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Blood plasma substrates and muscle lactic-acid response after exhaustive exercise in common carp and trout: indications for a limited lactate-shuttle

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Abstract—In a Blazka swim tunnel swim trials with individual carp (*Cyprinus carpio*, $N = 6$, approximately 176 g) and rainbow trout (*Oncorhynchus mykiss*, $N = 6$ approximately 123 g) were performed until exhaustion at six body lengths per second (BL/s). Control carp ($N = 6$) and control trout ($N = 6$) were sampled after a moderate swim exercise at 1.5 BL/s. Significant differences were demonstrated in the exhausted carp group in comparison with the control group for plasma sodium (108.8%), lactic acid in red muscle (RM) (375.5%), lactic acid in white muscle (WM) (484.5%), triglycerids (133.9%), total protein (126.7%) and phospholipids (116.8%). In trout only, potassium was significantly elevated in the exhausted group (129.2%). T3- and T4-plasma values, as well as the T3/T4 ratio, were unaffected by the exercise protocol in both fish species. Despite the high lactic acid values in muscle tissue (RM: range 5-7 mM, WM: range 4-9 mM) in the exhausted groups of both fish species, the lactate in blood plasma in both fish species was not elevated (range 1.5-1.6 mM). This indicates that lactate is not released from the muscle compartment towards the blood and led to the concept of a 'non-release' lactic acid mechanism in cyprinid and a salmonid fish species after strenuous exercise.

Keywords: blood substrates; carp; exhaustive exercise; lactate shuttle; 'non-release' lactic acid; swim-tunnel; T3; T4; trout.

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INTRODUCTION

Lactic acid, a product of 'anaerobic glycolysis', is mainly produced in the white muscle. Because approximately 65% of the body mass of Teleost fish consist of white muscle (Dalla Via et al., 1997), this muscle tissue plays an important role in the production of lactic acid, its release and elimination. In general, under conditions of extreme activity, when the energy demand exceeds the capacity of aerobic energy production, anaerobic metabolism is activated in order to produce additional ATP. The anaerobic glycolysis is of the low efficiency, high flux type. Due to a high free energy loss, the reaction has a fast reaction rate and is nearly independent of substrate and product levels. The ATP yield is only 2 mol ATP per mol glucose with a concomitant production of 2 mol lactate and 2 mol hydrogen ions (Stryer, 1988).

Little information is available about the release and timescale for excretion of lactic acid from the active tissues into the circulation. In addition, lactic acid may not solely be considered as an end product causing fatigue and acid-base disturbances, but also may be considered as a substrate for oxidative tissues such as red muscle and cardiac muscle. Also, in principle, using lactate as a substrate, hepatic gluconeogenesis (i.e., the Cori cycle) and in situ glyconeogenesis in white and red muscle may also occur. The first objective of this study was, therefore, to study the metabolic changes associated with exhaustive exercise in fish, with emphasis on the compartmentalisation of lactate. The second objective was to discuss, based on literature data, the possible routes of recycling of lactate in fish species. Finally we were interested in the effect of exhaustive exercise on hormones (T3 and T4), substrates (inter alia glucose, free fatty acids (= FFA), total plasma protein), and ions (sodium, potassium and chloride) in order to study the effect of our exercise protocol on metabolism and homeostasis of the animals.

In this study, two different fish species, rainbow trout and common carp, which have different habitats, were used to study response to exhaustive exercise and its effect on blood substrates and muscle lactic acid. Rainbow trout (*Oncorhynchus mykiss*) are more frequently found in areas of high altitude, while common carp (*Cyprinus carpio*), are found at lower altitudes. Reasons for this include the stronger currents and colder water in the mountain rivers that form the trout's habitat. Rainbow trout are the most frequently studied fish species in terms of exercise physiology (Milligan, 1996). They have an impressive swimming and migratory capacity and can be considered, with other salmonid fish species, as a high performance fish (Webb, 1994). In contrast to rainbow trout, the common carp is a more sluggish fish, more accustomed to slow moving, muddy rivers in relatively flat land (Banarescu et al., 1971).

