Depletion of high energy phosphates implicates post-exercise mortality in carp and trout; an in vivo $^{31}$P-NMR study

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Abstract

As in vivo $^{31}$P-Nuclear Magnetic Resonance spectroscopy is currently the state of the art method to measure continuously intracellular pH (pHi) and energy status of muscle tissue, we used this method to study the recovery from exhaustive exercise. The biochemical changes during recovery are not well understood and it was suggested that post-exercise mortality could be caused by low pHi; other studies however indicate that energy depletion might be more important. To analyse the mechanism of post-exercise recovery pHi, ATP, Pi, and PCr must be measured at the same time, which is possible using in vivo $^{31}$P-NMR. Common carp and rainbow trout of about 100 g were exercised to exhaustion in a swim tunnel. After swimming 10 h at 1.5 body lengths (BL)/s (aerobic control), 50% of the fish were forced to swim at 6 BL/s until exhaustion. Recovery of energy rich phosphates was found to be faster in carp (1.2–1.9 h) than in trout (1.5–2.3 h). The same applied for the recovery from acidosis, which took 1.75 h in carp and 5.75 h in trout. In parallel experiments the energy phosphates and lactate levels were measured in liver, red muscle, and white muscle. Exhaustion caused a significant drop in the energy status of red and white muscle tissue of trout and carp (corroborates NMR data), while no change at all was observed in liver tissue. The lactate levels were increased in the muscle but not in liver and blood. While all experimental animals looked healthy after exhaustion, 40–50% of the carp as well as trout died during the recovery phase. The energy status of those individuals measured by $^{31}$P-NMR was much lower than that of the survivors, while in contrast there was no difference in pHi. Thus, it appears that not acidosis but depletion of high energy phosphates disabled muscle function and therefore may have been the cause of death of the non-survivors.

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Keywords: $^{31}$P-NMR; Exercise; White muscle; Liver; Trout; Carp; Anaerobic metabolism; Creatine kinase; Collapse; Burst activity; Energy metabolism; Energy status; Intracellular pH; Acidosis

1. Introduction

Exhaustive exercise in fish is primarily powered by white anaerobic musculature. Burst-type exercise in fish generally involves the use of three endogenous fuels stored within the white muscle: glycogen, ATP and phosphocreatine (PCr). Energy is in the early stages of activity mainly derived from the breakdown of ATP and PCr. A similar amount of anaerobic energy can be produced by glycolysis, resulting in the accumulation of lactate. The net conversion of glycogen to lactate causes tissue acidosis, which is however, modulated by the alkaline reaction of creatinephosphate (PCr) and the acidic reaction of ATP hydrolysis (van den Thillart and van Waarde, 1996). Peak levels of lactate and inorganic phosphate are an indication for anaerobically produced energy during burst exercise. Following a bout of exhaustive exercise the recovery of PCr is generally quite rapid, usually within 1 h post-exercise. Unlike the recovery of PCr however, the removal of muscle lactate via the monocarboxylate transporter, oxidation and resynthesis of glycogen is in fish much slower (Kieffer, 2000).

After exercise till exhaustion significant mortality of fish occurs which may be as high as 40% (Wood et al., 1983).
high mortality rate during post-exercise is not immediate but happens several hours after collapse suggesting that it is mainly due to an impaired capacity to recover. It certainly must be due to extreme physiological disturbances. Major metabolic changes under those conditions are: a) depletion of glycogen and high energy phosphates, b) accumulation of lactate and concomitant metabolic acidosis, c) ionic and osmotic imbalance, and d) a surge in plasma catecholamines and cortisol. Wood et al. (1983) suggested that of these factors the intracellular acidosis of the white muscle compartment might be the proximate cause of post-exercise mortality in fish. Although acidosis inhibits many cellular processes, several studies suggest that a change in membrane potential might be the major factor leading to cell death (Buck et al., 1993). This change is set in motion by a low ATP level, which impairs ion fluxes and particularly results in uncontrolled high Ca$^{2+}$-influx.

In previous in vivo $^{31}$P-NMR studies we observed that when energy stores were severely depleted during anaerobiosis, i.e. when [ATP] <30% of normoxic values, the experimental fish could not recover (van den Thillart et al., 1989b; van Ginneken et al., 1995). Based on that observation we hypothesized that the proximate cause of death of fish after extreme exercise may be depletion of energy stores. This is in contrast with the suggestion of Wood et al. (1983) who stated that the proximate cause is extreme intracellular acidosis.

A first step to solve this question is to measure the dynamics of both pHi and energy status in the recovering white muscle. A most useful technique for this is $^{31}$P-NMR, as it has the advantage that it is non-invasive and non-destructive. In addition, artifacts caused by tissue sampling and extraction are eliminated and semi continuous monitoring is possible.

In vivo $^{31}$P-NMR has been applied to study the effects of environmental hypoxia and anoxia on energy metabolism in fish (van den Thillart et al., 1989a,b, 1990; van den Thillart and van Waarde, 1993, 1996; van Waarde et al., 1990, 1991; van Ginneken et al., 1995, 1996, 1999).

