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Effects of exercise on nitrogen excretion, carbamoyl phosphate synthetase III activity and related urea cycle enzymes in muscle and liver tissues of juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The purpose of this study was to determine if carbamoyl phosphate synthetase III (CPSase III) and related urea cycle enzyme activities in skeletal muscle tissue of juvenile rainbow trout (*Oncorhynchus mykiss*) increase during short- or long-term exercise, in parallel with changes in whole-body urea excretion rates. Urea excretion was elevated by 65% in fish that swam at high-speed (50 cm/s) vs. low-speed (20 cm/s) over a 2-h period, with no significant changes in CPSase III, ornithine transcarbamoylase or glutamine synthetase activities in muscle tissue. Fish that swam for 4 days at high-speed had higher rates of ammonia excretion and GSase activity in muscle and liver tissue relative to low-speed swimmers. Calculations showed that 47–53% of excreted urea, theoretically could be accounted for by total muscle CPSase III activity in juvenile and adult trout. The data indicate that increases in the rate of urea excretion during short-term high intensity exercise are not linked to higher activities of urea cycle enzymes in muscle tissue, but this does not rule out the possibility of increased flux through muscle CPSase III and related enzymes. Furthermore, these results indicate that urea cycle enzyme activities in skeletal muscle tissue can account for a significant portion of total urea excretion in juvenile and adult trout.

Keywords: Ammonia excretion; Glutamine synthetase; Nitrogen metabolism; Ornithine transcarbamylase; Swimming; Urea excretion

1. Introduction

Although the majority of teleost fishes excrete nitrogen wastes primarily as ammonia, a small but significant component is excreted as urea (5–40%; for review see Campbell and Anderson, 1991; Wood, 1993). Urea production in teleosts is thought to be a by-product of dietary arginine catabolism (argininolysis) and/or purine degradation (uricolysis) (for reviews see Forster and Goldstein, 1969; Mommsen and Walsh, 1989; Anderson, 1995a; Korsgaard et al., 1995; Walsh, 1998). The hepatic ornithine–urea cycle is responsible for de novo urea synthesis. The entire set of urea cycle enzymes are present in liver tissue of elasmobranch fishes (Anderson, 1980,

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1991), but their presence in liver tissue of teleosts has been documented in only a few species, including *Opsanus beta* (Read, 1971; Mommsen and Walsh, 1989; Walsh, 1997), *Heteropneustes fossilis* (Saha and Ratha 1987, 1989) and *Oreochromis alcalicus grahami* (Randall et al., 1989). The tilapia *O. alcalicus grahami* from Lake Magadi, Kenya is adapted to water of pH 10.5 and excretes all nitrogen wastes as urea. Recently, it was reported that all five urea cycle enzymes are present in skeletal muscle at levels of activity that surpass those present in the liver (Lindley et al., 1999). Hence, Lindley et al. (1999) proposed that the muscle was the major site for urea formation in this alkaline-adapted ureotelic teleost.

The first enzyme in the urea cycle in fish, carbamoyl phosphate synthetase III (CPSase III), utilizes glutamine as the nitrogen-donating substrate for carbamoyl phosphate synthesis (Anderson, 1995a,b). Hepatic CPSase III is absent in most adult teleost species studied (Anderson, 1980; Wright, 1993; Wright et al., 1995; Korte et al., 1997; Felskie et al., 1998). However, low but possibly significant levels of CPSase III activity and CPSase III mRNA, along with the activity of the second urea cycle enzyme, ornithine transcarbamoylase (OTCase) have been recently reported to be present in skeletal muscle tissue of rainbow trout (Korte et al. 1997), largemouth bass (*Micropterus salmoides*; Kong et al., 1998) and gulf toadfish (Julsrud et al., 1998; Kong et al., 2000). The enzyme activities have also been reported to be present in common carp (*Cyprinus carpio*) and bowfin (*Amia calva*) (Felskie et al., 1998).

In ammoniotelic teleosts, the presence of muscle CPSase III and OTCase activities may be important in ammonia detoxification. In white muscle during exercise, ammonia is released from the deamination of adenylates; AMP is converted to NH_3 and IMP via the enzyme AMP deaminase (Lowenstein and Tornheim, 1971; Dobson et al., 1987; Mommsen and Hochachka, 1988; Wright et al., 1988; Wang et al., 1994). Urea excretion rates are significantly elevated during high-speed (34–50 cm/s) compared to low-speed (20 cm/s) aerobic swims in rainbow trout (Lauff and Wood, 1996; Alsop and Wood, 1997). Although these 'aerobic' swims are thought to be powered mostly by red oxidative muscle fibers, more white muscle fibers may be recruited at higher swimming speeds (Wilson and Egginton, 1994) and consequently, adenylate metabolism would result in ammonia synthesis in muscle tissue. Excess ammonia could combine with glutamate to form glutamine, catalyzed by glutamine synthetase (GSase), providing the primary nitrogen-donating substrate for CPSase III. In the presence of OTCase, carbamoyl phosphate formed would be converted to citrulline.

