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Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during starvation in juvenile rainbow trout, *Oncorhynchus mykiss*

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Abstract Oxygen consumption, CO_2 excretion, and nitrogenous waste excretion (75% ammonia-N and 25% urea-N) were measured daily in 4-g rainbow trout over a 15-day starvation period. Oxygen consumption and CO_2 excretion declined while N excretion increased transiently in the mid-part of the starvation period but was unchanged from control levels at the end. Component losses (as percentage of total fuel used) of protein, lipid, and carbohydrate were 66.5, 31.1, and 2.4% respectively, as measured from changes in body weight and body composition, the latter relative to a control group at day 0. *Instantaneous* fuel use, as calculated from the respiratory quotients and nitrogen quotients, indicated that relative protein use rose during starvation, but contributed at most 24% of the aerobic fuel (as carbon). Lipid metabolism fell from about 68 to 37%, and was largely replaced by carbohydrate metabolism which rose from 20 to 37%. We conclude that the two approaches measure different processes, and that the *instantaneous* method is preferred for physiological studies. The *compositional* method is influenced by greater error, and measures the fuels depleted, not necessarily burned, because of possible interconversion and excretion of fuels.

Key words Starvation · Respiratory quotient · Nitrogen quotient · Fuel · Rainbow trout, *Oncorhynchus*

Introduction

Most of the presently available information on fuel utilization in fish has been obtained from measurements of changes in body composition during an experiment, or over the course of the fish's natural migration (Duncan and Tarr 1958; Idler and Clemens 1959; Johnston and Goldspink 1973; Robinson and Mead 1973; Mommsen et al. 1980; Jezierska et al. 1982; Virtanen and Forsman 1987). In general, the conclusion from such *compositional* studies is that the carbon source for ATP synthesis arises mainly from the direct metabolism of lipid and protein reserves, with minimal usage of carbohydrate. However, there are two potential limitations to the depletion approach: the first is that the method reveals the fuels which have been depleted, not necessarily the fuels which have been burned in metabolism. For example, fuels may be interconverted prior to respiration, or they may be excreted (e.g., mucus secretion) without being burned. Secondly, compositional measurements are necessarily destructive and terminal, so fuel utilization cannot be followed in individual fish. The necessity of detecting differences in composition between groups of fish may introduce considerable variability.

An alternate approach is to monitor the *instantaneous* fuel utilization by respirometry on single animals during a study. In theory, the proportions of O_2 consumption, CO_2 production, and nitrogenous waste excretion can be used to stoichiometrically calculate the particular combination of carbohydrate, protein, and lipid actually being oxidized at any point in time, as long as the animal is undergoing only aerobic metabolism and is in a steady state condition [i.e., measured fluxes and production/consumption rates are equivalent; Kleiber (1987, 1992)]. In practice, this approach has not yet been applied in full to fish, probably because of difficulties in measuring CO_2 and N-waste excretion in water. However, partial measurements have been made in a number of studies (Kutty 1968, 1972, 1978; Brett and Zala 1975; Sukumaran and Kutty 1977; van den Thillart and Kesbeke 1978; Wiggs et al. 1989; Wood et al. 1994). Results have been highly variable. For example, protein utilization, the parameter

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most often studied via the ratio of N-waste excretion to O_2 consumption, has been estimated to contribute 14–90% of total metabolism [references as above, and review by van Waarde (1983)].

Our goals in the present study were two fold: the first was to apply the respirometric ("*instantaneous*") method in full to evaluate fuel utilization over a 15-day starvation period in juvenile rainbow trout. Based on the work of Brett and Zala (1975), who measured O_2 consumption and N-waste excretion (but not CO_2 excretion) in sockeye salmon over 3 weeks starvation, we hypothesized that the relative use of protein as a metabolic fuel would increase during starvation, and the relative use of other fuels would decrease proportionately. Our second goal was to compare the *instantaneous* method with the classical *compositional* method. Changes in body composition over the same period were therefore determined. We hypothesized that if the results of the two methods were substantially different, it would indicate that excretion or interconversion of fuels are potentially significant complications in the use of the compositional approach.

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum, formerly *Salmo gairdneri* Richardsoni; 2–4 g) were obtained from Rainbow Springs Hatchery (Thamesford, Ontario) and were kept in 15 °C dechlorinated Hamilton city tap water for at least 2 weeks prior to experimentation. The water was moderately hard, with the following composition ($mEq \cdot l^{-1}$): Na^+, 0.6; Ca^{2+}, 1.8; Cl^-, 0.8; K^+, 0.04; Mg^{2+}, 0.5; titration alkalinity (to pH = 4.0), 1.9; total hardness, $\approx 140\ mg \cdot l^{-1}$ as $CaCO_3$; pH 8.0. The fish were maintained on a commercial trout feed (Martin Trout Food Pellets, Tavistock, Ont.), and were fed 1.0% of their body weight daily. The composition of the diet, as reported by the manufacturer, was 40% (minimum) crude protein, 12% crude fat (minimum), and 3% fibre (maximum). In addition, our analysis of the food indicated 8.4% as glucose plus glycogen.

Respirometry – the influence of water quality

Decarbonated water was used to provide a low background level of total CO_2 against which excreted CO_2 could be accurately measured. MCO_2 could not be reliably determined against the high background level of total CO_2 (≈ 2 mM, almost all as HCO_3^-) in normal Hamilton tap water (from Lake Ontario). Decarbonated water was made up in a 750 l tank by acidifying normal tap water to $\approx$pH 3 with concentrated HCl and bubbling vigorously overnight with air. The pH was then brought up to 6.8 with NaOH. The final product had a total CO_2 concentration of 20 μM, 3.2 $mEq \cdot l^{-1}$ of Cl^- and 1.1 $mEq \cdot l^{-1}$ of Na^+.

A simple experiment was performed to test whether the transfer from normal tap water to decarbonated water had any adverse effects on gas exchange. Fish were acclimated (unfed) for 24 h in individual small (70-ml), opaque respirometers served with a flow of $150\ ml \cdot min^{-1}$ of air-saturated tap water. Respirometry was performed under a closed system regime; a water sample was taken prior to the typical 10-min closure, and then again afterwards. The actual duration of the bouts was calculated to not let the PO_2 of the water drop below 120 torr. The water samples were analyzed for both O_2 and T_{amm} (total ammonia = $NH_4^+ + NH_3$; see below). Sampling periods were immediately before the changeover, as well as 10, 30 and 60 min, and 2, 4, and 6 h after the changeover.

