The Journal of Experimental Biology 207, 1993-2002
Published by The Company of Biologists 2004
doi:10.1242/jeb.00901

Dogmas and controversies in the handling of nitrogenous wastes: The effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout

Makiko Kajimura*, Sara J. Croke, Chris N. Glover and Chris M. Wood
Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada, L8S 4K1
*Author for correspondence (e-mail: kajimur@mcmaster.ca)

Accepted 23 January 2004

Summary

Ammonia and urea are the primary forms of nitrogen excretion in teleost fish. There exists, however, a discrepancy between the sum of ammonia plus urea nitrogen and total nitrogen, indicating that 'unknown' nitrogen end products may play an important role in nitrogen metabolism. The current study analysed a wide range of nitrogen end products in both fed and fasted juvenile rainbow trout. Ammonia-N (53–68%) and urea-N (6–10%) were confirmed as the most important forms of nitrogenous waste, but an interesting finding was the considerable excretion of nitrogen as amino acids (4–10%), *via* the gills, and as protein (3–11%), probably *via* the body mucus. Use of anal sutures delineated an important role for the gastrointestinal tract in the production of ammonia-N and urea-N in fed fish, but amino acid-N and protein-N output by this route were both negligible. Alternative nitrogen products – trimethylamine, trimethylamine oxide, uric acid, and nitrite + nitrate – were not excreted in detectable quantities. Creatine-N and creatinine-N outputs were detected but contributed only a small fraction to total nitrogen excretion (<1.4%). Despite the wide scope of nitrogenous end products investigated, a considerable proportion (12–20%) of nitrogen excretion remains unknown. Possible alternative end products and methodological considerations are proposed to explain this phenomenon. The findings described above were used to recalculate the nitrogen quotient (NQ=$\dot{M}_N/\dot{M}_{O_2}$) on trout that had been either fasted or fed various daily rations (1%, 3% or 5% dry food per unit wet body mass per day). Feeding increased oxygen consumption ($\dot{M}_{O_2}$) and total-N excretion ($\dot{M}_N$). The NQ is often used as a measure of protein utilisation in aerobic metabolism and assumes that all protein (and amino acid) fuels are converted by oxidation to nitrogenous waste products that are excreted. However, the results showed that calculation of the NQ based on total nitrogen excretion may overestimate protein utilisation in aerobic metabolism because of significant excretion of N in the form of proteins and amino acids, whereas the use of summed ammonia-N and urea-N excretion probably underestimates the contribution of protein towards aerobic metabolism. These errors increase as ration increases, because the discrepancy between total-N excretion and ammonia-N + urea-N excretion increases.

Key words: amino acid, protein, nitrogen excretion, fish, nitrogen quotient.

Introduction

Protein is used as a metabolic fuel by fish, although its quantitative importance remains somewhat controversial (reviewed by Wood, 2001). Regardless, the consequence of protein oxidation is the production of nitrogenous waste, which is excreted predominantly as ammonia together with a small amount of urea in teleost fish. The literature regarding modes of nitrogen metabolism and nitrogenous waste excretion has been extensively reviewed in a number of recent papers (Mommsen and Walsh, 1991, 1992; Wood, 1993; Korsgaard et al., 1995; Walsh, 1997). These reviews have primarily focused on the metabolism, excretion and toxicity of ammonia and urea, the major end products of nitrogen metabolism. However, it is well documented that the total nitrogen excretion, measured with a nitrogen oxidizer or by Kjeldahl digestion, is much higher than the combined ammonia plus urea concentrations (Smith, 1929; Wood, 1958; McCarthy and Whitledge, 1972; Beamish and Thomas, 1984; Cockcroft and Du Preez, 1989; Walsh et al., 2001; De Boeck et al., 2001). These published reports, and our own preliminary experiments, have found that this discrepancy may account for 10–60% of the total nitrogen excretion rate and is dependent on species, food condition and temperature (reviewed by Wood, 1995, 2001). For example, Walsh et al. (2001) reported that this discrepancy between ammonia plus urea concentrations *versus* total nitrogen levels is more prominent in feeding than in fasting midshipmen (*Porichthys notatus*).

The implication of these findings is that a considerable amount of excreted nitrogen is likely to be found in waste products other than ammonia and urea. While the importance of alternative nitrogen products such as creatine, creatinine, trimethylamine (TMA), trimethylamine oxide (TMAO), uric acid, amino acids and protein has been investigated sporadically, there has been no thorough, systematic examination of these compounds in a single set of experiments. The primary goal of the current research was to determine the nature and relative importance of these minor nitrogen end products in both fed and fasting rainbow trout.