MATERIAL AND METHODS

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 fish, which were used in the experiment to determine blood substrates in blood plasma and lactate in white and red muscle and liver tissue, were the following sizes. Carp ($N = 12$) had a mean body mass of 176.2 ± 26.3 g and a mean body length of 19.3 ± 1.4 cm, and rainbow trout ($N = 12$) had a mean body mass of 122.9 ± 22.5 g and a mean body length of 21.7 ± 1.05 cm. The animals were kept in a laboratory in local tap water for at least 1 month at 20°C, fed daily with Trouvit pellets (Trouw, Putten, The Netherlands), and acclimated to a light/dark cycle of 14/10 h. The day before the onset of the experiment, the fish were anaesthetised with 3-aminobenzoate ethyl ester methane sulphonate (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 Ginneken et al. (2002).

Swim trials

Individual fish were forced to swim overnight at 1.5 BL/s for a period of 10 h. The rotor speed to water flow was calibrated by Laser-Doppler techniques at the Hydraulics Laboratory TU, Delft, The Netherlands. At the end of the 10 h moderate exercise, the control group animals were anaesthetised, blood was collected and muscle samples were taken from white and red muscle or liver. To compose an exhausted group, the fish were forced to swim faster after the 10 h period. Over a period of 1 h, the rotor speed was slowly increased to a speed corresponding to 6 BL/sec. The fish were made to swim at this rate until exhaustion, indicated by failure to continue swimming. The fish were anaesthetised in 100 PPM MS-222, which took approximately 3 min, and killed for tissue and blood sampling. Trout swam at 6 BL/s for 30-120 min before collapse, while most carp ceased swimming at 20-30 min.

Handling and sampling of the fish for blood and metabolite measurements

After exposure to the exercise protocol, the fish were quickly anaesthetised with 100 PPM MS-222 (3-aminobenzoate ethyl ester methane sulphonate, Sigma). After 3 min the anaesthetised fish were taken out of the swim tunnel and blood was collected with a heparinised syringe (flushed with 3000 units heparin/ml blood) and directly centrifuged at 10 000 rpm for 5 min. The plasma was divided in eppendorf tubes (50, 20, 10, 20, 50, 33, 33, 33, 25 and 25 μl) for analysis of lactic acid, cholesterol, total protein, phospholipids, glucose, sodium, potassium, chloride, total thyroxin (TT4) and trijodothyronine (TT3) respectively and stored at -80°C pending analysis. For the glucose measurements, 50 μl plasma was

Table 1.
Sampling procedure of blood plasma and tissues of individual trout and carp.

Time (min)	Sampling procedure trout and common carp
T = −3	Anaesthetising of the fish in the swim tunnel
T = 0	Via special lid take fish out of swim tunnel
T = 1	Sampling blood out of caudal vein with syringe
T = 2	Killing fish by cutting spinal cord of fish and dissection of liver followed by directly freeze clamping
T = 3	Dissection of white muscle followed by directly freeze clamping
T = 4	Dissection of red muscle followed by directly freeze clamping

mixed with 200 μl 6% trichloric acid solution to precipitate plasma proteins and stored at −80°C. Glucose was determined by colorimetric assay (Sigma). Lactic acid, cholesterol, total protein and phospholipids were measured with Boehringer Mannheim kits (UV-method 139084, MPR1 CHOD-PAP 1442341, MPR3 124281 and MPR2 691844, respectively). Plasma sodium, potassium and chloride levels were measured by flame photometry and colorimetric procedures (Technicon). Total thyroxin (TT4) and tri-jodothyronine (TT3) were determined with commercial Amerlite kits (Amersham International PLC, Buckinghamshire, UK) modified for fish plasma. Liver, white muscle, and red muscle tissues of each fish were removed and freeze clamped within 2, 3 and 4 min, respectively, after complete anaesthetisation. A time schedule of the sampling procedure is given in table 1. After sampling, the tissues were stored at −180°C until extraction. Tissue extraction for lactic acid measurements was performed as described in earlier papers (Van Ginneken et al., 1997, 1998).