Respirometry – the influence of starvation

Eight Blaźka-style swimming respirometers of known volume (≈ 3.2 l) were used to house individual fish for the duration of the experiment (Blaźka et al. 1960, in Beamish 1978). Except for the periods of closed respirometry, air-saturated water flowed through each respirometer at a rate of approximately $150\ ml \cdot min^{-1}$. The water from the head tank of a recirculating system (total volume 500 l) entered each respirometer via one port and exited via the sampling port, from where the water drained directly into a wet table. To ensure thermal equilibrium the respirometers were submerged in the water of the wet table. About 300 l of the water was replaced on a daily basis.

Eight fish (4.5 ± 0.1 g, mean $\pm$ SEM) were quickly blotted dry, weighed to the nearest 0.1 g and then transferred to individual respirometers. An opaque sheet floated on the water surface to minimize visual disturbance; the sampling port and tubing from the head tank passed through holes in the sheet. The fish were not fed at any time during the tests, including the 24-h acclimation period.

Over the subsequent 15-day test period, all fish underwent one or two closed respirometry trials each day. Following the withdrawal of a 30-ml water sample, the respirometer was sealed for 3 h, after which an end sample was taken. PO_2 did not typically drop below 120 torr. Each aliquot was immediately divided into three subsamples. The 8-ml subsample for CO_2 analysis filled a precooled glass vial which was quickly capped to prevent diffusive exchange of CO_2, and stored at 4 °C for analysis later the same day (see below). A 15-ml subsample for analysis of nitrogenous wastes was immediately frozen and stored at −20 °C. Oxygen (as PO_2) was measured immediately on the balance of the water sample with a water jacketed O_2 electrode (Radiometer E5046) attached to a Cameron O_2 meter (OM-200) and thermostatted to the temperature of the test system (15 ± 1 °C). PO_2 was converted to oxygen concentration (CO_2) using tabulated solubility coefficients for freshwater (Boutilier et al. 1984). MO_2 could then be calculated via the equation:

$$MO_2 = \frac{\Delta CO_2 \cdot vol}{m \cdot t} \quad (1)$$

where MO_2 is the molar oxygen consumption in $\mu mol\ O_2 \cdot g^{-1} \cdot h^{-1}$, ΔCO_2 is the difference in oxygen concentration ($\mu mol \cdot l^{-1}$) between start and end of the test period, *vol* is the volume (l) of the respirometer, *m* is the initial mass of the fish (g), and *t* is the time (h) of the run. Analogous equations were used for CO_2, T_{amm}, and urea excretion rates.

The water samples for CO_2 analysis were rewarmed to 15 °C and measured in duplicate on a Shimadzu GC-8A gas chromatograph equipped with a Poropak Q column; the output was displayed on a Shimadzu-CR3A integrator. A series of $NaHCO_3$ standards were made up in the 0–200 $\mu mol \cdot l^{-1}$ range using the test medium.

The salicylate-hypochlorite assay was used to analyze total ammonia in the water (Verdouw et al. 1978). Urea was measured as in Rahmatullah and Boyd (1980) with a modification for increased sensitivity; the colour reagent consisted of 10 mg thiosemicarbizide and 500 mg of diacetyl monoxime per 20 ml deionized water (T.P. Mommsen, pers. comm.). Due to the relatively low levels of urea production relative to the volume of the respirometer, it was necessary to freeze-concentrate water samples five-fold prior to urea analysis. Samples (5 ml) were freeze-dried and rehydrated to a volume of 1.0 ml. Standards (0–10 μM) were treated in the same way.

Body composition

At the end of the experiment, the fish were sacrificed with neutralized tricaine methanesulfonate (MS222; final concentration 1 $g \cdot l^{-1}$), removed from the respirometers, blotted, weighed, freeze-clamped with aluminum tongs in liquid N_2, and stored at −70 °C for analysis of proximate body composition. A second set of seven fish from the same stock were sacrificed after going through the same acclimation period as the test fish. The mean weight of these control fish (4.2 ± 0.3 g) was not significantly different from the starting weight of the test fish (4.5 ± 0.1 g; $P > 0.05$). The bodies of the test and control fish were individually ground in an Ika electric tissue grinder (Staufen, Germany) which was cooled with methanol/dry ice (~ −77 °C). The resulting powder was lyophilized to determine water content. All assays were done in duplicate on these freeze-dried samples.

Lipids were extracted from 100 mg of sample powder using 10 ml of chloroform-methanol (2:1) and overnight incubation in the dark at 4 °C. Then, 2.6 ml of 0.9% NaCl was added with mixing, and the tubes were again allowed to incubate overnight. All water-soluble tissue components and methanol partitioned into the saline, leaving only the lipid in the lower chloroform phase (approximately 7 ml). A 4-ml aliquot of the chloroform phase was evaporated to dryness, held in a dessicator for 1 h, and lipid content was determined gravimetrically. Protein was determined via the Lowry method (Miller 1959) using bovine serum albumin (Sigma) as a standard. Glucose, glycogen and lactate were assayed as in Bergmeyer (1985). Due to mechanical constraints of removing the fish from their respirometers, there was a delay of approximately 1 min between anoesthetization and freeze-clamping. This delay could have resulted in decreased glycogen, and increased both glucose and lactate (Black et al. 1962; Barton et al. 1985); therefore, the sum of glucose, glycogen and lactate is reported as total carbohydrate. Ash content was determined by heating the freeze-dried tissue to 750 °C until a constant weight was obtained (approximately 4 h).

Fuel use calculations

The respiratory quotient $RQ = \frac{MCO_2}{MO_2}$ and nitrogen quotient $NQ = \frac{MN}{MO_2}$ were determined for each fish at each time. Note that $M_{urea\text{-}N}$ was used in the calculation of NQ, not simply M_{urea}. Therefore,

$$NQ = \frac{2 \cdot M_{urea} + M_{T_{amm}}}{MO_2}$$

In the rare instances (5–10% overall) where the RQ of an individual fish during a particular test period was found to be greater than 1.0, that RQ was not used to calculate fuel use. Only values of $RQ \leq 1.00$ were used to determine *instantaneous* fuel use since an RQ of 1.00 represents the upper limit obtainable during aerobic metabolism (Kleiber 1987). The potential bias introduced by this procedure is addressed in the Discussion. Only T_{amm} and urea were measured for the calculation of NQ since they represent the vast majority of N-waste products. The error associated with neglecting other possible N-products will also be addressed in the Discussion.