In the present study we applied the same method to study the recovery from exhaustive exercise by rainbow trout, *Oncorhynchus mykiss* and common carp (*Cyprinus carpio*). With the use of swim tunnels, we exposed carp and trout to a controlled exhaustive swimming protocol. In order to make a clear distinction between the aerobic and anaerobic type of swimming, we let the fish swim overnight at low speed. The combination of swim tunnel exercise followed by in vivo $^{31}$P-NMR-measurements provided new in vivo data on post-exercise recovery of fish muscle.

2. Materials and methods

2.1. Animals and handling

The experiments were performed with rainbow trout, *Oncorhynchus mykiss* (de Keyzerberg — Blitterwijk, The Netherlands) and common carp, *Cyprinus carpio*, (Agricultural University Wageningen, The Netherlands). The animals were kept in the laboratory in local tap water for at least 1 month at 18 °C, fed daily with Trouvit pellets (Trouw, Putten, The Netherlands), and acclimated to a light–dark cycle of 14–10 h.

2.2. Swim trials

The day before the onset of the experiment, the fish were anaesthetized with 3-aminobenzoate ethyl ester methanesulphonate (MS-222, Sigma, St. Louis, MO, USA) at a final concentration of 100 ppm and placed in a large Blazka swim tunnel as described by van den Thillart et al. (2004). The relation between rotor speed and water flow was determined by Laser-Doppler technique at the Hydraulics Laboratory TU, Delft (The Netherlands), the set up was calibrated and almost linear between 0.2 and 1.0 m/s.

In order to reduce stress incurred by forced swimming, we used (overnight) 10 h swimming at 1.5 BL/s as a starting condition. This is a rather low speed which both species can endure for long periods. At the end of this standardized low exercise load, the control animals (CO) were anaesthetized and placed in the flow-through cell for the NMR-measurements or were sacrificed and sampled. A second group was forced to exhaustion after the 10-h period. This was reached by a stepwise increase of the speed to 6 BL/s, which was maintained until collapse. Starting at 1.5 BL/s the speed was increased every 30 min with 0.5 BL/s. After failure the fish were anaesthetized with 100 ppm MS-222, and placed in the NMR flow-through cell or killed for tissue sampling. In this protocol trout swam at 6 BL/s for 30–120 min before collapse, carp swam at 6 BL/s for 20–30 min.

2.3. $^{31}$P-NMR-measurements

After the swim trial the anaesthetized fish were placed in a Perspex flow-through cell, which fitted in a modified Bruker bioprobe as described before (van den Thillart et al., 1989a). The fish was immobilized with an inflatable plastic bag filled with water and pressed with the left body side against the flat window of the flow-through cell. At the same time a tube was placed in the mouth of the fish and the gills were irrigated with a constant water flow of 170 mL min$^{-1}$. The temperature of the experimental set up was controlled by a water bath at 18 °C, while the total experimental set up was placed in a thermostatted room. The animals awoke within 5 min after transfer and stayed quiet in the dark of the magnet, which could be deduced from NMR spectra. Placing the fish in the flow-through cell took approximately 3 min. Optimizing the NMR signal by shimming and tuning took less than 10 min by an automatic routine.

In vivo $^{31}$P-NMR spectra of the lateral musculature were obtained with a 9.4 T Bruker MSL-400 NMR spectrometer. The signal was picked up with an 18-mm surface coil, double-tuned to hydrogen (400 MHz) and phosphorus (162 MHz) frequencies. The coil was placed about 2 cm behind the operculum while the total experimental set up was placed in a thermostatted room. The animals awoke within 5 min after transfer and stayed quiet in the dark of the magnet, which could be deduced from NMR spectra. Placing the fish in the flow-through cell took approximately 3 min. Optimizing the NMR signal by shimming and tuning took less than 10 min by an automatic routine.

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18 mm from the center of the surface coil), an acquisition time of 0.4 s and a 4-s relaxation delay. Measurements of the longitudinal relaxation time ($T_1$)-values of the metabolites of interest demonstrated that the resonances of ATP, PCr, and Pi were almost fully relaxed under these conditions, $\exp(-t/T_1) < 0.1$. After baseline correction, the peak areas of the different high energy phosphate compounds were determined and corrected for saturation. Changes in the steady-state concentrations of metabolites as a result of exercise were expressed as the changes of their relative resonance intensities (RRIs). The pattern of the [ATP] during the course of the experiment is determined from the peak areas of the $\beta$-phosphate resonance of ATP. For each individual of the exhausted trout- and carp-group $^{31}$P-NMR spectra were obtained during the first 10 h of recovery. Similarly spectra were taken from the controls over 10 h to determine the background level.

2.4. Handling and sampling

After exercise, the fish were quickly anaesthetized with 100 ppm MS-222 (3-aminobenzoate ethyl ester methane sulphonate, Sigma-Aldrich, St. Louis, MO, USA). After 3 min the anaesthetized fish were taken out of the swim tunnel and blood was collected with a heparinized syringe (flushed with 3000 units heparin per mL blood) and directly centrifuged at 13,000 g for 5 min. The plasma was divided in microcentrifuge tubes (50 μL) for analysis of lactate. Lactate was measured with Boehringer Mannheim kits (UV-method 139084). Liver, white muscle, and red muscle tissues of each fish were removed and freeze clamped within 2, 3, and 4 min respectively after complete anaesthesia. The tissues were stored in liquid nitrogen until extraction. Tissue extraction was carried out as described before (van den Thillart et al., 1990). ATP, ADP, and PCr were measured according to Harmsen et al. (1982) with some modifications (van Ginneken et al., 1997).