In summary, in plasma of both fish species lactic acid, cholesterol, total protein, phospholipids, glucose, sodium, potassium, chloride, total thyroxin (TT4) and tri-jodothyronine (TT3) were measured, while lactic acid was also measured in tissue extracts of liver, white- and red muscle.

Statistics and calculations

Data are presented as means ± SD. Statistics were performed using a one-way ANOVA. $P \leqslant 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogrov-Smirnov and $F_{\max}$ tests, respectively.

RESULTS

Differences between species for blood parameters

Comparing the control groups of carp and trout for blood parameters, the following characteristics can be considered. In comparison to carp, trout had higher concentrations of potassium (120.6%), sodium (109.2%), and chloride (124.0%). Lactic

Table 2.
Parameters measured in blood plasma of trout and carp groups (control and exhausted). Each group consists of six animals. $P \leqslant 0.05$ denotes a significant difference between exhausted and control groups. Only lactate values in white-, red muscle and liver were measured in perchloric acid extracts.

	Carp control	Carp exhausted	*P*-value	Trout control	Trout exhausted	*P*-value
Potassium (meq/l)	2.67 (0.55)	3.35 (0.31)	0.0508	3.22 (0.48)	4.16 (0.50)	0.0126*
Sodium (meq/l)	158.32 (5.62)	172.32 (9.63)	0.0286*	172.92 (16.29)	177.06 (12.59)	0.6342
Chloride (meq/l)	118.67 (5.46)	127.96 (13.07)	0.1578	147.14 (12.37)	144.26 (8.85)	0.6526
Glucose (mmol/l)	2.920 (1.758)	3.666 (0.847)	0.3789	2.599 (0.954)	2.852 (1.012)	0.6637
Triglycerids (mM)	1.683 (0.326)	2.253 (0.362)	0.0170*	2.146 (0.535)	2.241 (0.714)	0.7999
Total Protein (g/l)	33.57 (6.43)	42.54 (6.97)	0.0432*	40.66 (1.63)	34.95 (8.88)	0.1786
Cholesterol (mmol/l)	3.59 (0.37)	3.38 (0.68)	0.5308	5.72 (0.59)	5.03 (1.88)	0.8640
Phospholipids (mmol/l)	5.19 (0.24)	6.06 (0.79)	0.0430*	7.66 (0.96)	6.13 (1.85)	0.1144
FFA (mM)	0.50 (0.12)	0.19 (0.06)	0.0018*	0.44 (0.11)	0.38 0.05)	0.2901
Total-T3 (nmol/l)	0.645 (0.348)	0.948 (0.428)	0.213	2.111 (0.672)	1.688 (0.687)	0.369
Total-T4 (nmol/l)	2.386 (2.161)	2.915 (2.760)	0.756	22.42 (28.05)	20.69 (27.66)	0.843
T3/T4 ratio	0.482 (0.434)	0.182 (0.168)	0.219	0.361 (0.338)	0.529 (0.592)	0.560

acid in RM was much higher in trout than in carp (4.40 vs 1.51 mM) indicating that this type of muscle is used at 1.5 BL/s in trout. Lactic acid values for plasma, white muscle and liver for both species were of the same order (between 1-4 mM), while this was also the case for glucose. Triglycerids, total protein, cholesterol and phospholipids were significantly higher in trout in comparison with carp (respectively, 127.5%, 121.1%, 159.3% and 147.6%). Trout also had a higher total-T3 (327.3% of the value for carp) and a higher total-T4 (939.6% of the value for carp) (table 2). The T3/T4 ratio was not significantly different between carp and trout for rest ($P \leqslant 0.606$) and exhausted groups ($P \leqslant 0.393$).

Differences between control and exhausted groups for blood parameters

After exhaustion most blood parameters were increased for carp while free fatty acids (FFA) in plasma were significantly decreased in this species. Significant increases were demonstrated for sodium (108.8%), lactic acid in red muscle

(375.5%), lactic acid in white muscle (484.5%), triglycerids (133.9%), total protein (126.7%) and phospholipids (116.8%). A decrease was observed with FFA (0.50 to 0.19 mM). The other measured parameters, potassium, chloride, glucose, total-T3 and total-T4, increased insignificantly in carp, while lactic acid in the liver and cholesterol dropped (not significantly). For the exercised trout group only potassium and lactic acid in red and in white muscle increased significantly in comparison to the control group (129.2%, 158.2% and 432.0%, respectively), while lactic acid in blood plasma dropped significantly (113.7%). The other measured parameters, sodium, glucose and triglycerids increased insignificantly in trout, while chloride, lactic acid in the liver, total protein, cholesterol, phospholipids, total-T3 and total-T4 dropped insignificantly in trout (table 2).