Since the fish were found to be neither strictly ureotelic nor strictly ammoniotelic, but rather excreted 75% T_{amm}-N and 25% urea-N, traditional values of RQ representing protein use ($RQ_{protein}$) for 100% ammoniotelism or 100% ureotelism could not be used. The calculation used to determine the $RQ_{protein}$ (0.94) in this study is the same as that used by Kleiber (1987, 1992); the theory behind this is explained in the Discussion.

Instantaneous fuel usage was then determined as follows:

$$P + L + C = 1.0 \quad (3)$$

where P, C, and L represent the fraction of the total fuels burned arising from protein, carbohydrate and lipid, respectively. The protein component could be determined using,

$$P = \frac{NQ}{0.27} \quad (4)$$

where 0.27 is the theoretical maximum for NQ [i.e. when protein is the sole fuel source; van den Thillart and Kesbeke 1978]. The carbohydrate and lipid fractions could then be determined using the respective fuel-specific RQs:

$$RQ = P \cdot 0.94 + C \cdot 1.0 + L \cdot 0.71 \quad (5)$$

Since the value of P has been determined (Eq. 4), only C and L need be determined. Since (from Eq. 3),

$$L = (1.0 - P - C) \quad (6)$$

by substituting Eq. 4 into Eqs. 6 and 5:

$$L = \left(1.0 - \frac{NQ}{0.27} - C\right) \quad (7)$$

$$RQ = \frac{NQ}{0.27} \cdot 0.94 + C \cdot 1.0 + L \cdot 0.71 \quad (8)$$

Finally, substituting Eq. 7 into Eq. 8:

$$RQ = \frac{NQ}{0.27} \cdot 0.94 + C \cdot 1.0 + \left(1.0 - \frac{NQ}{0.27} - C\right) \cdot 0.71 \quad (9)$$

which simplifies to:

$$RQ = 0.85 \cdot NQ + 0.29 \cdot C + 0.71 \quad (10)$$

RQ and NQ were measured in the experiment, so the equation can be solved for C; lipids can be then determined by difference (Eq. 6).

The above calculations yield the relative contributions of the individual fuels to MO_2. The percentage contribution of each fuel based on the *consumption of* O_2 was then converted to a percentage based on *carbon usage* via the fuel-specific RQs. The total carbon usage was reflected in the MCO_2 data of Fig. 1, which was then apportioned to absolute carbon expenditures of the three fuel types using these C-based percentages.

Compositional fuel usage was calculated in the traditional manner from the depletion of reserves over the 15-day starvation period, based on measured changes in body composition and body weight. The test fish declined from 4.5 to 3.8 g (average masses) over the experimental period. The concentrations of each component were converted to absolute amounts using the average 4.5 g (starting) or 3.8 g (terminal) body weights at the end of starvation in the test fish. The difference was taken to obtain the mean loss of each component. The composition of the control fish was used to represent that of the test fish at the start of the experiment, since body compositions could not be determined pre- and post-test on the same fish. Total depletions of each fuel were tallied and percentages calculated. The carbon mass of each fuel depleted was calculated based on the percentage weight of carbon in each fuel (Kleiber 1987).

As an internal check between the two methods of determining fuel use, the *measured* total O_2 consumed and N excreted were summed directly by taking the area under the MO_2 and M_N graphs (Figs. 1, 2) and multiplying by the mean weight of the fish. These values could then be compared with *predictions* of the total O_2 consumed and N excreted, based on the fuel depletions from the measured changes in body composition.

Statistics

Data are expressed as means ± 1 SEM(n). Regressions were fitted by the method of least squares and tested for significance using the Pearson linear correlation and the appropriate t-test (Fig. P

graphics package, Biosoft, Ferguson, Mo., USA). A one-way ANOVA was used to check for any variation between points over time in the starvation experiment and in the water changeover experiment. If the F value indicated significance, Bonferroni's modification (for multiple comparisons) of Student's paired t-test was used to evaluate individual points of interest. An independent t-test was used to determine statistical significance between control and test fish for the body composition measurements. A $P < 0.05$ was considered significant for all tests.

Results

The influence of water quality

The changeover from normal tap water to decarbonated tap water had no effect ($P > 0.05$) on either MO_2 or MNH_3 over the 6-h test (Table 1). Ammonia excretion remained stable at $\sim 0.17\ \mu mol \cdot g^{-1} \cdot h^{-1}$, whereas MO_2 remained stable at $\sim 5.0\ \mu mol \cdot g^{-1} \cdot h^{-1}$. It was then assumed that working in decarbonated water would not give rise to any complicating effects.

Respirometry and N-waste excretion

Starting MO_2 was $7.5\ \mu mol \cdot g^{-1} \cdot h^{-1}$ for the trout in the Blaźka respirometers. Both MO_2 and MCO_2 decreased over the 15-day test period, though this was only significant ($P < 0.001$) for O_2 consumption (Fig. 1a, b). In both cases, values fluctuated about the line of regression with decreased amplitudes over the second half of the experiment. MO_2 decreased from approximately 7.5 to $5.3\ \mu mol \cdot g^{-1} \cdot h^{-1}$, while MCO_2 dropped from 6.8 to $5.5\ \mu mol \cdot g^{-1} \cdot h^{-1}$. Since the slope of CO_2 excretion was less than that for O_2 consumption, a positively sloped (though not significantly so) RQ resulted (Fig. 1c). Since this study focused on aerobic metabolism, only aerobic values of RQ (i.e. < 1.0) were included in the means in Fig. 1c.

Nitrogenous waste excretion was triphasic over the duration of the experiment (Fig. 2a). The first phase (80 h) showed a MN which averaged $0.23\ \mu mol \cdot g^{-1} \cdot h^{-1}$. During the second phase (90 h) the fish exhibited a relatively stable 50% increase ($0.34\ \mu mol \cdot g^{-1} \cdot h^{-1}$) which was significant relative to most points in the first phase. In the final phase (170 h), the excretion rate dropped to a level not significantly different from the first phase and averaged $0.25\ \mu mol \cdot g^{-1} \cdot h^{-1}$. Total ammonia ($NH_3 + NH_4^+$) represented about 75% of the total N excretion. Neither any one phase, nor the entire curve taken as a whole, showed a significant slope.