2.5. NMR derived parameters

The intracellular pH (pHi) was estimated by observing the difference in chemical shift between PCr and Pi. The pH measurements were calibrated using several model solutions as described previously (van den Thillart, 1989b). The pHi was calculated using the formula:

$$
pHi = 6.72 + \log \frac{((\sigma - 3.27)/(5.69 - \sigma))}{[\text{ADP}]_{\text{free}}}
$$

Where $\sigma$ corresponds to the chemical shift between PCr and Pi. $[\text{ADP}]_{\text{free}}$ was calculated according to Lawson and Veech (1979):

$$
[\text{ADP}]_{\text{free}} = \frac{[\text{ATP}] \cdot [\text{Creatine}]}{[\text{PCr}] \cdot [\text{H}^+] \cdot K_{eq}}
$$

Values for [ATP] determined with HPLC in freeze clamped white muscle tissue of carp and trout were 3.61 ± 0.46 and 5.29 ± 0.37 μmol/g respectively. [PCr] and [Pi] were estimated from the ratio of the relative resonance intensities (RRIs) of PCr, Pi, and ATP. The free creatine concentration was obtained by subtracting [PCr] from colorimetrically determined total creatine concentration. Total creatine concentration in freeze clamped dorsal white muscle of carp and trout was respectively 29.9 ± 3.0 μmol/g wet mass (van Waarde et al., 1990) and 19.8 ± 2.3 μmol/g wet mass (Richards et al., 2002). The $[\text{H}^+]$ is derived from pHi ($pHi = -\log [\text{H}^+]$). The $[\text{ADP}]_{\text{free}}$ was converted from nanomoles per gram intracellular water to nanomoles per gram wet weight by correcting for intracellular water content of 56.6% for teleosts (Withers, 1992). The equilibrium constant ($K_{eq}$) of the CK reaction is 1.47 × 109 at 20 °C, and $[\text{Mg}^{2+}] = 1$ mM (van Waarde et al., 1990). The PCr/Pi ratio was used as an index of the steady-state capability of the oxidative phosphorylation. The ratio is a reflection of the ADP control system of the creatine kinase (CK) equilibrium reaction.

2.6. Statistics

Data are presented as means ± SD. Statistics were performed using one-way ANOVA. $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov–Smirnov and Levene tests respectively. In case of no homogeneity of the variances (Levene $P \leq 0.05$), nonparametric Kruskal Wallis tests were used instead, after which the data were reciprocally compared with Mann–Whitney statistics.

3. Results

3.1. Animals

Weight and length data of trout and carp used in the NMR-experiments are presented in Table 1. The swimmers were swum till exhaustion at 6 BL/s after overnight swimming at 1.5 BL/s. During recovery in the magnet however, about 50% of the swimmers died. Thus we split each swim group in an exhausted survivor (ES) and an exhausted non-survivor group (ENS). The fish, which were used in a parallel experiment to measure metabolites in white muscle, red muscle and liver, had the following size: carp ($n=12$) 176.2 ± 26.3 g, 19.3 ± 1.4 cm; rainbow trout ($n=12$) 122.9 ± 22.5 g, 21.7 ± 1.05 cm.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mass (g)</th>
<th>Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trout</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (CO)</td>
<td>100.2 ± 13.2</td>
<td>20.4 ± 1.52</td>
</tr>
<tr>
<td>Exhausted-survivors (ES)</td>
<td>114.2 ± 13.0</td>
<td>21.4 ± 1.14</td>
</tr>
<tr>
<td>Exhausted-non-survivors (ENS)</td>
<td>98.5 ± 24.3</td>
<td>19.8 ± 1.70</td>
</tr>
<tr>
<td><strong>Carp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (CO)</td>
<td>100.6 ± 7.9</td>
<td>16.4 ± 0.96</td>
</tr>
<tr>
<td>Exhausted-survivors (ES)</td>
<td>92.0 ± 9.0</td>
<td>16.0 ± 0.86</td>
</tr>
<tr>
<td>Exhausted-non-survivors (ENS)</td>
<td>94.9 ± 15.4</td>
<td>15.9 ± 0.89</td>
</tr>
</tbody>
</table>

After overnight swimming at 1.5 BL/s, some of the swimmers were transferred immediately to the NMR-magnet, and used as controls (CO), others were swum till exhaustion at 6 BL/s. During recovery about 50% of the swimmers did not recover, resulting in an exhausted survivor (ES) and an exhausted non-survivor group (ENS). Each group consisted of 5 animals.
3.2. $^{31}$P-NMR

Fig. 1 shows a series of $^{31}$P-NMR spectra (stacked plots) from a typical experiment during the first 2 1/2 h of recovery after exhaustive swimming. The patterns for both carp and trout are rather similar. The major peaks are from sugar phosphates (SP), inorganic phosphate (Pi), phosphocreatine (PCr), and ATP ($\alpha$, $\beta$, $\gamma$). From the start of the $^{31}$P-NMR measurements, which is about 10 min after the collapse, we see a decline of the Pi and a concomitant increase of the PCr peaks. From each experiment NMR parameters (peak position and area) were calculated every 10 min over 10 h. The means with the 95% confidence interval of $\beta$-ATP, PCr, pHi and the PCr/Pi ratio is presented for carp in Fig. 2 and for trout in Fig. 3.