Lactic acid responses, tissue vs plasma

Lactic acid values in the different tissue compartments (red muscle (RM), white muscle (WM), liver) and blood plasma of control and exhausted carp and trout groups are depicted in figure 1. Briefly summarised, lactic acid in WM of control carp and trout group were both low, 1.87 and 1.00, respectively. But these values were higher than in complete resting groups. In cannulated resting common carp, plasma values of 0.30 ± 0.15 mmol l^{-1} were found, while in cannulated resting trout, these values were 0.48 ± 0.11 mmol l^{-1} (Vianen et al., 2001). In our control group, plasma lactic acid values were higher because our group was swimming overnight at 1.5 BL/s.

In the exhausted carp vs trout group, lactic acid increased in WM, respectively, 9-fold and 4-fold. In RM tissue, lactic acid was low in the control carp group (1.51 mM) but significantly higher in RM of trout (4.40 mM). In addition, in the exhausted carp vs trout group, lactic acid increased in WM, respectively, 9-fold and 4-fold. Lactic acid is low (around 2 mM) in liver tissue of carp and trout groups (control and exhausted). Also in blood plasma, lactic acid is low, around 1.5 mM of control and exhausted groups of both fish species.

DISCUSSION

From studies with other fish species it was concluded that the 50% fatigue time during a sustained swimming effort was 4 BL/s (Brett, 1967) or 3 to 4 BL/s (Blaxter, 1969). In our study we observed that trout were exhausted at 6 BL/s speeds after 30-120 min, while most carp ceased swimming after less than 30 min at this swimming speed. Hence a major difference between both fish species is the time interval before reaching fatigue and exhaustion. Other differences between the two fish species in response to exhaustive exercise were observed for substrates, blood ions and lactic acid in muscle tissue.

In carp a significant rise of triglycerids, total protein and phospholipids was observed in the blood plasma of the exhausted group while this was not the case for