Using the decreasing and fluctuating MO_2 and the changing MN, a linear ($r = 0.662$) and increasing ($P < 0.001$) relationship for the NQ was obtained (Fig. 2b). The NQ rose from 0.037 at $t = 0$ h to 0.064 at $t = 344$ h.

Table 1 The effect of the changeover from normal tap water to decarbonated tap water on oxygen consumption ($n = 12$) and T_{amm} excretion ($n = 10$) in juvenile rainbow trout. The changeover occurred at $t0$; there was no significant difference in either parameter. Means ± SEM

Time (hours)	Oxygen ($\mu mol\ O_2 \cdot g^{-1} \cdot h^{-1}$)	Ammonia ($\mu mol\ T_{amm} \cdot g^{-1} \cdot h^{-1}$)
−0.025	5.04 ± 0.81	0.16 ± 0.03
0.167	5.02 ± 0.51	0.21 ± 0.05
0.5	5.19 ± 0.58	0.16 ± 0.03
1.0	4.93 ± 0.60	0.16 ± 0.03
2.0	5.31 ± 0.93	0.19 ± 0.04
4.0	4.75 ± 0.56	0.18 ± 0.03
6.0	5.23 ± 0.68	0.17 ± 0.03

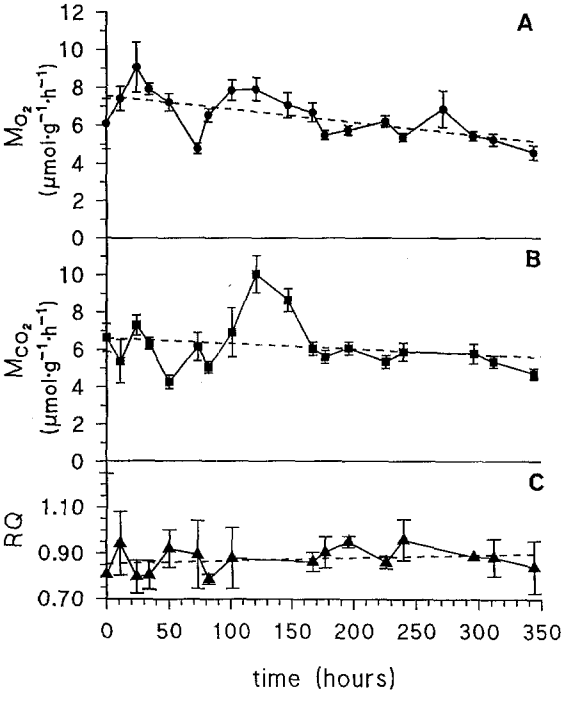

Fig. 1A–C (**A**) Oxygen consumption, (**B**) carbon dioxide excretion and (**C**) aerobic respiratory quotient over 15 days of starvation in juvenile rainbow trout. The *dashed line* is the regression. Means ± SEM, $n = 8$

Instantaneous fuel use

Relative protein use can also be elucidated from Fig. 2b (right axis) due to the proportionality between it and the NQ (Eq. 4). At 0 h protein made up only 14% of the total fuels; at 344 h protein use had risen significantly by almost 10%. When the NQ and the regressed values of aerobic RQ were used to calculate the generalized fuel use picture, two distinct areas of fuel use were found (Fig. 3a). During the first quarter of the experiment lipids averaged approximately 68% of all fuels burned, whereas carbohydrates represented

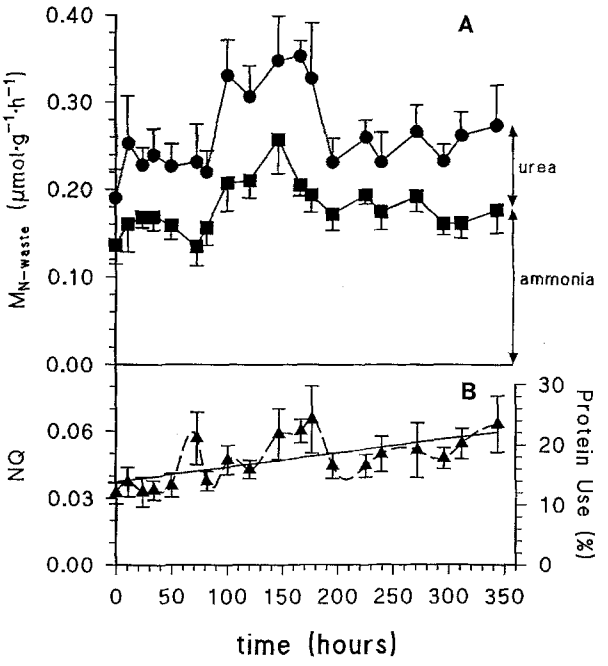

Fig. 2A, B (**A**) Total nitrogenous waste excretion (total-nitrogen as the sum of ammonia-N and urea-N, *circles*; T_{amm}, squares) and (**B**) nitrogen quotient (*left axis*) and relative protein use (*right axis*) over 15 days of starvation in juvenile rainbow trout. Means + SEM, $n = 8$

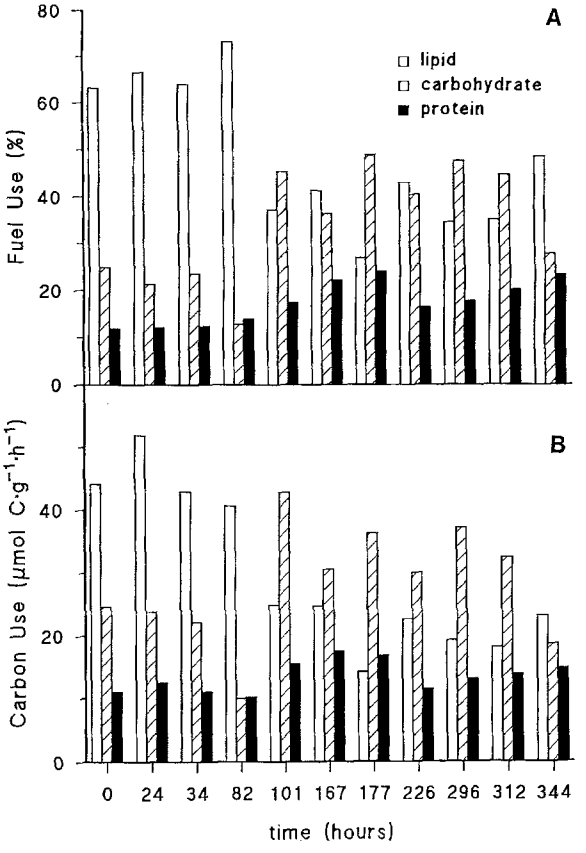

Fig. 3A, B (**A**) Relative use and (**B**) absolute carbon use rate of lipid (*open bars*), carbohydrate (*hatched bars*), and protein (*solid bars*) as calculated from respirometry and nitrogenous waste excretion data, over 15 days of starvation in juvenile rainbow trout

about 20%, and proteins only about 12%. Over the remainder of the starvation period, the contributions of the two N-free fuels in supporting MO_2 tended to equal out at about 37% each, with protein making up the balance (15–24%).