Just after swim failure, the PCr peaks are low and those of Pi are high, they return slowly to resting levels during recovery. In the control carp and trout, the [PCr] remained at approximately 100% during the whole experimental period of 10 h (not depicted). In the exhausted carp the initial PCr value was 57.5% of the control value, while for the exhausted trout this value was 64.8%. In both species the [PCr] returned to 100% within 1.75 h (Fig. 1). The pH of control carp and trout remained between 7.2 and 7.3 for a period of 10 h (figures not depicted). The initial pH values of exhausted carp and trout however, were significantly decreased. The pH of carp started at 6.64 and recovered to 7.22 in 1.75 h (Fig. 2). The pH of the exhausted trout started at 7.66, but recovered rather slowly over a period of 5.75 h to 7.2 (Fig. 3).

3.3. Metabolites in white muscle, red muscle and liver

Lactate values in plasma, white muscle, red muscle and liver of both fish species are given in Table 3. In the plasma of the exhausted carp and trout, lactate levels were hardly elevated, as shown by values of $1.64\pm0.40$ mM and $1.48\pm0.09$ mM respectively. In trout plasma the lactate levels even decreased significantly. No changes were observed in the liver tissue; in contrast many small increases were found in the red and white muscle of exhausted carp and trout. In the white muscle lactate levels increased by more than 4 fold for respectively carp and trout. For red muscle the lactate increase was a bit smaller: in carp levels went up from $1.51\pm0.49$ mM to $5.67\pm1.83$ mM, and in trout from $4.40\pm1.22$ mM to $6.96\pm1.98$ mM (Table 3).

In Table 4, PCr and nucleotide levels measured by HPLC are given for 3 tissues (red muscle, white muscle, and liver) of carp and trout after swimming at low and exhaustive swim speeds. No changes were observed in the liver tissue; in contrast many...
changes occurred in the muscle tissue, particularly with respect to the energy phosphates. In the red muscle a marked decrease of PCr was observed in both species; in carp the PCr level fell from 9.79 to 3.66 μmol/g, and in trout from 5.81 to 2.96 μmol/g. A similar but more pronounced decline was observed in the white muscle of the exhausted fish; in carp the PCr level fell from 14.92 to 2.52 μmol/g, in trout from 21.1 to 1.5 μmol/g. The ATP level in red muscle of both species declined as well; in carp from 2.69 to 1.89 μmol/g, in trout from 1.87 to 1.02 μmol/g. A more pronounced decline was apparent in the white muscle; in carp ATP declined from 3.61 to 1.48 μmol/g, in trout from 5.29 to 1.36 μmol/g. The IMP levels of red and white muscle changed in the opposite way as the ATP levels. In the carp red muscle the IMP levels increased from 0.26 to 0.96 μmol/g, in trout from 0.79 to 2.62 μmol/g. In the carp white muscle IMP levels increased from 0.09 to 3.67 μmol/g, in trout from 0.19 to 4.32 μmol/g. In red and white muscle no changes occurred in the ADP level, while AMP levels stayed below the detection level of 3 nmol/g.

4. Discussion

4.1. Experimental set up

In order to distinguish between aerobic endurance swimming and exhaustive anaerobic swimming, we used the initial 10 h swimming at 1.5 BL/s as control condition. This condition is better than resting as at low fixed swim speeds all animals are at the same metabolic rate. The endurance swimming at low speed also allowed the animals to accustom to swimming in the swim tunnel, which reduced stress incurred by the transfer to a new environment. At speeds up to 60% Ucrit lactate and glycogen levels do not change in trout, indicating that metabolism...
remains aerobic (Richards et al., 2002). From the behavior of the animals as well as from the metabolite patterns it is obvious that 10 h swimming did not activate anaerobic processes. The lactate levels remained low in plasma and muscle tissue (Table 3) and the high energy phosphates were high (Table 4). The fish had apparently no problems to keep up with the water flow, nor were there signs of stress or irregular modes of swimming.

**In vivo** NMR spectroscopy has the advantage of a non-invasive measurement, which reduces artifacts due to sampling. The technique is particularly suited for measuring dynamics in energy metabolism, as the signals from the same tissue sample can be followed over time (van den Thillart and van Waarde, 1996). We were, however, not able to measure at the same time in all relevant tissues, nor could we obtain NMR data over the first 10 min of recovery after collapse. So, additional data were obtained from freeze clamped tissues in parallel studies. Comparison between both methods demonstrates that especially for PCr 40–50% of the recovery occurred during the first 10 min, a period required for placing the animal in the magnet and for optimizing the NMR signal. The freeze clamped samples showed a 90% depletion of PCr in white muscle of trout and 75% depletion in white muscle of carp (Table 4). The NMR data however, showed that after 10 min the depletion of the PCr stores in both species was about 40%, and 10% at 1 h after collapse (Figs. 2 and 3). This clearly indicates a rather fast bi-phasic recovery process of PCr in fish muscle.

