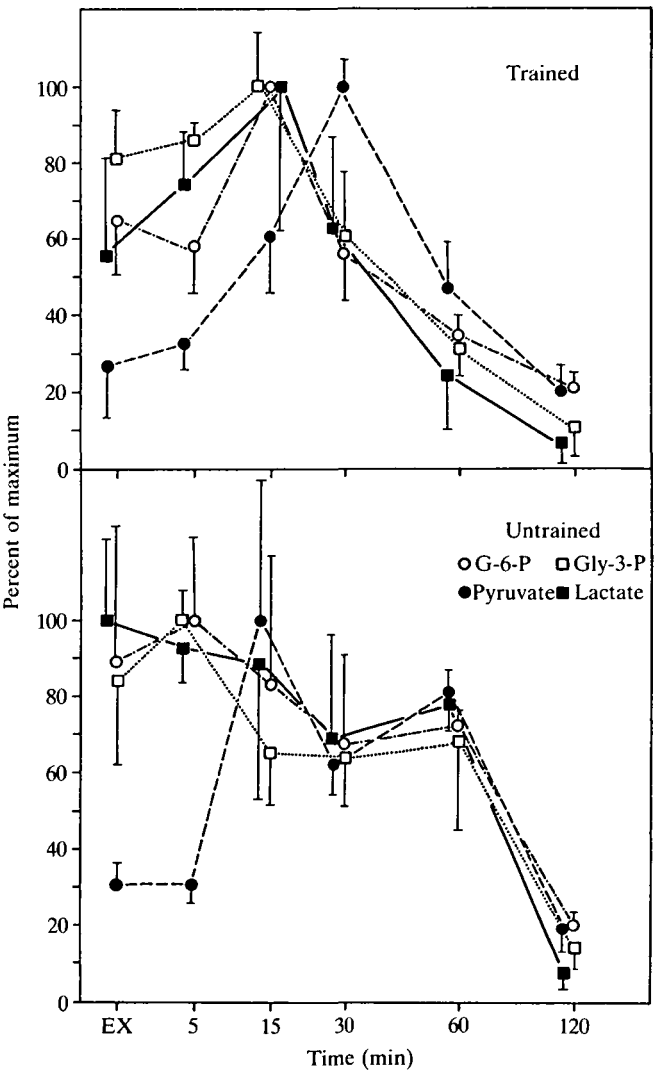

Fig. 4. Relative changes of concentration of four key metabolites of glycolysis in trained and untrained *Leuciscus cephalus* during recovery from exhaustion (EX). Relative concentrations were obtained by setting the maximum value at 100%. The time axis is not to scale. Means and standard deviations of 2–5 individual fish (see Table 1). G-6-P, glucose-6-phosphate; Gly-3-P, glycerol-3-phosphate.

Discussion

Our experiments allow us to define two characteristic features of the metabolic consequences of endurance exercise training in juvenile fish. First, the trained fish are *metabolically* less exhausted by mechanical stimulation than the untrained fish. Second, metabolism returns to its pre-exercise steady state faster in the trained than in the untrained group.

Table 2. *Time course of recovery of trained and untrained* Leuciscus cephalus *as described by the two time constants,* k1 *and* k2*, of equation 1, as well as by two calculated characteristics of the curve*

	*k*1	*k*2	t_{max}	$y_{1\,h}$
Untrained				
G-6-P	<0·05	1·10	0·08	0·36
F-6-P	0·05	1·52	0·10	0·35
Gly-3-P	<0·05	0·94	0·07	0·35
Pyruvate	0·41	0·72	0·60	0·49
Lactate	<0·05	1·32	0·09	0·42
Trained				
G-6-P	0·28	0·28	0·20	0·30
F-6-P	0·26	0·26	0·18	0·07
Gly-3-P	0·16	0·34	0·15	0·21
Pyruvate	0·53	0·54	0·45	0·51
Lactate	0·29	0·29	0·21	0·32
Probability (α)				
without pyruvate	≤0·05	≤0·05	≤0·05	≤0·05
with pyruvate	≤0·10	≤0·01	≤0·20	≤0·20

t_{max} is the time (in hours) when the maximum concentration of the metabolite was observed: $y_{1\,h}$ represents the relative concentration of the metabolite after 1 h of recovery.