Carbon use from proteins was lowest over the first 82 h, where the average contribution was 10.5 µg $C \cdot g^{-1} \cdot h^{-1}$ (Fig. 3b). At the same time, carbon use from lipids was at its highest at an average of 44 µg $C \cdot g^{-1} \cdot h^{-1}$. The period from 101 to 177 h showed higher protein C use at 17 µg $C \cdot g^{-1} \cdot h^{-1}$ after which it decreased and averaged 14 µg $C \cdot g^{-1} \cdot h^{-1}$. Thereafter, lipids only contributed between 15 and 25 µg $C \cdot g^{-1} \cdot h^{-1}$. Carbon from carbohydrate was oxidized initially at 23 µg $C \cdot g^{-1} \cdot h^{-1}$; following a brief drop to 10 µg $C \cdot g^{-1} \cdot h^{-1}$ at 82 h, carbohydrates supplied carbon over the range of 19 to 43 µg $C \cdot g^{-1} \cdot h^{-1}$ with an average use of 32 µg $C \cdot g^{-1} \cdot h^{-1}$.

Body composition and compositional fuel use

Mean body weight declined from 4.5 to 3.8 g over the 15-day period. The percent water content of the starved fish was significantly higher ($P < 0.005$) than in the controls, though total water content went down (Table 2). The concentration of ash also increased significantly ($P < 0.005$) though again, actual content dropped slightly. Since there was no dietary source of minerals, presumably much of the ash decrease was due to Ca^{2+} release from bone and subsequent loss to the environment. The three fuels all dropped in concentration, though only total carbohydrates and proteins did so significantly ($P < 0.005$ and $P < 0.025$, respectively; Table 2a). The component losses of protein, lipid and carbohydrate by weight were 66.5, 31.1, and 2.4%, respectively (Table 2b), though if the fuels losses are expressed in terms of their carbon component, then the contributions to total fuel by protein, lipid and carbohydrate were 58.4, 40.0, and 1.6%, respectively (Table 2c). Finally, if a nitrogen quotient is calculated from the *predicted* O_2 consumptions and N excretions (based on the measured depletion of fuels), a value of 0.125 results, in contrast to the mean of 0.042 by respirometry. This "*compositional*" NQ then converts to a protein use of 46.3% in contrast to the 15.4% by respirometry (Table 3).

It should be noted that the discrepancy in the percentage contributions of protein to fuel use [58.4% (Table 2c) versus 46.3% (Table 3a)] is *apparent*, rather than real, and results from the different bases used in the two calculations. The prediction arising from the NQ (compositional data, Table 3a) is based on the fate

Table 2 Body compositions (mg · 100mg^{-1}, wet weight) of pre- and post-starvation fish. (a) Total carbohydrates includes glucose, glycogen and lactate. $n = 7$ (controls) or 8 (test fish).

	Lipid	Total carbohydrates	Protein	Inorganics	Water
Prestarvation (controls)	7.6 ± 0.5	0.25 ± 0.03	14.0 ± 0.7	2.40 ± 0.03	75.37 ± 0.33
Poststarvation (test fish)	7.0 ± 0.1	0.14 ± 0.001**	12.3 ± 0.4*	2.68 ± 0.05**	76.67 ± 0.26**
(b) Absolute mass of components (mg), based on average 4.5 g (prestarvation) and 3.8 g (poststarvation) fish.					
Prestarvation	341.2	11.3	628.6	107.8	3384
Poststarvation	264.6	5.3	464.9	101.3	2898
Difference	76.6	6.0	163.7	6.5	486
Total fuel lost (percentage)	31.1	2.4	66.5		
(c) Carbon mass in the fuels (mg).					
Prestarvation	259.3	4.5	326.9	total carbon	
Poststarvation	201.1	2.1	241.8	lost	
Difference	58.2	2.4	85.1	145.7	
Total C lost (%)	40.0	1.6	58.4	100.0	

* $P < 0.025$, ** $P < 0.005$, indicate significant difference from controls. Means ± SEM

Table 3 A comparison of predicted oxygen consumption, nitrogenous waste excretion, nitrogen quotient and protein use from body compositional changes and respirometry

a) Body Composition	Fuels lost (mg)	Caloric equivalent (kcal · g^{-1})	Oxycaloric equivalent (kcal · mol O_2^{-1})	O_2 for combustion mmol	N excreted as waste (mmol)	NQ	Protein (%)
Protein	163.7	5.7	107	8.72	1.98		
Lipid	76.6	9.5	105	6.93	–		
Carbohydrate	6.0	4.0	112	0.21	–		
Total	246.3			15.86	1.98	0.125	46.3
b) Respirometry			O_2 consumption (mmol)	N excretion (µmol)	NQ	Protein (%)	
			9.96	415.1	0.041	15.4	

of the O_2, not on the source of the CO_2 as is the case in Table 2c. Conversion of the depleted carbon masses from Table 2c to total O_2 consumption through their respective RQs, followed by the calculation of NQ, yields a protein usage (50.2%) virtually equivalent to the 46.3% figure; the difference is due to rounding errors in the constants used.