### 4.2. Correspondence with literature data

PCr recovered almost completely in 30 min in white muscle of Pacific spiny dogfish (*Squalus acanthias*) (Richards et al., 2003), while for rainbow trout this was within 45 min (Wang et al., 1994). Our recovery data were different, i.e. 90% recovery in about 60 min, and 100% in 90 min. Differences are also apparent with respect to ATP recovery. In the Pacific spiny dogfish ATP remained depressed at ⅓ of the initial value even...
Table 4; high levels in white muscle and lower levels in red tissue used at slow swimming speeds, up to 70% of the species and exercise protocol. However the tendency remains the same, [PCr] showed a rapid recovery within 1 h, intracellular pH fluctuated between 7.2 and 6.7 and showed a recovery over a period of 6–8 h while the [ATP] showed not such a steep decline like [PCr] and recovered within a period of 15 min up to 2.5 h.

4.3. Anaerobic vs. aerobic capacity

Trunk musculature in fish species is spatially divided into separate regions of red-oxidative and white-glycolytic fibres that are differentially recruited to propel the fish at different swimming speeds (Richards et al., 2002). Red muscle is the tissue used at slow swimming speeds, up to 70–80% of the critical swimming speed. The differences in musculature function are reflected in the phosphorylated compounds shown in Table 4; high levels in white muscle and lower levels in red muscle. High energy content corresponds with high anaerobic power of white muscle. The loss in high energy phosphates was particularly high in trout white muscle; 23.5 μmol/g compared to 14.5 for carp. In the red muscle this was much less, i.e. 3.7 for trout and 6.9 μmol/g for carp. Rainbow trout had higher energy stores (PCr, ATP) in white muscle than carp i.e. 1.5 times higher. This is partly in correspondence with the time to fatigue. While carp and trout were of the same weight, and swam at the same speed (6 BL/s), rainbow trout could swim 2–4 times longer than carp. The longer endurance of trout at those high speeds may however also be due to a higher partitioning of aerobic red muscle tissue to swimming.

The increase of lactate in the muscle tissues of both species was moderate when compared with changes in PCr. Actually it is quite remarkable to find that white muscle lactate levels in trout were 4.3 μmol/g while the concentration in carp went up to 9.1 μmol/g. This correlates with the difference in pHi observed with 31P-NMR; after exhaustion the pHi in trout is 6.7, while in carp the muscle was more acidified; intracellular pH was 6.4.

Table 3

Lactate levels (μmol/g) measured in plasma, red muscle and white muscle of trout and carp after swimming overnight at 1.5 BL/s (control group) or after an additional trial at 6 BL/s till exhaustion (exhausted survivor group). The experiments were run in parallel with the NMR-experiments. Animals were sampled immediately after the swim protocol. Each group consists of 6 animals.

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Statistical significance of differences between groups: *P ≤ 0.05; **P ≤ 0.01; **P ≤ 0.001.

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After 4 h of recovery (Richards et al., 2003). In rainbow trout white muscle ATP levels fell with 15–20% and recovered within 15 min to initial values (Wang et al., 1994), while in our studies with rainbow trout recovery took 2–3 h.

The intracellular pH in trout of white muscle measured with the DMO technique had an initial value of 7.2, which corresponds well with our data (Fig. 3), and reached an intracellular pH value of 6.5, 4 h after exercise (Arthur et al., 1997). Our lowest pHi value at 30 min recovery was higher: pHi of 6.7, after 4 h the pHi was already 7.1 and recovery was complete within 6 h.

So there are large differences in the dynamics of recovery of white muscle between the different studies dependent on fish species and exercise protocol. However the tendency remains the same, [PCr] showed a rapid recovery within 1 h, intracellular pH fluctuated between 7.2 and 6.7 and showed a recovery over a period of 6–8 h while the [ATP] showed not such a steep decline like [PCr] and recovered within a period of 15 min up to 2.5 h.
TAN 0.90±0.19 0.81±0.16 1.05±0.32 1.17±0.19
AMP 0.73±0.04 0.67±0.06 0.68±0.09 0.72±0.10
ADP 0.66±0.12 0.56±0.11 0.63±0.21 0.77±0.15
ATP 0.23±0.17 0.25±0.08 0.42±0.18 0.40±0.20

b

⁎

IL 0.31±0.15 1.58±0.78
TAN 2.69±0.44 1.80±0.46 3.46±0.48 2.69±0.76
⁎

AMP

ADP 0.82±0.11 0.77±0.07 0.77±0.14 0.80±0.32

⁎

PCr 5.81±1.57 2.96±0.64
IL 0.03±0.009 2.33±1.14
⁎

IMP 0.19±0.05 4.32±1.06
b

⁎

ADP 0.97±0.04 0.74±0.09 0.92±0.20

⁎

Table 4

Nucleotides (means±S.D.) in white muscle, red muscle and liver of trout and carp sampled after overnight swimming at 1.5 BL/s (Control) and after additional exhaustive swimming at 6 BL/s (exhausted)