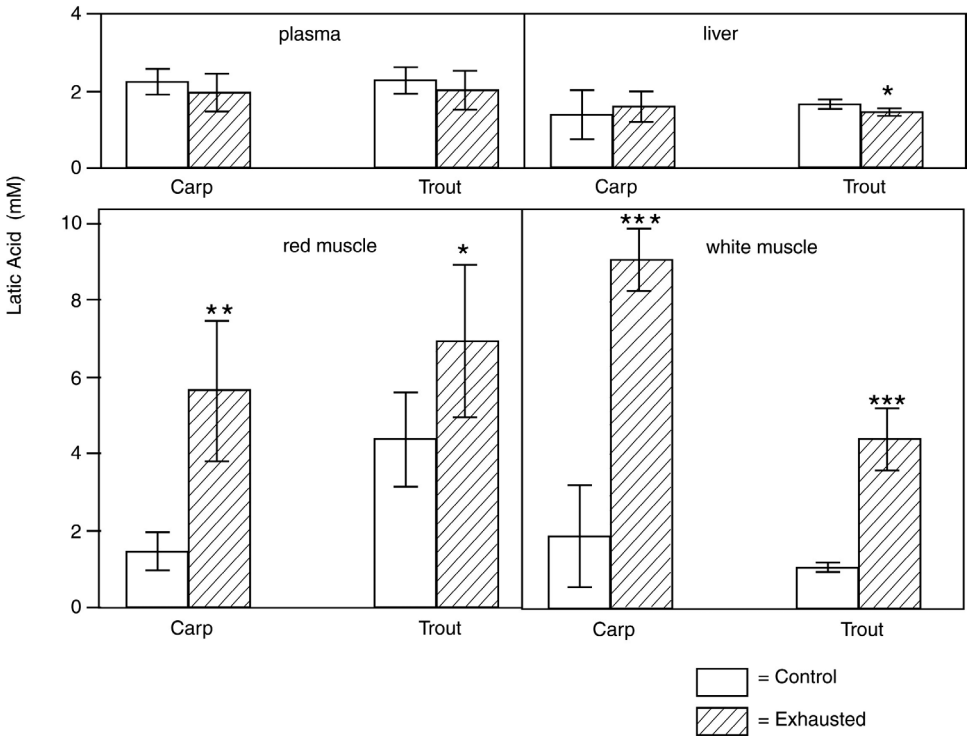

Figure 1. Lactate concentrations (mM) in white-, red muscle, liver tissue and blood plasma of common carp (control ($N = 6$), vs exhausted ($N = 6$)), and rainbow trout (control ($N = 6$), vs exhausted ($N = 6$)). Individual fish of the control groups were forced to swim overnight at 1.5 body length per second (BL/s). For the exhausted groups the fish were made to swim at 6 BL/s until collapse.

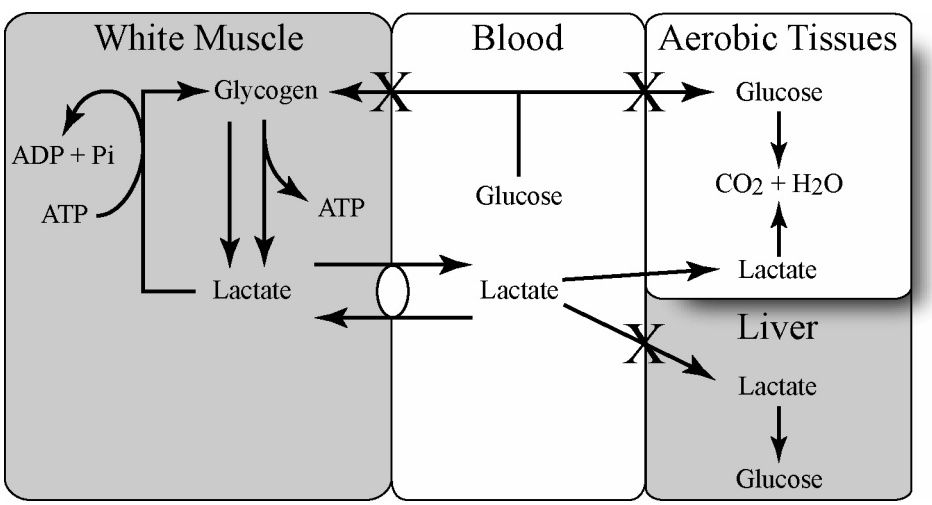

Figure 2. Schematic diagram describing the quantitative recycling of lactate by muscle following exercise in fish (modified from: Milligan, 1996). See text for further details.

exhausted trout (table 2). This may be indicative of an increased substrate mobilisation. However, in the carp group, sodium, chloride and potassium concentrations were also elevated, which may point to problems in osmoregulation. A water efflux from the blood compartment is more probable than increased substrate utilisation. In trout this is not the case.

Another explanation for the rise of potassium in the carp and trout group may be the result of an efflux of potassium from the muscle tissue by the exercise protocol. Nielsen and Boesgaard (1994) also observed this result in rainbow trout (*Oncorhynchus mykiss*). In a moderately exercised trout group (1.5 BL/s), potassium was significantly increased from controls after 2 h of exercise until the end of the experiment after 24 h (Nielsen and Boesgaard, 1994). Two explanations can be given for this observation: (1) The leakage of potassium from the muscle compartment to the blood plasma may be the result of the leakiness of membranes and a diminished expression of energy utilising pumps. A failure or lower activity level of the sodium/potassium pumps may be the result of a shortage in ATP production due to exercise. (2) Thomas et al. (1987) observed that the level of the increased potassium concentration is dependent on the intensity level of exercise in rainbow trout. Thus the increased potassium levels in blood plasma of exercise-exposed fish may be a direct result of the activity level of the muscle. The latter explanation is supported by the observation that muscular contraction can induce an efflux of potassium ions from the myoplasma into the extracellular fluid (Sjogaard, 1990).