A comparison of the instantaneous and compositional methods for calculating fuel use

The very different conclusions arising from the instantaneous versus compositional methods for calculating fuel utilization (e.g., 15.4% for protein from respirometry versus 46.3–50.2% from the compositional approach) leads to further critical assessment (Table 3). Based on the changes in body composition, it would take 15.9 mmol O_2 to completely burn the substrates. The measured total O_2 consumption was actually 9.96 mmol, 38% lower. A similar internal check of the N budget reveals an even larger discrepancy. Theoretically, a fish should excrete 1.21 mol N for every 100 g protein used [12.1 µmol N · mg protein^{-1}; Kleiber 1987)]. From Table 2b an average 4.5-g fish lost 163.7 mg of protein over 368 h. Therefore, a total of 1.98 mmol N should have appeared in the water, assuming all of it was excreted (Table 3a). However, from the data on N-waste excretion (Fig. 2a), a total of only 415 µmol N was excreted, 79% lower. This unaccounted balance of 1.56 mmol N would have arisen from 129 mg of protein. It is very clear that the two methods yield a fundamentally different picture of the fuel use budget.

Discussion

Critical assessment of theory for fuel use by respirometry

The respirometric approach for determining *instantaneous* fuel use assumes that the fish are in a steady state such that measured fluxes of O_2, CO_2, and N-wastes between the animal and the water are equal to production/consumption rates at the tissues. Given the long duration (15 days) and multiple measurements in the present study, it is highly probable that this condition was satisfied on average. The approach also assumes that only aerobic metabolism is occurring. In this regard, it is noteworthy that Kutty (1972) found mean RQs of *Tilapia mossambica* to be 1.03 during routine metabolism. Since the fish were exposed to high concentrations of O_2, he interpreted the result to mean that about 20% of the CO_2 was being produced anaerobically despite aerobic conditions. Similarly, in this study, fish periodically exhibited spontaneous bouts of apparent anaerobic metabolism (perhaps spontaneous bursts of exercise) reflected in a few RQ values greater than 1.0. Such values were eliminated to satisfy the assumptions of the fuel usage analysis (see Materials and methods). If some of these instances of high RQ were not due to anaerobiosis but rather to a short-term absence of steady state, then the effect of omitting these data would have been to bias the analysis towards lipid (RQ = 0.71) and away from carbohydrate (RQ = 1.0). Protein usage (calculated from NQ) would have been unaffected. Given the surprising finding of this study that carbohydrate use was higher than previously believed (discussed below), the bias would have been a conservative one.

The $RQ_{protein}$ value employed is a matter of critical concern which has not been fully addressed in previous studies. Several studies on fish metabolism have used the ureotelic $RQ_{protein}$ [0.83; Kleiber (1987)] in the calculation of fuel use with the assumption that the different nitrogenous waste products would have no effect on the value (Kutty 1972). It was later demonstrated theoretically that the real $RQ_{protein}$ for ammoniotelic animals was 0.97 (van den Thillart and Kesbeke 1978). However, very few "ammoniotelic" organisms, including trout, excrete exclusively ammonia. In a classic paper, Smith (1929) found urea to be a secondary, though important N-excretory product in teleosts; this has been subsequently confirmed in this study and by many others (e.g. Brett and Zala 1975; Jobling 1981; Kikuchi et al. 1990; Wilkie and Wood 1991; Jayaram and Beamish 1992). Since the amount of carbons and hydrogens excreted with the N are different for ammonia and urea, the $RQ_{protein}$ will change with different proportions (Kleiber 1987):

$$RQ_{protein} = -0.001363U + 0.9729 \quad (11)$$

where U is the percentage of the N-wastes excreted as urea. This relationship assumes ammonia and urea are the only two N-waste products.

While the degree of ammoniotely versus ureotely directly alters the true $RQ_{protein}$, it has no direct effect on the NQ value (0.27) employed for 100% protein metabolism. However, if in fact urea is not coming from protein breakdown, then including urea-N in the NQ will tend to overestimate protein usage. In general, it is likely that under the resting, aerobic conditions of the present experiments, net adenylate and nucleic acid breakdown would have been small, and that most urea-N would have come from protein metabolism. Even though most of the urea likely arises from uricolysis of purines (Van Waarde 1983), the N in the purines are thought to arise originally from protein (Forster and Goldstein 1969). Walton and Cowey (1982) concluded that purine N is derived from glycine, aspartate and glutamate, and that uricolysis is important in linking amino acid degradation to urea synthesis. Urea may also be synthesized from arginine [via arginase; Kaushik (1980)]. Overall, it appears probable that most of the excreted urea arose from protein catabolism, so the NQ was the more accurate index of protein usage than the ammonia quotient (AQ), a point also emphasized by Kutty (1978). In the extreme scenario that no waste products other than ammonia were associated with protein breakdown, then an overestimate of protein use would have resulted. Again the bias would have been conservative, as another surprising finding of the present study was the relatively low reliance on protein as a fuel (discussed below).

Other nitrogenous waste products do exist [e.g., creatine, creatinine, trimethylamine oxide, amino acids; Forster and Goldstein (1969)], though Olson and Fromm (1971) demonstrated for 50-g *Oncorhynchus mykiss* that urea, T_{amm} and water-borne protein (not a metabolic end product) accounted for essentially all the N excreted to the water. Jayaram and Beamish (1992) found urea and T_{amm} to account for all (within statistical limitations) of the excreted N in 200-g lake trout, *Salvelinus namaycush*. Other end products then, at least under non-extreme conditions, appear quantitatively negligible.

Instantaneous fuel use during starvation

The surprising conclusions from the *instantaneous* approach were that protein usage was much lower (14–24%) and carbohydrate usage much higher (20–37%) during starvation than traditionally believed (see Introduction). This belief has been based on the *compositional* approach, which is supported by the present compositional measurements (Table 2). However, there exists previous instantaneous evidence of lower protein usage which has been generally overlooked. For example, in his review on ammonia pro-

duction by fish, van Waarde (1983) presented a summary of reported ammonia quotients, noting that "the contribution of protein catabolism to energy metabolism is . . . over 40%". Presumably this was phrased in such a way as to reflect conventional thought. An equally true statement about the summary would be that "four of the five reports show that the contribution of protein catabolism to energy production ranges from 14–45%". Particularly noteworthy are relatively low values in other salmonids: 19–36% in starved sockeye salmon *Oncorhynchus nerka* (Brett and Zala 1975) and about 26% in fasted Atlantic salmon *Salmo salar* (Wiggs et al. 1989). In contrast, protein utilizations over 65% appear to be the norm in various *Tilapia* spp. (Kutty 1972; Wood et al. 1994).