<table>
<thead>
<tr>
<th></th>
<th>Trout (N=6) control</th>
<th>Trout (N=6) exhausted</th>
<th>Carp (N=6) control</th>
<th>Carp (N=6) exhausted</th>
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<tr>
<td><strong>White muscle</strong></td>
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<tr>
<td>PCr</td>
<td>21.09±1.30</td>
<td>1.52±0.87*</td>
<td>14.92±1.70</td>
<td>2.52±0.89*</td>
</tr>
<tr>
<td>ATP</td>
<td>5.29±0.37</td>
<td>1.36±0.59*</td>
<td>3.61±0.46</td>
<td>1.48±0.49*</td>
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<tr>
<td>ADP</td>
<td>0.97±0.04</td>
<td>0.74±0.09*</td>
<td>0.82±0.06</td>
<td>0.92±0.20*</td>
</tr>
<tr>
<td>AMP</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
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<tr>
<td>IMP</td>
<td>0.19±0.05</td>
<td>4.32±1.06*</td>
<td>0.09±0.03</td>
<td>3.67±0.77*</td>
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<tr>
<td>TAN</td>
<td>6.26±0.39</td>
<td>2.10±0.63*</td>
<td>4.43±0.51</td>
<td>2.40±0.66*</td>
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<tr>
<td>IL</td>
<td>0.03±0.009</td>
<td>2.33±1.14*</td>
<td>0.02±0.008</td>
<td>1.69±0.63*</td>
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<tr>
<td><strong>Red muscle</strong></td>
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</tr>
<tr>
<td>PCr</td>
<td>5.81±1.57</td>
<td>2.96±0.64*</td>
<td>9.79±2.11</td>
<td>3.66±1.33*</td>
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<td>1.87±0.40</td>
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<td>ADP</td>
<td>0.82±0.11</td>
<td>0.77±0.07</td>
<td>0.77±0.14</td>
<td>0.80±0.32</td>
</tr>
<tr>
<td>AMP</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>IMP</td>
<td>0.79±0.31</td>
<td>2.62±0.88*</td>
<td>0.26±0.14</td>
<td>0.96±0.32*</td>
</tr>
<tr>
<td>TAN</td>
<td>2.69±0.44</td>
<td>1.80±0.46</td>
<td>3.46±0.48</td>
<td>2.69±0.76</td>
</tr>
<tr>
<td>IL</td>
<td>0.31±0.15</td>
<td>1.58±0.78*</td>
<td>0.07±0.04</td>
<td>0.39±0.16*</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCr</td>
<td>&lt;0.007</td>
<td>&lt;0.007</td>
<td>&lt;0.007</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>ATP</td>
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<td>0.25±0.08</td>
<td>0.42±0.18</td>
<td>0.40±0.20</td>
</tr>
<tr>
<td>ADP</td>
<td>0.66±0.12</td>
<td>0.56±0.11</td>
<td>0.63±0.21</td>
<td>0.77±0.15</td>
</tr>
<tr>
<td>AMP</td>
<td>0.73±0.04</td>
<td>0.67±0.06</td>
<td>0.68±0.09</td>
<td>0.72±0.10</td>
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<tr>
<td>IMP</td>
<td>0.08±0.03</td>
<td>0.07±0.02</td>
<td>0.19±0.10</td>
<td>0.15±0.07</td>
</tr>
<tr>
<td>TAN</td>
<td>0.90±0.19</td>
<td>0.81±0.16</td>
<td>1.03±0.32</td>
<td>1.17±0.19</td>
</tr>
<tr>
<td>IL</td>
<td>0.08±0.03</td>
<td>0.07±0.02</td>
<td>0.22±0.16</td>
<td>0.12±0.05</td>
</tr>
</tbody>
</table>

All parameters are expressed in μmol/g except for the IMP-load (IL) which is dimensionless. TAN=[(ATP+ADP+AMP)], IL=[(IMP)/(ATP+ADP+AMP)]. * denotes significant difference at P≤0.05.

Anaerobic glycolysis was in both species clearly not used to its maximum, as values as high as 40 μmol/g have been found in trout muscle after exhaustion (Kieffer, 2000). Possibly the applied protocol — e.g. swimming at 6 BL/s till collapse — did not cause maximal glycolytic flux, while in contrast at the same time the high energy stores were almost depleted.

It is remarkable that burst swimming using the swim tunnel method as applied in this study and that of Schulte et al. (1992) results in a 5–6 times longer endurance than with the prodding method applied by Wood et al. (1983). The protocol of Schulte et al. was based on the maximal speed of the fish which varied during the burst swim period. Our protocol was set at a fixed 6 BL/s. Still in both protocols the fish were swimming at a rather constant high frequency, which obviously differs from the intermittent bursts from the prodding method. The observed difference in endurance may be explained by two factors i.e. handling stress and oxygen extraction. The stress during the exercise in the swim tunnel will certainly be much less than that for the fish prodded in a open circular tank, as the animals in the tunnel were not handled and already habituated to their environment. Furthermore swimming in a straight line at constant speed allows the animals to apply ram ventilation and optimise oxygen extraction, which may not occur when they have to dash away each time from the prodding stick. It is likely that even at high swim speeds red muscle tissue can operate aerobically as this muscle is highly vascularized. Under all conditions lactate is a prime oxidative substrate (van den Thillart, 1986), hence active red muscle tissue will keep the plasma lactate levels low, thus reducing metabolic acidosis in the white muscle and postponing the moment of collapse. Indeed we did observe in this study rather low lactate levels in the plasma and the red muscle of the exhausted animals. This indicates that a significant part of the energy for locomotion was still generated by aerobic red muscle.