The results were compared using the Wilcoxon–Mann–Whitney U-test and the probability α is given that the results for trained and untrained fish do not differ.

Owing to its specific metabolic position, pyruvate follows a time course which differs from that of the other metabolites. Thus, excluding pyruvate from the U-test leads to lower probabilities.

G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; Gly-3-P, glycerol-3-phosphate.

Although both groups gave the impression of being *behaviourally* exhausted at the end of stimulation, PCr had dropped to only 54 % of pre-exercise level, and lactate increased to only $3{\cdot}83 \pm 1{\cdot}83\,\mu mol\,g^{-1}$ in the trained group, compared with 28 % and $6{\cdot}28 \pm 1{\cdot}36\,\mu mol\,g^{-1}$, respectively, in the untrained group. The most likely explanation of this difference is that in both groups exhaustion was precipitated after about the same duration of mechanical stress by a failure of some central (neurohormonal?) mechanism, even though the metabolism of the trained fish was more aerobic than that of the untrained fish when this event took place.

A weakness of our approach is that the degree of mechanical stress could not be quantified except by noting the time when complete exhaustion occurred (this state being defined by the cessation of reactions of the fish to touch or prodding). No difference in this respect was apparent between the trained and the untrained group. However, in two previous publications it was shown that a similar discrepancy between metabolic and behavioural exhaustion can be induced by temperature, and that in this case the same result was achieved by unquantified mechanical stress (J. Dalla Via, M. Huber, W. Wieser & R. Lackner, in preparation) and by exercising the fish at a defined speed for different lengths of

time (Wieser *et al.* 1986). The second feature of metabolic training effects observed by us appears to be the result of a well-known characteristic of exercise physiology in man and other vertebrates, termed *anaerobic recovery* (Cerretelli & Ambrosoli, 1973; di Prampero *et al.* 1973; Cerretelli *et al.* 1975). Anaerobic recovery is based on the ability of muscles to produce lactate in the presence of oxygen, thus more quickly replenishing energy stores that have been depleted in the course of the foregoing exercise.

The excess lactate formed in the muscle can be used as a quick source of carbon and energy in certain oxidative tissues, such as the heart (Driedzic *et al.* 1985) or, more controversially, the red muscle fibres (Wittenberger & Diaciuc, 1965; Wieser *et al.* 1987). This ability of tissues to form lactate under fully aerobic conditions, thus providing a carbon source for other tissues, has been called the *lactate shuttle* (Brooks, 1986). An increase in the rate of this shuttle also implies accelerated removal of lactate from blood plasma. This is quite clearly shown to be the case by comparing the lactate kinetics of trained and untrained *L. cephalus* (Fig. 1; Table 2). As the values of $k2$ in Table 2 indicate, lactate is removed about four times faster from the tissues of trained fish than from those of untrained fish. A higher lactate clearance rate from plasma has also been reported for trained rainbow trout (Hammond & Hickman, 1966).

In *L. cephalus* the lower concentration of lactate and the higher concentration of PCr at the end of mechanical stimulation indicate that the long period of endurance training has led to an increase in the aerobic capacity of the swimming muscle, probably because of hypertrophy and hyperplasia of red muscle fibres (Broughton *et al.* 1981; Greer-Walker & Emerson, 1978) and not because of an increase in the activities of oxidative enzymes (Johnston, 1982; S. Hinterleitner, M. Huber & W. Wieser, unpublished observations). At the same time, training has enabled the fish to maintain a high rate of glycolysis during the first phase of recovery which seems to be the prerequisite for increasing the rate of the lactate shuttle. In this way the time required for the aerobic metabolism to return to its pre-exercise steady state was shortened considerably in trained compared with untrained *L. cephalus*. The conclusion appears justified that, despite the differences in underlying mechanisms, endurance training leads to similar types of metabolic reorganization in juvenile fish and in mammals. In both cases the aerobic capacity of the animals is enhanced, although in fish, as far as we know, this response is based entirely on the increase of the mass of slow oxidative fibres without concomitant change of enzyme activities, whereas in mammals a marked increase in mitochondrial density and in the activities of the enzymes of aerobic metabolism are observed.

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