Van den Thillart (1986) employed a partial version of the *instantaneous* approach (MO_2 + MCO_2 measurements only) to calculate that fasted rainbow trout used an instantaneous fuel mix of 80% protein and 20% lipid, based on a measured RQ of 0.91. Almost exactly the same RQ was measured in the present study (Fig. 1C), but with a very different conclusion. Van den Thillart (1986) assumed a complete absence of carbohydrate use in his calculation, based on the observation that exogenous glucose (administered via catheter) was not metabolized, and the report of Black et al. (1962) that glycogen levels in the liver and muscle remained constant during moderate exercise. However, constant glycogen reserves may be representative of a maintenance level turnover, not necessarily a lack of turnover (cf. Bever et al. 1981). Had N excretion been measured, and the possibility of carbohydrate usage not been excluded, it is quite possible van den Thillart (1986) might have reached conclusions similar to those of the present study.

MO_2 declined more or less linearly with time during starvation (Fig. 1a) while MN exhibited three distinct phases, but no significant difference from the starting value after 15 days (Fig. 2a). These results are very similar to those of Brett and Zala (1975) for sockeye salmon starved over 22 days; both *instantaneous* studies thereby indicate a progressively increasing reliance on protein during starvation, though at much lower percentages than assumed from *compositional* studies. An increased dependence on proteins during starvation in mammals is known (Walton and Cowey 1982) and the trend is also now apparent in fish, though only in a relative, not an absolute sense (Fig. 3a,b).

It should be noted that while this and other effects seen in the present study have been attributed to starvation, it remains possible that other factors may have contributed. For example, the control data have been taken from fish at the start of the starvation period, rather from a group fed throughout the 15-day period, because of the impracticality of feeding the fish in the respirometers. It is therefore possible that the effects seen in the starved fish reflect the influence of long term confinement in the respirometers and/or greater age, in addition to that of starvation alone.

The metabolism of protein C transiently increased (Fig. 3b) during the midphase of the 15-day starvation in concert with the rise in MN (Fig. 2a) and the decline in lipid C usage at this time. However, a rise in carbohydrate metabolism proved to be the more substantial and persistent replacement (up to ~40% of total fuel usage) for the drop in lipid metabolism (Fig. 3b). In contrast, *compositional* studies have generally dismissed carbohydrate metabolism as negligible during starvation, a conclusion with which the present *compositional* data are in accord (contribution ~2%; Table 2). Furthermore whereas respirometry indicated that protein oxidation contributed only 14–24% of MO_2, measured protein depletion suggested a protein contribution of about 50% over 15 days of starvation. A likely explanation for a disagreement of this magnitude is that proteins, while supplying a basal level of carbon for direct entry into the Krebs cycle, also underwent gluconeogenesis (and perhaps lipogenesis). In the kelp bass *Paralabrax*, Bever et al. (1981) found a rapid rate of disappearance of injected labelled alanine with a concomitant increase in labelled glucose. Gluconeogenesis with a corresponding turnover of glycogen stores (Driedzic and Hochachka 1978) may contribute a significant portion of the discrepancy.

Discrepancies between instantaneous and compositional estimates of fuel usage during starvation

The test fish in this study used 38% less O_2 than predicted from the measured depletion of fuels (Table 3a). A similar problem was encountered by Krueger et al. (1968) who found that the caloric value of the material losses in rapidly swimming coho salmon (*Oncorhyncus kisutch*) was about threefold higher than predicted from literature values of MO_2. In the current study, the predicted cumulative MO_2 was calculated on the assumption that the differences measured in body composition all went directly towards powering metabolism. As explained in the previous section, it is possible that much of the protein was converted to glucose before being catabolized for energy. If this in fact occurred (as indicated by the respirometry), a substantial overestimate of cumulative MO_2 via the compositional method would have occurred since the caloric equivalent of carbohydrate is 30% less than that of protein. An additional possible reason for the discrepancy is outlined below, namely the excretion of fuels (e.g., protein) without oxidation. This would also explain the dilemma faced by Krueger et al. (1968).

A fivefold surplus of N excretion was predicted from the body composition data over that actually measured via respirometry (Table 3). A very likely source of some of the "missing" N was direct excretion as protein or

amino acids. Indeed, Olson and Fromm (1971) reported that 25% of all N-waste excretion in rainbow trout (an amount equal to the urea excretion) occurred in the form of protein, presumably as a component of mucus. Bever et al. (1981) followed the disappearance of radio-labelled amino acids and found that some of the amino acids were in fact incorporated into the mucus. This, together with any amino acid excretion which occurred, could have accounted for a substantial portion of the N discrepancy between the respirometry data and the protein depletion data. It would also help explain the discrepancy between the respirometry data for total O_2 consumption and the total fuel depletion, because excreted proteins and amino acids would be measured as fuel depletion, but of course would *not* have been associated with O_2 consumption.

The other possible reason for discrepancies between respirometry data and fuel depletion data is experimental error, which was undoubtedly greater for the depletion measurements. Firstly, because of technological limitations, it is impossible to measure body composition on the *same* fish at the beginning and end of starvation; differences in composition between two different groups of fish must be used. In contrast, respirometry data were collected from the same fish throughout the experiment. Secondly, the measurement of body weight changes is critical, but for fish of the size used in the present study, it was not practical to measure weight with an accuracy of more than 0.1 g (two significant figures) without causing undue stress to the fish through excessive drying. This was 14% of the average body weight change (0.7 g)!

In conclusion, the *instantaneous* method, based on respirometry, and the *compositional* method, based on depletion, yield different answers because they measure different processes. The former indicates the mix of substrates which are actually being oxidized at that point in time, whereas the latter measures net losses from the fish. Since fish have a great ability to convert their proteins to carbohydrates and lipids, and may excrete substantial amounts of protein unmetabolized, neither type of fuel use alone can be used as a predictor of the other. The compositional approach is probably best suited for very long term ecophysiological studies employing large numbers of fish to minimize variability. The instantaneous approach based on respirometry appears far more suitable for short term physiological studies.