Assuming that CPSase III and OTCase catalyze citrulline synthesis in muscle tissue, then the liver may be responsible for converting citrulline to urea, as sufficient levels of activities of the remaining urea cycle enzymes are present in the trout liver (Korte et al., 1997). In mammals, urea cycle intermediates are shuttled between organs, for example, citrulline synthesized in the small intestine (Raijman, 1974; Windmueller and Spaeth, 1974, 1981) is utilized by the kidney to make arginine (Featherston et al., 1973; Bergman et al., 1974). Hence, an increase in urea synthesis during high-speed swimming (Lauff and Wood, 1996; Alsop and Wood, 1997) may be related to ammonia detoxification via urea cycle enzyme activities in muscle tissue and other organs.

The purpose of our study was to test the hypothesis that urea cycle enzyme activities in skeletal muscle tissue of juvenile trout increase during exercise and that this correlates with increased rates of urea excretion. Juvenile rainbow trout swam at two exercise intensities (20 and 50 cm/s) for 2 h or 4 days, and nitrogen (ammonia and urea) excretion rates and CPSase III, OTCase, and GSase activities in muscle and liver tissues were determined. If constitutive levels of enzyme activity are not sufficient to handle increased urea synthesis during exercise, then an increase in enzyme activity would be observed (i.e. due to synthesis of new enzyme and/or modification of existing enzyme), at least after 4 days. We also assessed whether the level of activity of muscle CPSase III (the urea cycle enzyme typically with the lowest level of activity) was sufficient to account for whole-body urea production in either juvenile or adult trout, as has been observed in largemouth bass (Kong et al., 1998).

2. Materials and methods

2.1. Animals

Juvenile (mean weight 25 ± 2 g) and adult rain-

bow trout (478 ± 16 g) of both sexes were obtained from the Silver Creek Aquaculture company (Erin, Ontario). The fish were held in the Aqualab facility at the University of Guelph (11 ± 1°C, photoperiod 12L:12D) for 2 weeks prior to experimentation. Fish were fed trout pellets (Martin's Feed Mills, Elmira, Ontario) at 2% of their body weight for 5 days each week. Feeding was terminated 24 h prior to the 2-h exercise experiments and 48 h prior to the 4-day exercise experiments to reduce fouling of the respiratory chambers.

2.2. Experimental protocol

Three different experimental protocols were conducted:

1. Short-term (2-h) swimming experiments in juvenile trout at low-speed (20 cm/s, control) or high-speed (50 cm/s).
2. Long-term (4-day) aerobic swimming experiments in juvenile trout at low-speed (20 cm/s, control) or high-speed (50 cm/s).
3. Resting urea excretion experiments in adult trout.

2.2.1. Swim respirometer experiments

Juvenile fish were weighed and then placed in individual Blazka-type swim respirometers (~3.4 l; Fry, 1971). With the exception of the closed chamber flux periods, flow-through aerated water was supplied (100–150 ml/min) to each respirometer. Relatively constant water temperature (12°C) was achieved by immersing the respirometers in flowing water on a wet table in order to measure nitrogen excretion rates; the respirometers were closed for 2-h flux periods. In preliminary experiments, water O_2 levels were monitored during the flux period with an O_2 electrode (JSB Clark) and transducer connected to a standard chart recorder. In the absence of aeration, water O_2 levels fell below 70% by the end of the 2-h flux period. To ensure water oxygen levels were above 80% saturation at all swimming speeds, respirometers were gently bubbled with air during the flux period. Water samples (20-ml) were removed immediately after the respirometers were sealed (0 h) and following the 2-h flux period. Samples were frozen (−20°C) for later analyses of water ammonia and urea concentrations (< 1 week).

Respirometers were cleaned prior to each swimming trial to eliminate any possible contribution of microbial activity to nitrogen excretion rates. Blank trials of ammonia and urea excretion rates in respirometers without fish were negligible.

In the 2-h aerobic swimming experiment, all fish were initially acclimated for 1 h to an exercise rate of 20 cm/s. Following the acclimation period, fish were equally divided into a low-speed and a high-speed swimming group. Low-speed fish were maintained for an additional hour at 20 cm/s. The flow of water for the high-speed group was increased in a step-wise manner by 10 cm/s every 20 min (Hammer, 1995) to achieve a final swimming speed of 50 cm/s. Fish that were unable to maintain swimming at 50 cm/s were eliminated from the experiment. Respirometers were then sealed and aerated for a 2-h flux period. Immediately following the flux period, the experiment was terminated, fish were removed from the respirometers, killed by a sharp cephalic blow, and liver and muscle tissues were excised and frozen in liquid N_2. Tissue samples were stored (−86°C) for later enzyme analysis (< 6 months).