This suggests that the mode of swimming and particularly its intensity determines the activation of the different biochemical processes. The activity of the glycolysis is determined by ATP/AMP, Pi, pH, and particularly in this case by the availability of glucose-6P, which depends on the activity of glycogen phosphorylase. The latter is predominantly controlled by circulating catecholamines. Thus it is quite possible that a large difference can be found in the anaerobic metabolism between stressed and unstressed animals. The latter will likely produce lactate at a later stage than the former. Thus we suggest that the substrate flow for lactate production is mainly controlled by hormones, while the depletion (and recharging) of phosphorylated compounds is controlled by intracellular biochemical mechanisms. This interpretation would explain the large differences between burst swim experiments with respect to depletion of PCr+ATP and accumulation of lactate.

4.4. Free [ADP]

The [ADP]free in white muscle of rested fish calculated from measured data in this study (Fig. 4) is consistent with resting data from other 31P NMR studies where the same approach (CK equilibrium reaction) was used: 18–20 μM in carp, tilapia and goldfish at 20 °C (van Waarde et al., 1990), 20 and 32 μM in goldfish muscle at 5 and 20 °C, respectively (van Waarde et al., 1991), and 80, 72, 76 μM in goldfish, tilapia and carp respectively at 20 °C (van Ginneken et al., 1995). In contrast, the ADP levels measured by HPLC in extracted tissues are always much higher, as also observed in this study. This can be explained by minor breakdown of ATP during sampling and furthermore for a large part by extraction from the F-actin bound ADP (Walliman et al., 1992). So the measured ADP-concentrations from the tissue extracts have a limited value for thermodynamic interpretations in contrast to the [ADP]free values calculated from the CK equilibrium reaction.

In this study we observed that during recovery from exhaustion [ADP]free in trout falls within 0.5 h from 27 μM to the control value of 10 μM. The [ADP]free level in carp was already very low (5 μM), it dropped over 1 h to <1 μM, coming back to the control level during the second hour. The [ADP]free is a very important trigger for mitochondrial oxidation, the very low levels of [ADP]free in carp as well as in trout muscle indicate that the tissue is well oxygenated during recovery, which shifts the adenyl/kinase equilibration towards ATP generation. The driving force for this is actually the phosphorylation potential (PP):

\[
PP = [ATP]/([ADP]_{free} \cdot [P_i] \cdot [H^+]^\frac{1}{2})
\]

As all 3 parameters in the denominator have a dynamic range of 10 fold, the driving force of ATP hydrolysis (ΔG) may vary...
widely (van den Thillart and van Waarde, 1993). During recovery the parameters $[P_i]$ and $[H^+]$ are for a long time 5–10 fold higher than in resting conditions, therefore very low $[ADP]_{free}$ is the only way to drive ATP synthesis (from a thermodynamic point of view). This process occurs more efficiently in carp than in trout (Fig. 4), as in carp the $[ADP]_{free}$ falls during recovery to a 10 fold lower level than the control. A possible explanation might be that the perfusion of the muscle in carp is better than that in trout during recovery. It is well known that in many fish species lactate remains for long periods in the tissues, likely due to restricted perfusion (Wardle, 1978). So, the faster recovery in carp muscle could be due to a better perfusion, a higher oxygen availability and corresponding higher mitochondrial activity.

4.5. Mortality

In the literature hardly any attention is given to mortality occurring during the recovery from exhaustive exercise. In the FAO report “Mortality of fish escaping trawl gears” (Suuronen, 2005), the author aims to assess and summarize the principal factors affecting the stress, injury and mortality of fish that arise from fishing processes, particularly when fish escape from trout gears. In the case of mortality due to swimming exhaustion (Suuronen, 2005) concludes that more work is needed to assess the importance of stress and swimming exhaustion on the survival of escaping fish. The paper of Wood et al. (1983) observed a mortality rate of about 50%, with most animals dying between 4 and 8 h after collapse. This observation suggests that mortality is not due to the collapse per se, but to a defective capacity to recover. Also our protocol, where we swum the animals till exhaustion at 6 BL/s for about 30 min, resulted in about 50% mortality in both species. The usage of a swim tunnel may be a less stressing event for the fish, still this did not reduce mortality.

It is remarkable that trout collapsed from prodding had very low initial cortisol levels (Milligan et al., 2000), while other stress hormones adrenalin and noradrenalin were rather high. Thus far no hormone measurements were published differentiating survivors and non-survivors from exhaustive exercise. The effect of hypoxia exposure on stress hormones cortisol and catecholamines was measured by Van Raaij et al. (1996) in surviving and non-surviving trout during exposure to hypoxia. In that study it was shown that even before lactate levels started to rise the stress hormone levels of the non-survivors were already much higher than those of the survivors. As catecholamines are known to speed up metabolism by stimulating circulation and muscle tension, it is likely that the non-survivors had a much higher energy consumption rate than the survivors. In addition as explained above catecholamines stimulate anaerobic glycolysis, which may result in severe acidosis.