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References

Barton BA, Weiner GS, Schreck CB (1985) Effect of prior acid exposure on physiological responses of juvenile rainbow trout (*Salmo gairdneri*) to acute handling stress. Can J Fish Aquat Sci 42: 710–717

Beamish FWH (1978) Swimming capacity, In: Hoar W, Randall DJ (eds) Fish physiology, vol VII. Academic Press, New York, pp 101–187

Bergmeyer HU (1985) Methods of enzymic analysis. Academic Press, New York

Bever K, Chenoweth M, Dunn A (1981) Amino acid gluconeogenesis and glucose turnover in kelp bass (*Paralabrax* sp.). Am J Physiol 240: R246–R252

Black EC, Robertson AC, Lam K-C, Chiu W-G (1962) Changes in glycogen, pyruvate and lactate in rainbow trout (*Salmo gairdneri*) during and following muscular activity. J Fish Res Board Canada 19: 409–436

Boutilier RG, Heming TA, Iwama GK (1984) Physico-chemical parameters for use in fish respiratory physiology, In: Hoar W, Randall DJ (eds) Fish physiology, vol 10A. Academic Press, New York, pp 401–430

Brett JR, Zala CA (1975) Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. J Fish Res Board Can 32(12): 2479–2486

Driedzič WR, Hochachka PW (1978) Metabolism in fish during exercise, In: Hoar W, Randall DJ (eds) Fish physiology, vol VII. Academic Press, New York, pp 503–543

Duncan DW, Tarr HLA (1958) Biochemical studies on sockeye salmon during spawning migration III Changes in the protein and non-protein nitrogen fractions in muscles of migrating sockeye salmon. Can J Biochem Physiol 36: 799–803

Forster RP, Goldstein L (1969) Formation of excretory products, In: Hoar W, Randall DJ (eds) Fish physiology, vol I. Academic Press, New York, pp 313–350

Idler DR, Clemens WA (1959) The energy expenditures of Fraser River sockeye salmon during the spawning migration to Chilko and Stuart Lakes. International Pacific Salmon Fisheries Commission, New Westminster

Jayaram MG, Beamish FWH (1992) Influence of dietary protein and lipid on nitrogen and energy losses in lake trout, *Salvelinus namaycush*. Can J Fish Aquat Sci 49: 2267–2272

Jezierska B, Hazel JR, Gerking SD (1982) Lipid mobilization during starvation in the rainbow trout, *Salmo gairdneri* Richardson, with attention to fatty acids. J Fish Biol 21: 681–692

Jobling M (1981) Some effects of temperature, feeding and body weight on nitrogenous excretion in young plaice *Pleuronectes platessa* L. J Fish Biol 18: 87–96

Johnston IA, Goldspink G (1973) Some effects of prolonged starvation on the metabolism of the red and white myotomal muscles of the plaice *Pleuronectes platessa*. Mar Biol 19: 348–353

Kaushik SJ (1980) Influence of nutritional status on the daily patterns of nitrogen excretion in the carp (*Cyprinus carpio* L) and the rainbow trout (*Salmo gairdneri* R). Reprod Nutr Dev 20: 1751–1765

Kikuchi K, Takeda S, Honda H, Kiyono M (1990) Oxygen consumption and nitrogenous excretion of starved Japanese flounder. Nipp Suis Gak 56: 1891

Kleiber M (1987) The fire of life. Krieger, Malabar

Kleiber M (1992) Respiratory exchange and metabolic rate In: Geiser SR (ed) Handbook of physiology. Am Physiol Soc, Bethesda, pp 927–938

Krueger HM, Saddler JB, Chapman GA, Tinsley IJ, Lowey RR (1968) Bioenergetics, exercise and fatty acids of fish. Am Zool 8: 119–129

Kutty MN (1968) Influence of ambient oxygen on the swimming performance of goldfish and rainbow trout. Can J Zool 46: 647–653

Kutty MN (1972) Respiratory quotient and ammonia excretion in *Tilapia mossambica*. Mar Biol 16: 126–133

Kutty MN (1978) Ammonia quotient in sockeye salmon (*Oncorhynchus nerka*). J Fish Res Board Can 35: 1003–1005

Miller GL (1959) Protein determination on larger sample sizes. Anal Chem 31: 964

Mommsen TP, French CJ, Hochachka PW (1980) Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. Can J Zool 58: 1785–1799

Olson KR, Fromm PO (1971) Excretion of urea by two teleosts exposed to different concentrations of ambient ammonia Comp Biochem Physiol 40A: 999–1007

Rahmatullah M, Boyd TRC (1980) Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. Clin Chem Acta 107: 3–9

Robinson JS, Mead JF (1973) Lipid absorption and deposition in rainbow trout (*Salmo gairdneri*). Can J Biochem 51: 1050–1058

Smith HW (1929) The excretion of ammonia and urea by the gills of fish. J Biol Chem 81: 727–742

Sukumaran N, Kutty MN (1977) Oxygen consumption and ammonia excretion in the catfish *Mystus armatus*, with special reference to swimming speed and ambient oxygen. Proc Indian Acad Sci 86B: 195–206

Van den Thillart G (1986) Energy metabolism of swimming trout (*S. gairdneri*). J Comp Physiol B 156: 511–520

Van den Thillart, G Kesbeke F (1978) Anaerobic production of carbon dioxide and ammonia by goldfish *Carassius auratus* (L.). Comp Biochem Physiol 59A: 393–400

Van Waarde A (1983) Aerobic and anaerobic ammonia production by fish. Comp Biochem Physiol 74B: 675–684

Verdouw H, Van Echted CJA, Dekkers EM (1978) Ammonia determination based on indophenol formation with sodium salicylate. Water Res 12: 399–402

Virtanen E, Forsman L (1987) Physiological responses to continuous swimming in wild salmon (*Salmo salar* L.) parr and smolt. Fish Physiol Biochem 4: 157–163

Walton MJ, Cowey CB (1982) Aspects of intermediary metabolism in salmonid fish. Comp Biochem Physiol 73B: 59–79

Wiggs AJ, Henderson EB, Saunders RL, Kutty MN (1989) Activity, respiration, and excretion of ammonia by Atlantic salmon (*Salmo salar*) smolt and postsmolt. Can J Fish Aquat Sci 46: 790–795

Wilkie MP, Wood CM (1991) Nitrogenous waste excretion, acid-base balance, and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. Physiol Zool 64: 1069–1086

Wood CM, Bergman HL, Laurent P, Maina JN, Narahara A, Walsh PJ (1994) Urea production, acid-base regulation, and their interactions in the Lake Magadi *Tilapia*, a unique teleost adapted to a highly alkaline environment. J Exp Biol 189: 13–36

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