In the 4-day aerobic swimming experiment, all fish were initially acclimated for 20 h at zero water velocity in the respirometers. The pre-exercise fish were then killed and muscle and liver tissues were excised, frozen in liquid N_2, and stored at (−86°C) for later enzyme analysis (< 6 months). The low-speed fish swam at 20 cm/s for the remainder of the experiment. The high-speed fish swam at 20 cm/s for 1 h and then the water flow was gradually increased by 10 cm/s per h to achieve a final swimming speed of 50 cm/s within 3 h. The respirometers were then closed and nitrogen excretion was determined over a 2-h flux period (day 1). Note that both the low- and high-speed groups swam for 4 h prior to the first flux period on day 0. On each consecutive day (day 2–4), at 24-h intervals, nitrogen excretion was measured over 2-h flux periods. Following the final flux period, fish were killed and tissues stored for later enzyme analyses, as above.

2.2.2. Nitrogen excretion in adults

Nitrogen excretion was measured daily over 5

consecutive days (at the same time each day) in individual fish in separate darkened perspex chambers (~10 l). Measurements were taken on consecutive days to ensure a representative excretion rate was determined. At the beginning of each 2-h flux period, the chamber was flushed with fresh water and the volume reduced to a minimum (~3.5 l) so as to maximize the sensitivity with which changes in water urea concentration could be measured. This caused no apparent disturbance to the fish.

Control blank experiments were conducted to determine if microbes in the water or attached to the experimental chambers had any influence on measured nitrogen excretion rates. These control experiments revealed that microbial contamination was not a significant concern.

2.3. Measurements

2.3.1. Water samples

Water ammonia levels were measured with the salicylate–hypochlorite assay (Verdouw et al., 1978) and urea levels, by the diacetyl–monoxime method (Rahmatullah and Boyde, 1980). The changes in water urea levels were relatively small over the 2-h flux periods and therefore water samples (5-ml) were freeze-dried and then reconstituted in a smaller volume of water (1 ml) to increase the resolution of the analyses. For each urea assay, urea standards were also lyophilized to account for any potential loss of urea during the drying process (estimated to be less than 8%). Urea-N and ammonia-N excretion rates were calculated as the difference in water ammonia or urea concentration (μmol.N/l) multiplied by the volume of the system in l, and then divided by the elapsed time in hours and the weight of the fish in grams. Urea excretion rates were multiplied by two to account for the two nitrogen (N) atoms in each urea molecule.

2.3.2. Enzyme assays of trout tissues

Enzyme activities were measured on skeletal muscle and liver tissue at 26°C. The white muscle sample was excised from the dorsal surface below the vertebral column and above the lateral line to avoid contamination with red muscle tissue near the lateral line. Tissues were homogenized with six (muscle) or 10–18 (liver) volumes of extract buffer (0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.7, 0.4 mM ethylenedinitrilo tetra acetic acid (EDTA), 0.1 M KCl, 1 mM dithiothreitol (DTT)) at 4°C, subjected to brief sonication, and centrifuged at $14000 \times g$ for 10 min. The resulting supernatant was passed through a Sephadex G-25 column equilibrated with extract buffer before measuring enzyme activities (Korte et al., 1997). CPSase was measured as previously described (Korte et al., 1997). γ-Glutamyltransferase activity of glutamine synthetase was determined by measuring γ-glutamylhydroxamate formed after 40 min of reaction using 0.2 ml of extract in a final reaction volume of 0.8 ml (Shankar and Anderson, 1985). The reaction mixture for measuring OTCase activity contained 10 mM ornithine, 5 mM carbamoyl phosphate, 0.05 M Hepes, pH 7.7, 0.05 M KCl, 1 mM DTT, 0.6 mM EDTA, and an appropriate volume of extract in a final volume of 0.5 ml. Reaction was stopped at zero time (control) and after 30 min by adding 70 μl of 2 N $HClO_4$. After centrifugation, citrulline formed in 0.25 ml of supernatant was determined by adding 1 ml of color reagent, boiling for 20 min, and measuring the A_{466} (Xiong and Anderson, 1989). Enzymes activities were expressed in units/g liver tissue and units/mg protein, where unit represents μmol/min. Protein was measured by the dye-binding method of Bradford (1976) using Bio-Rad Laboratories reagents and bovine serum albumin as a standard.

2.4. Statistics

In the short-term aerobic swimming experiment, excretion rates and enzyme activities of the low- and high-speed swimmers were compared by a one-way analysis of variance ($P < 0.05$). In the long-term swimming experiment, excretion data was first analyzed by a one-way repeated measure analysis of variance with time (day) as a factor. Where time was not a factor, excretion rates for the low- and high-speed swimmers were compared for each day by a two-sample t-test ($P < 0.05$). Time was a factor only for the urea excretion values in the low-speed (20 cm/s) swimmers (specifically, day 1 and day 2 were significantly different by the Student–Newman–Keuls method). Enzyme activities between the three

groups (non-swimmers, low-speed swimmers and high-speed swimmers) were compared by a single factor ANOVA. In cases where a significance was found, a Bonferoni test was applied to determine which specific parameters were significantly different.