The blood data from the non-survivors in the prodding experiment of Wood et al. (1983) showed that during recovery the HCO$_3^-$ pool was not replenished, and the arterial pH hardly recovered from the initial fall (7.85 to 7.35). The authors concluded that a low intracellular pH of the muscle must have been the proximal cause of death, because the blood pH was
only partially recovered at the time of death and no other parameters were significantly different from the survivors. They stated that the preferential retention of metabolic protons results in a prolonged depression of muscle pH, ultimately leading to the death of the animal. Since then many authors studied recovery from exhaustive exercise, unfortunately without paying attention to the non-survivors. Still direct measurement of intracellular pH in surviving and non-surviving animals is required to test this hypothesis.

Causes for mortality can be many, low phosphorylation potential, low pH, low membrane potential, and low energy status, even suffocation due to impaired oxygen transport could be possible. As the energy consumption level determines the time to exhaustion, it is likely that the extent of depletion of energy stores as well as the concomitant metabolic changes must be related to mortality. Low phosphorylation potential (PP) may be the first candidate, as it is the driving force behind all physiological processes. Low pH has also an effect on the energy status as it is a contributing factor in the ATP-hydrolysis reaction. However, the PP is mainly determined by three parameters — P<sub>i</sub>, [ADP]<sub>free</sub>, and H<sup>+</sup> — that all have a similar potential in shifting the equilibrium position, each having a dynamic range up to 10 fold (van den Thillart and van Waarde, 1993).

Apart from the PP other factors may play an additional role. In a study with trout, it was demonstrated that the activity of white muscle phosphofructokinase (PFK) is very sensitive to low pH (Ferguson and Storey, 1992). Purified preparations of the enzyme show essentially zero activity at pH 6.5. In our study we show that the pH<sub>i</sub>, in both species falls to pH=6.6–6.7. This would imply that post-exercise animals have an arrested glycolytic flux and cannot rely any further on anaerobic glycolysis for energy generation due to internal acidosis. Because the energy content of the muscle was almost depleted, it is clear that inactivation of PFK at low pH may have contributed to an early collapse.

Apart from its effect on the PP, low pH<sub>i</sub> has also a strong inhibiting effect on the muscle contraction force, the latter however, only at high P<sub>i</sub> levels. Actually it is the H<sub>2</sub>P<sub>O</sub><sub>4</sub> species of P<sub>i</sub> that is known to bind with high affinity to myosin ATPase (Potma et al., 1994; van den Thillart and van Waarde, 1996). Thus depletion of ATP and PCR stores results in high tissue P<sub>i</sub> levels. Hydrolysis of ATP has an acidification effect, which is however by far overruled by the alkaline reaction of PCR hydrolysis (van den Thillart and van Waarde, 1993), in addition as can be seen in our experiments, the depletion of ATP is quantitatively negligible compared to the depletion of PCR. Thus we expect an alkaline reaction; acidification occurs therefore only when anaerobic glycolysis is activated and enough acid is produced to compensate for the alkaline effect of the PCR hydrolysis. Thus only in combination with lactate production, high [H<sub>2</sub>P<sub>O</sub><sub>4</sub>] levels are reached which can inhibit muscle contraction.

The results from our 31P-NMR study demonstrate that in the survivors as well as the non-survivors of both carp and trout the intracellular pH was not significantly different and remained close to pH=6.7 (Table 2). Comparing survivors with non-survivors, there is no change in pH<sub>i</sub> immediately after collapse from burst swimming. This clearly shows that the pH<sub>i</sub> cannot be the proximal cause of death, and therefore rejects the hypothesis of Wood et al. (1983). In contrast, the most remarkable changes are the 10 fold higher P<sub>i</sub> and the 10 fold lower PCR levels in the non-survivors. Thus it seems more likely that the combination of excessive depletion of energy stores and the high concentration of H<sub>2</sub>P<sub>O</sub><sub>4</sub> (which impairs muscle contraction) impaired the recovery of the non-survivors.

One may wonder why animals go beyond their point of no return; this certainly has no survival value for the animal. In this respect it is interesting to note that trout are able to recover much faster when they are allowed to swim at low speeds after collapse (Milligan et al., 2000). While during resting cortisol levels rise to very high levels, during swimming cortisol does not accumulate at all, and even blood lactate levels remain below 5 mM. Furthermore, blood pH and muscle glycogen recover in 2 h, which otherwise lasts for 8–12 h. Swimming has at least the advantage that it activates blood perfusion of most tissues, and thus allows rapid oxidation of the accumulated lactate that quickly replenishes the HCO<sub>3</sub><sup>−</sup> pool. The resynthesis of lactate to glycogen will also help to restore metabolic acidosis. So, possibly slow swimming may be the natural way of recovery from severe exercise, therefore we assume that mortality after exhaustive exercise will not happen when the animals are allowed to move freely.

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References