For the thyroid hormones, T4 is the main hormone secreted by the thyroid gland of the fish while e.g., in trout, up to 70% of the plasma T4 may be deiodinated rapidly by 5′-monodeiodinase (5′D) extra-thyroidally (e.g., in liver) to T3. The latter is the more biologically active form of thyroid hormone (Kiessling et al., 1994). Therefore the T3/T4 ratio may give information about 5′monodeiodinase-activity. The thyroid hormones (T3 and T4) were not affected by exercise in our study. This is in correspondence with the study of Nielsen and Boesgaard (1994) where T3 and T4 also were not affected in exercising rainbow trout (1.5 BL/s). Also in the study of Pagnotta et al. (1994), no consistent effect of exercise was observed on circulating T4 levels of rainbow trout. The only exception to these reports is the study of Himick and Eales (1990) who reported that circulating T4 levels increase in rainbow trout in response to exercise. They also suggest that a T4 increase is associated with an increase in plasma glucose levels (Himick and Eales, 1990). Although glucose levels in carp plasma were slightly elevated, they were not significantly increased in both fish species. This may be indicative that catecholamine levels were probably not elevated, suggesting that the exercised fish were not stressed.

Differences in T3 and T4 levels between the different studies mentioned earlier can probably be ascribed to the swimming speed and the time period of exposure to an exercise protocol. This is illustrated in a study where three groups of Chinook salmon (*Oncorhynchus tshawaytscha*) were subjected to different swimming speeds of 0.5, 1.0 and 1.5 BL/s in seawater for a period of 212 days. The swimming speed

did not alter the plasma T4 concentration but increased the plasma T3 concentration and the T3/T4 ratio (Kiessling et al., 1994). The authors conclude that alterations in T3 titres were caused by changes in 5′monodeiodinase activity and/or in plasma T3 clearance (Kiessling et al., 1994). Thus, in conclusion, our study showed that exhaustive exercise had no effect on the T4 and T3 or the T3/T4 ratio for carp or for rainbow trout.

Plasma protein concentration was significantly elevated in the exhausted carp group ($P \leqslant 0.0432$). Recent respirometric analysis of fuel use during aerobic swimming in rainbow trout indicated that, in contrast to earlier beliefs, the relative contribution of proteins as an aerobic fuel is low (<30%) and that the major fuel exploited by fish during exercise is lipids (Kieffer et al., 1998). In this context, it is generally accepted that, at a sustained cruising speed, fish use lipid metabolism to drive red muscle (Bilinski and Jonas, 1972; Blaxter, 1969). The control FFA values for carp correspond to a former study where a value of 0.59 mM was reported (Van Ginneken et al., 1998). In the control trout plasma, FFA values were lower than in carp. In correspondence to this observation, Larsson and Fänge (1977) suggested that the FFA level is dependent on the actual site of lipid storage which is species-dependent. High FFA levels are found in fish species which store most of their lipids in the liver or mesenteric fat (e.g., *gadids* and *cyprinid*). In these species transport of FFA via the blood is necessary to supply the tissues utilising FFA for oxidation. In contrast, more active species, such as salmonids and mackerel, store significant amounts of lipids in muscle tissue where they are catabolised near the site of origin without being released to the circulation (Van Raaij, 1994). Our results corroborate this view. Also, higher plasma FFA were found in the common carp, a cyprinid species, than in the trout, a salmonid species. The drop of the FFA in the exhausted carp group probably indicates that they provide energy for burst swimming and are not fully replenished. Further, exhaustive exercise is a stressful situation leading to high levels of catecholamines, especially noradrenaline. It was suggested earlier that the ratio of noradrenaline to adrenaline is the major factor to determine the effect of catecholamines on plasma FFA. Elevated levels of adrenaline result in a rise of FFA through β-mediated stimulation of lipolysis, whereas plasma FFA may decrease at elevated noradrenaline levels due to a predominant stimulation of $\alpha 2$ adrenoreceptors, resulting in a reduction of lipolysis (Van Raaij et al., 1995). A recent in vivo study with cannulated carp indicated that not only $\alpha 2$ but also β-adrenoreceptors are responsible for a reduction of FFA levels via noradrenaline (Van den Thillart et al., 2001).