3. Results

3.1. Short-term (2-h) swimming experiment

The rate of urea excretion was elevated by 65% in the high-speed compared to the low-speed swimmers (Fig. 1a) and there were no significant differences in ammonia excretion rates (Fig. 1b). Consequently, the percentage of nitrogen excreted as urea [urea-N excretion/(urea-N excretion + ammonia excretion)] was 10% ± 2 in the low-speed group and 15% ± 2 in the high-speed fish.

There were no significant differences in muscle tissue CPSase III, OTCase or GSase activities when high vs. low-speed swimmers were compared (Table 1). OTCase activity in liver tissue, however, was significantly higher in the high-speed compared to low-speed swimmers (Table 1).

3.2. Long-term (4-d) swimming experiment

In the low-speed swimmers or control fish, the daily rate of urea excretion was relatively variable. Urea excretion between day 1 and 2 was significantly different in the low-speed swimmers (Fig. 2a). Ammonia excretion rates were elevated in the high-speed compared to the low-speed swimmers on days 2–4 (2.3-fold, day 2; 3.7-fold, day 3; 5.7-fold, day 4; Fig. 2b). The percentage of nitrogen excreted as urea was 37% ± 8 (20 cm/s) vs. 13% ± 6 (50 cm/s) on day 1, 13% ± 5 (20 cm/s) vs. 12% ± 5 (50 cm/s) on day 2, 38% ± 7 (20 cm/s) vs. 15% ± 5 (50 cm/s) on day 3, and 43% ± 9 (20 cm/s) vs. 17% ± 8 (50 cm/s) on day 4. Of these % urea excretion values, the differences between the 20 cm/s and 50 cm/s fish were statistically significant on day 1, 3 and 4.

GSase activities in skeletal muscle (both U/g tissue and U/mg protein) were significantly higher by 2.6-fold in the high- vs. low-speed swimmers and the pre-exercised fish (Table 2). Liver GSase

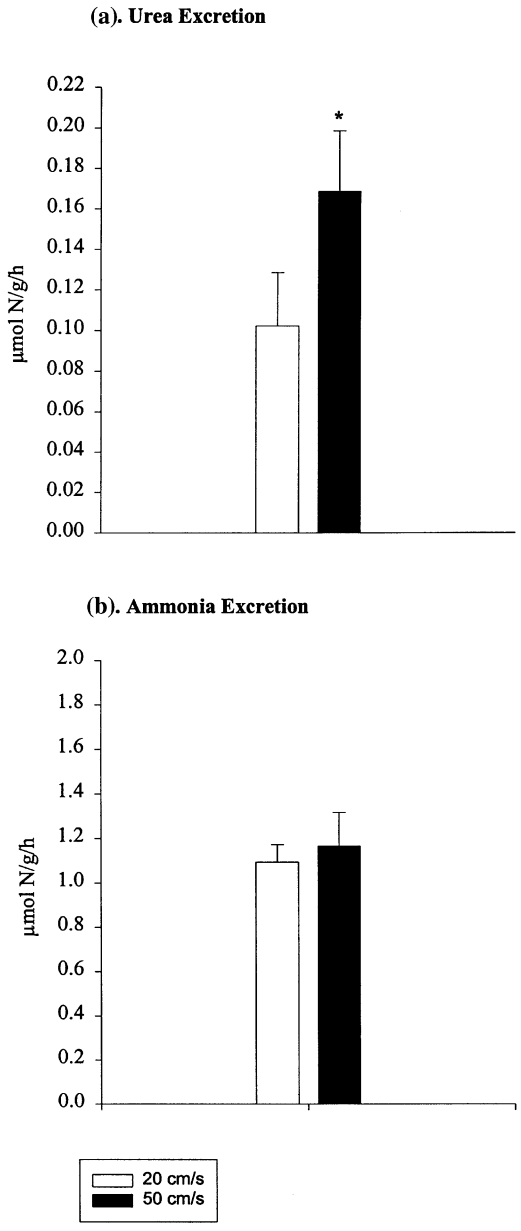

Fig. 1. Nitrogen excretion rates in low-speed (20 cm/s, gray bar, $n = 9$) and high-speed swimmers (50 cm/s, black bar, $n = 7$) during a short-term (2 h) aerobic swimming trial a. Urea and b. Ammonia. Asterisk indicates significant difference from low-speed (20 cm/s) swimmers ($P < 0.05$). Means ± S.E.

activity (U/mg protein) was elevated by 1.6-fold in the high-speed swimmers compared to the pre-exercised fish (Table 2). OTCase activity (U/g tissue) was significantly higher in liver tissue of the low-speed swimmers relative to the pre-exercised fish and the high-speed swimmers (Table

Table 1
Carbamoyl phosphate synthetase III (CPSase III), ornithine carbamylase (OTCase) and glutamine synthetase (GSase) activities in liver and muscle tissues after short-term (4-h) low-speed (20-cm/s) or high-speed (50-cm/s) aerobic swimming experiments in juvenile trout[a]

	Low-speed		High-speed	
	Muscle	Liver	Muscle	Liver
CPSase III				
(nmol/min/g tissue)	0.6 ± 0.1 (9)	BLD	0.5 ± 0.1 (8)	BLD
(nmol/min/mg protein)	0.01 ± 0.00 (9)	BLD	0.01 ± 0.00 (7)	BLD
OTCase				
(μmol/min/g tissue)	0.357 ± 0.053 (8)	0.007 ± 0.001 (8)	0.404 ± 0.101(8)	0.012 ± 0.002[b] (7)
(μmol/min/mg protein)	0.007 ± 0.002 (9)	0.00006 ± 0.00001 (8)	0.006 ± 0.001 (7)	0.00011 ± 0.00003 (7)
Gsase				
(μmol/min/g tissue)	0.036 ± 0.007 (5)	0.861 ± 0.138 (5)	0.023 ± 0.004 (7)	0.865 ± 0.117 (7)
(μmol/min/mg protein)	0.0006 ± 0.0002 (5)	0.0072 ± 0.0009 (5)	0.0005 ± 0.0001 (7)	0.0074 ± 0.0006 (7)

[a] Means ± S.E. (n). BLD: below level of detection.
[b] Significantly different from low-speed enzyme activity ($P < 0.05$).

2). CPSase activity was unchanged between the three groups of fish (Table 2).

3.3. Muscle urea cycle enzyme activity and urea synthesis

To discern whether urea cycle enzyme activities in muscle tissue were sufficient to make a significant contribution to whole-animal urea excretion rates, we compared carbamoyl phosphate synthetase III (CPSase III) activity and urea excretion rates in juvenile and adult fish (Table 3). The level of CPSase III activity in muscle of juvenile and adult fish (Tables 1 and 4) was considerably lower compared to OTCase and GSase activities and, therefore, we based our calculations on CPSase III because it would clearly be rate-limiting. CPSase III, OTCase and GSase in adult tissues (Table 4) were similar to juvenile values (Tables 1 and 2). The rates of urea excretion in juvenile fish were taken from Fig. 1a (20 cm/s). The rates of urea excretion in adult fish measured on 5 consecutive days were not significantly different (day 1, 0.072 ± 0.028; day 2, 0.066 ± 0.025; day 3, 0.055 ± 0.022; day 4, 0.055 ± 0.025; and day 5, 0.057 ± 0.027 μmol. N/g/h, $N = 6$) and therefore an average value was used for these calculations. Muscle mass for juvenile and adult trout was estimated to be 66% of total body weight (Stevens, 1968). Based on these measured or estimated parameters, total muscle CPSase III activity in juvenile and adult fish could account for 47–53% of the total urea excretion rate (Table 4).

4. Discussion

4.1. The role of muscle urea cycle enzymes in urea synthesis

Urea cycle-related CPSase III activity was present only in skeletal muscle tissue, not liver, in juvenile and adult trout (Tables 1, 2 and 4), supporting previous findings of CPSase III activity and mRNA levels in trout (Korte et al., 1997). Although the measured level of enzyme activity per gram muscle tissue is relatively low compared to hepatic activities in ureotelic fish (e.g. Randall et al., 1989; Anderson and Walsh, 1995), given the proportionally large mass of skeletal muscle tissue in trout, the total CPSase activity per fish may be significant. The same conclusion was reached by Kong et al. (1998) who reported low CPSase III activity in skeletal muscle tissue of largemouth bass but significant overall levels when tissue mass was considered.

In the ureotelic *O. alcalicus* grahami, Lindley et al. (1999) concluded that the level of activity of CPSase III was sufficient to account for the rate