The rise of lactate in WM and RM in the exhausted group demonstrates that the metabolism became anaerobic and swimming was strenuous. Interestingly the lactate in blood plasma in both fish species is not elevated in the exhausted groups. This is indicative that lactate is not fully released from the muscle compartment towards the blood. This mechanism for retaining lactate, produced during exercise in fish, corroborates with earlier data produced by other authors (Milligan, 1996; Laberee and Milligan, 1999). A recent *in vitro* study with sarcolemmal vesicles

from rainbow, Sharpe & Milligan (2003) demonstrated the enormous potential of the muscle energy store for retaining lactate with minimal lactate loss.

Two processes can, to some extent, affect blood lactate levels. Firstly, lactate can enter the sacrolemmal vesicles partly via a low-affinity carrier that is pyruvate sensitive (Wang et al., 1997) and possibly also via other routes such as passive diffusion (Laberee and Milligan, 1999). Because passive processes or fascilated processes via a carrier mainly determine re-uptake of lactate from the blood, it can be expected that, quantitatively, no large amounts of lactate will be taken up by the muscle tissue. Secondly, the small amounts released into the bloodstream are probably oxidised by other tissues such as liver, heart, red muscle, gill and red blood cells (Milligan, 1996). All these observations corroborate the presented data of this study with low lactate plasma levels in two exhausted fish species.

The non-release or slow release of lactate from the white or red muscle is mainly thought to be advantageous to fish for the following reason. The fish body is composed of as much as 65% white muscle (Dalla Via et al., 1997), and extracellular fluid is poorly buffered and of low volume compared with muscle intracellular fluid (Wang et al., 1997). Leakage of lactate to body fluids and other tissue compartments would have major consequences for the homeostasis of the animal.

The general view for regulation of the plasma-lactate in fish is, in principle, different from the mammalian situation. Brooks (1998) proposed the concept of the 'lactate shuttle' for mammals. In this concept lactate was considered as a metabolic intermediate, exchanging rapidly between tissue compartments. In principle, lactic acid can be removed by oxidation in the white muscle itself, and in oxidative tissues such as red- or cardiac muscle, by hepatic or muscle glycogen resynthesis (Cori cycle). It is thought that in fish there are some major differences to this concept (see fig. 1). First, from in vivo radiotracer studies in fish, it is apparent that the Cori-cycle activity in fish is low and blood-borne glucose is not readily taken up by skeletal muscle and makes little contribution to muscle glycogen resynthesis (review: Milligan, 1996). The second possibility of removal of lactate, oxidation in aerobic tissues such as red- and cardiac muscle, has the disadvantage that lactate must be released to the blood space, transported to the oxidative tissues and finally catabolised. This exchange between tissue compartments is disadvantageous for the animal, mainly because, as stated earlier, the extracellular fluid is poorly buffered and low in volume compared with muscle intracellular fluid (Wang et al., 1997).

Therefore, the major route of diminishing lactate levels is in situ glycogenesis and oxidation (fig. 1). Support for this theory comes from the following observations. First, the bulk of lactate and H^+ after exercise is still found in the white muscle. More than 80% of the lactic acid generated by the exercised muscle is retained in the muscle mass (Dalla Via et al., 1997; Milligan and Wood, 1986) Second, in an in vivo study with swimming cannulated trout injected with 1-^{14}C lactate, it was demonstrated that 99% of the lactic acid was oxidised to CO_2 (Van den Thillart, 1986).

In conclusion, despite the high lactic acid values in muscle tissue of fish exhausted in a swim tunnel (RM: range 5-7 mM, WM: range 4-9 mM), the lactate in blood plasma in both fish species is not elevated (range 1.5-1.6 mM). This indicates that lactate is not released from the muscle compartment towards the blood and led to the concept of a 'non-release' lactic acid mechanism. By retaining the lactate within the muscle and preventing 'leakage' of lactate to the blood, homeostasis of fish is maintained after strenuous exercise.

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