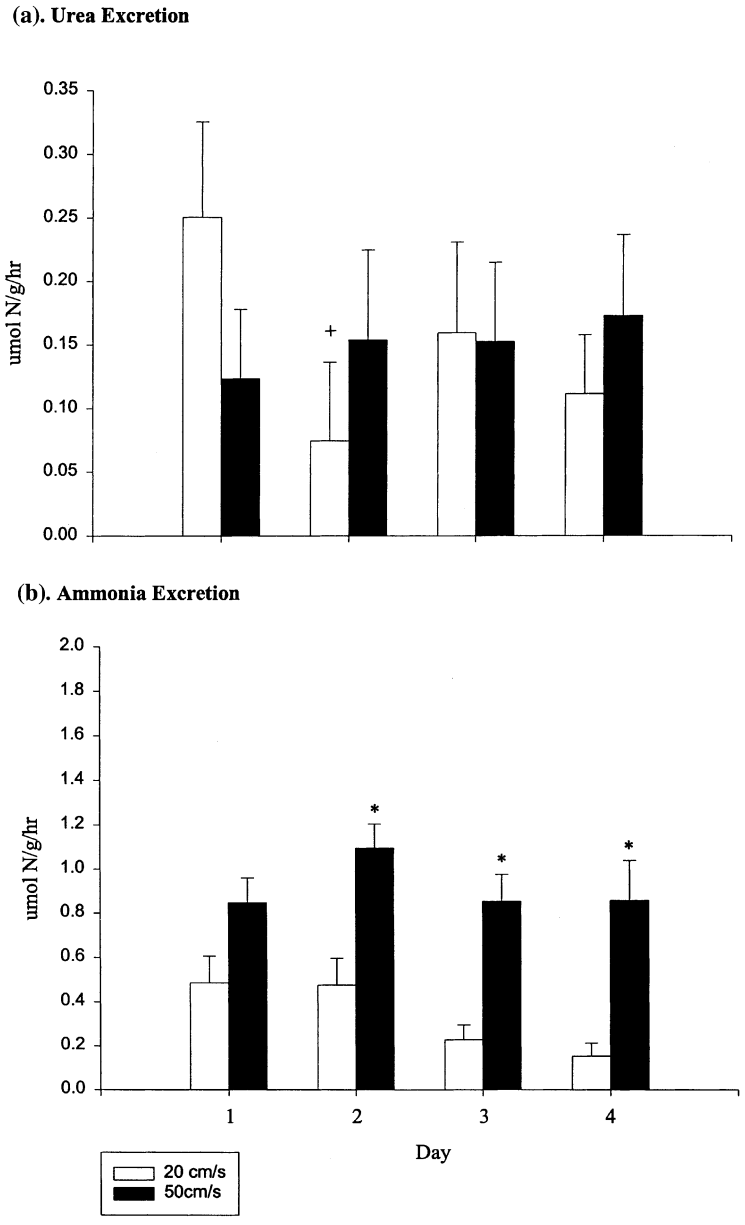

Fig. 2. Nitrogen excretion rates in low-speed (20 cm/s, $n = 6$) and high-speed swimmers (50 cm/s, $n = 6$) during a continuous long-term aerobic swimming trial A. Urea and B. Ammonia. Plus sign (+) indicates significant difference between day 1 and day 2 urea excretion rates in low-speed swimmers. Asterisk (*) indicates significant difference from low-speed (20 cm/s) swimmers ($P < 0.05$). Means ± S.E.

of urea excretion. In the present study, muscle CPSase III activity can account for 47–53% of urea excretion in ammoniotelic trout (Table 3). The absolute levels of CPSase III can be difficult to evaluate because there is often relatively high activities of the pyrimidine pathway-related CPSase II in most fish tissues (Felskie et al., 1998). Although every precaution was taken to differentiate between type I and II as outlined by Felskie et al. (1998), the in situ levels of CPSase III in skeletal muscle may have been higher than those reported here, accounting for a greater proportion of the total urea excreted (Table 3). Alternatively, approximately 50% of urea not accounted for by the urea cycle may have been derived from uric acid and/or arginine catabolism (see Section 1).

If the urea cycle is operative in rainbow trout,

Table 2
Carbamoyl phosphate synthetase III (CPSase III), ornithine carbamylase (OTCase) and glutamine synthetase (GSase) activities in liver and muscle tissues before the swimming experiment (pre-exercise), after long-term (4-day) low-speed (20 cm/s) or high-speed (50 cm/s) aerobic swimming in juvenile trout[a]

	Pre-exercise		Low-speed 4 days		High-speed 4 days	
	Muscle	Liver	Muscle	Liver	Muscle	Liver
CPSase III						
(nmol/min/g tissue)	0.8 ± 0.1 (10)	BLD	0.6 ± 0.1 (9)	BLD	0.5 ± 0.1 (7)	BLD
(nmol/min/mg protein)	0.01 ± 0.00 (10)	BLD	0.01 ± 0.00 (9)	BLD	0.01 ± 0.00 (7)	BLD
OTCase						
(μmol/min/g tissue)	0.504 ± 0.083 (8)	0.009 ± 0.001 (8)	0.480 ± 0.073 (9)	0.019 ± 0.001[b] (5)	0.460 ± 0.038 (7)	0.009 ± 0.003[c] (7)
(μmol/min/mg protein)	0.019 ± 0.010 (8)	0.00008 ± 0.00001 (8)	0.010 ± 0.002 (7)	0.00016 ± 0.00001 (3)	0.009 ± 0.001 (7)	0.00008 ± 0.00002 (7)
GSase						
(μmol/min/g tissue)	0.043 ± 0.003 (10)	0.855 ± 0.093 (8)	0.040 ± 0.006 (7)	0.797 ± 0.07 (5)	0.102 ± 0.022[b,c] (7)	1.088 ± 0.132 (7)
(μmol/min/mg protein)	0.0008 ± 0.0001 (10)	0.0070 ± 0.0007 (8)	0.0008 ± 0.0001 (7)	0.0072 ± 0.0017 (5)	0.0021 ± 0.0004[b,c] (7)	0.0115 ± 0.0019[b] (5)

[a] Means ± S.E. (n). BLD: below level of detection.
[b] Significantly different from pre-exercise values, $P < 0.05$.
[c] Significantly different from low-speed values, $P < 0.05$.

Table 3
Carbamoyl phosphate synthetase III (CPSase III) in muscle tissue, whole animal urea excretion rates and the % ratio of the two values in juvenile and adult fish[a]

	CPSase III activity (μmol/h)	Urea excretion rate (μmol/h)	(CPSase III activity/urea excretion) × 100 (%)
Juvenile (20 cm/s)	0.59	1.25	47
Adult (control)	7.57	14.34	53

[a] In juvenile fish that swam for 2 h, the rate of urea excretion was 0.10 μmol. N/g/h or 1.25 μmol/h (20 cm/s; Fig. 1) for a 25-g fish. Mean CPSase III was 0.0006 μmol/min/g tissue (Table 1), and if skeletal muscle mass was approximately 66% (Stevens, 1968), then maximal output would be 0.59 μmol/h. In control adult trout, the urea excretion rate was approximately 0.06 μmol. N/g fish/h or 14.34 umol urea/h for a 478-g fish. Mean CPSase III for this group was 0.0004 μmol/min/g tissue (Table 4) and if muscle mass is approximately 66%, then maximal output would be 7.57 μmol/h.

then we propose that the first two steps occur in the muscle. Most of the OTCase activity (> 98%) is found in muscle tissue and not liver (present study), intestine or kidney tissue (Korte et al., 1997). GSase activity is primarily in liver tissue (> 93%), however, with lower levels in muscle tissue (Tables 1, 2 and 4). The same ratio of liver/muscle GSase activities was also reported in lake char (*Salvelinus namaycush*; Chamberlin et al., 1991) and alkaline-adapted tilapia (Lindley et al., 1999). The relatively high levels of GSase activity in liver tissue are not surprising because glutamine is an important substrate in various metabolic reactions (Chamberlin et al., 1991). Regardless, GSase activity in the muscle is in excess of CPSase III activity by eight-fold (assuming that the biosynthetic rate of GSase is 20 times less than the assayed transferase rate; Shankar and Anderson, 1985; Anderson and Walsh, 1995). Moreover, GSase activity levels measured here were very similar to those reported for mammalian muscle tissue when the assay temperature difference is accounted for (King et al., 1983), and muscle is thought to be the major site of glutamine formation in mammals (Felig, 1975; Schrock and Goldstein, 1980; Jungas et al., 1992). It should also be noted that if total activity in the muscle tissue (~ 66% of body mass; Table 3) is compared to liver tissue (~ 1% of body mass), then the units of GSase in juvenile and adult fish are three- to four-fold higher in muscle tissue relative to liver. Thus, the possibility exists for significant glutamine formation, and in turn, citrulline synthesis in trout muscle tissue.

Table 4
Carbamoyl phosphate synthetase III (CPSase III), ornithine transcarbamylase (OTCase) and glutamine synthetase (GSase) activities in muscle and liver tissues of adult trout[a]

	Muscle	Liver
CPSase III		
(nmol/min/g tissue)	0.4 ± 0.0	BLD
(nmol/min/mg protein)	0.01 ± 0.00	BLD
OTCase		
(μmol/min/g tissue)	0.518 ± 0.090	0.042 ± 0.026
(μmol/min/mg protein)	0.008 ± 0.002	0.00013 ± 0.00003
GSase		
(μmol/min/g tissue)	0.054 ± 0.010	0.723 ± 0.078
(μmol/min/mg protein)	0.0007 ± 0.0001	0.0052 ± 0.0011

[a] Means ± S.E. ($n = 7$). BLD: below level of detection.

4.2. Urea excretion rates and exercise

It is clear from our findings that changes in nitrogen metabolism and excretion during exercise are complex and depend on the intensity and duration of the exercise period. The rates of ammonia and urea excretion, as well as the % urea excretion were markedly different between the short- and long-term exercise regimes and the low-speed (20 cm/s) and high-speed (50 cm/s) protocols.

The rate of urea excretion was significantly enhanced in juvenile trout that swam at high-speed for 2 h (Fig. 1a), in agreement with a study by Alsop and Wood (1997). They proposed that with more intense exercise there is an increase in the recruitment of white muscle fibers (Webb, 1971; Wilson and Egginton, 1994; Moyes and West, 1995; Lauff and Wood, 1996) and in turn, increased adenylate turnover, ammonia production and ammonia detoxification via ureagenesis. There were no significant changes in skeletal muscle tissue GSase, CPSase III, or OTCase activities in the high-speed swimmers. In this rela-

tively short exercise period, we would not expect profound changes in enzyme activity due to new protein synthesis, but rather changes related to more rapid cellular events, such as enzyme phosphorylation, allosteric effects or changes in the concentration of low molecular weight effectors. In this latter category, changes in the levels of the activator *N*-acetyl glutamate (AGA) are known to enhance flux through the CPSase III reaction in toadfish (Julsrud et al., 1998). In mammals, AGA and ornithine concentrations, as well as substrate availability (i.e. ammonia), regulate the citrulline-synthesizing capacity of liver mitochondria in the short-term (Carey et al., 1993). Hence, it is possible that changes in the enzyme microenvironment may have been responsible for rapid changes in urea production and excretion.

In contrast to the short-term experiment, ammonia excretion rates were two- to six-fold higher in fish that swam at 50 cm/s over 4 days, but urea excretion rates were not enhanced (Fig. 2b). Lauff and Wood (1996) reported a consistent elevation of urea excretion but not ammonia excretion in high-speed over low-speed swimmers each day of a 3-day aerobic swimming experiment. Furthermore, the average percentage of N-wastes appearing as urea reported by Lauff and Wood (1996) was 18% (low-speed) vs. 34% (high-speed), whereas in the present study, the values were reversed, 33% (low-speed) vs. 14% (high-speed). The explanation for the differences between the two studies may relate to (1) the divergent swimming performance between different stocks of hatchery-reared juvenile rainbow trout and/or (2) different swimming speeds designated as 'high-speed' between the two studies (~34 cm/s, Lauff and Wood, 1996 vs. 50 cm/s, present study). Recently, experiments on Nile tilapia (*Oreochromis niloticus*) demonstrated that ammonia excretion rates were elevated in fish that swam at 45–50 cm/s (short-term) or at 25 cm/s for 50 h (Alsop et al., 1999).

The long-term elevation of ammonia excretion was probably due to protein catabolism via transdeamination in the liver and/or muscle tissues, similar to white muscle proteolysis in spawning salmon (Mommsen et al., 1980; French et al., 1983). Although the progressive recruitment of white 'anaerobic' muscle fibers at higher swimming speeds and the accompanying rapid turnover of ATP probably makes a transitory contribution to ammonia production (~2–4 h; see above), a high rate of ATP turnover could not be maintained over such a long period of time (i.e. 4 days; C.M. Wood, pers. comm.). The importance of protein as a fuel during exercise in fish is controversial and species-specific (e.g. Kutty, 1972; Moyes and West, 1995; Weber and Haman, 1996). Lauff and Wood (1996) reported that in unfed rainbow trout, the relative protein contribution to fuel usage decreased as the swimming speed was increased from low-speed (~20 cm/s) to 'medium' swimming speeds (~34 cm/s). Unfortunately, there are no comparable studies in the literature where trout swam at a relatively high-speed (50 cm/s) for a prolonged period (4 days), as in the present study. Nevertheless, the data strongly suggest that amino acid and protein catabolism played a significant role in providing fuel for the 4-day high-speed exercise regime in this study.

Elevated ammonia excretion rates in fish that swam at 50 cm/s for 4 days were correlated with a 1.6- to 2.6-fold increase in liver and muscle GSase activities, respectively. The elevation of GSase activity may have been related to higher tissue ammonia levels in fish that swam at 50 cm/s and the conversion of excess ammonia (and glutamate) to glutamine. There are a variety of metabolic and environmental conditions where GSase activity is elevated in fish tissues for purposes of ammonia detoxification (e.g. Jow et al., 1999; for a review, see Korsgaard et al., 1995).

Small, but significant changes in liver OCTase activity were observed in both the short-term and long-term protocols. These results are perplexing because most of the OTCase activity was found in muscle, not liver tissue, and we could detect no CPSase III activity in liver tissue. Clearly, there is still much to learn about the role of urea cycle enzymes in liver and muscle tissue and the possibility of interorgan transport of intermediates in fish.

Urea excretion rates increase significantly in short-term high-speed swimmers (Fig. 1a) but were unchanged in the long-term high-speed swimmers on day 1 of the experiment (Fig. 2a), an apparent contradiction. The relatively high rate of urea excretion in the 'control' fish (20 cm/s) on day 1 of the long-term experiment may have exaggerated differences in the data. The differences may have been also due to slightly different exercise regimes. In the short-term experiment, the trout swam for 2 h prior to the measurement

of urea excretion compared to 4 h in the long-term experiment (see Section 2). It is evident from this study and others that N-waste excretion is very sensitive to changes in the intensity and duration of the exercise period.

In summary, CPSase III activity in skeletal muscle can account for much of the total urea excreted by rainbow trout. There was no significant induction of urea cycle enzymes, at least CPSase III and OTCase, in muscle of fish that swam for several days at high speed, but the activity of the accessory enzyme, GSase, was elevated by two-fold. There are numerous questions concerning the role of skeletal muscle tissue in fish nitrogen metabolism. Infusion of radio-labeled substrates for the urea cycle, and recovery of radio-labeled products may help to unravel the role of CPSase III and related enzymes in muscle tissue of rainbow trout.

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