The breakdown of protein, as with all fuels, is associated with an increase in metabolic rate as oxygen is consumed. Therefore, the ingestion of food is followed by an increase in metabolic rate in most animals (Kleiber, 1961; Garrow, 1974). According to standard metabolic theory (Kleiber, 1992), the excretion of waste nitrogen ($\dot{M}_N$) can be related to the consumption of oxygen ($\dot{M}_{O_2}$) to provide a quantitative measure of protein utilisation, the nitrogen quotient ($NQ=\dot{M}_N/\dot{M}_{O_2}$). From knowledge of the typical composition and metabolism of fish protein, an NQ of 0.27 represents the condition in which aerobic respiration is fueled entirely by protein (van den Thillart and Kesbeke, 1978), so under experimental conditions, the percent of metabolism fueled by protein oxidation can be calculated by the ratio of the measured NQ to 0.27. Traditionally, NQ has been calculated from the sum of ammonia-N + urea-N excretion. On this basis, protein has been estimated to contribute 14–36% in fasted salmonids (Brett and Zala, 1975; Wiggs et al., 1989; Lauff and Wood, 1996a,b; Alsop and Wood, 1997; Kieffer et al., 1998), with similar values usually determined in other species (16–34%; Kutty and Peer Mohamed, 1975; Jobling, 1980; Alsop et al., 1999). Values may increase greatly, sometimes close to 100%, in actively feeding fish (reviewed by Wood, 2001).

This calculation assumes that all the nitrogen excretion products result from the oxidation of protein. This is a valid conclusion when examining the production of ammonia and urea, both of which are oxidized waste products. However, as stated above, it is likely that a significant proportion of nitrogen excretion is achieved by alternative N-forms, some of which may not be oxidized. Considerable excretion of amino acids and protein, for example, could greatly influence the nitrogen output of the fish, without having contributed to oxygen consumption. Alternatively, there may be significant excretion of other oxidized N-products that are missed by the measurement of only ammonia-N + urea-N. The second focus of the present research was to critically re-examine measures of protein utilisation, using the knowledge gained regarding the nature and concentration of nitrogen excretory products determined under the primary research aim.

Materials and methods

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 18–49 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada). Fish were kept at 11±2°C in dechlorinated Hamilton tap water (hardness 140 mg l^{-1} as $CaCO_3$, pH 7.6, $[Ca^{2+}]$ 1.0 mmol l^{-1}, $[Mg^{2+}]$ 0.2 mmol l^{-1}, $[Na^{+}]$ 0.6 mmol l^{-1}, $[Cl^{-}]$ 0.7 mmol l^{-1}) for at least two weeks prior to experimentation under a constant photoperiod of 16 h:8 h L:D . They were maintained on a commercial trout feed (Silver Cup, Nelson & Sons, Inc., Murray, UT, USA). In series 1, fish were fed 1% of their body mass every other day for two weeks. In series 2, fish were fed a ration of 3% of their body mass every day for two weeks, with half of the food being given in the morning and the other half in the evening. The higher ration and more frequent feeding in series 2 was to prepare the fish for an experimental variation in daily ration, as well as for a longer period of starvation.

Experimental protocols

Series 1 – quantification of the components of nitrogen excretion

After the initial holding period, juvenile rainbow trout (19–49 g) were anesthetised by immersion in neutralised tricaine methanesulphonate (MS-222; 100 mg l^{-1}), weighed and then transferred individually to aerated containers (22 cm×22 cm) filled with 2 liters of dechlorinated Hamilton tap water (Day 0) thermostatted to the acclimation temperature. This holding water was changed every other day. Individual fish were randomly allocated into one of four groups: sutured fasting (N=16), sutured feeding (N=16), unsutured fasting (N=12) and unsutured feeding (N=12). Feeding groups were fed 2% of their body mass every other day, while fasting fish were not fed at all. On Day 9, fish designated to the sutured groups were anesthetised by neutralised MS-222 and the anus was sutured closed with a needle and silk thread (Ethicon, Somerville, NJ, USA). On day 10, fish in both sutured and unsutured feeding groups were fed 2% of their body mass as usual. The sutured fish ate normally, and virtually all food was consumed by both groups. After 4 h, both groups of sutured fish were placed individually in clean containers with 1.5 liters of dechlorinated Hamilton tap water for 24 h, and samples for nitrogen analysis were collected. Unsutured groups were transferred to identical clean conditions at the same time, and again water samples were taken for nitrogen analysis. For both unsutured groups, water samples were filtered through cheese cloth to remove any feces. Water samples were taken at the beginning and end of the 24-h period and frozen at –20°C for later analysis of nitrogen waste products.

Series 2 – respirometry

After the initial holding period, juvenile rainbow trout (18–43 g) were divided into three tanks (211 liters each, N=104 per tank) and fed a ration of 1%, 3% or 5% body mass day^{-1} for 7 days. A further tank contained fish that were initially fed on the 3% ration but thereafter starved for 13 days. On day 8 for fed fish and day 14 for the fasting group, a subset of fish were placed in Blazka-style swimming respirometers (3 liter) under flow-through conditions. Thus, the fed fish had been starved since the previous evening, ~12 h). The water velocity was set to 10 cm s^{-1}, a speed at which the fish would maintain

orientation but not swim actively. Fish were allowed to settle for one hour, then water flow was shut off while the current was maintained, and initial water samples were taken. The oxygen partial pressure (P_{O_2}) of the water in the respirometer was measured sequentially until saturation had dropped to ~70%, using a temperature-controlled Cameron E101 oxygen electrode connected to a Cameron OM-200 O_2 meter. The appropriate solubility coefficients from Boutilier et al. (1984) were used to convert P_{O_2} to O_2 concentration. Thereafter, aeration was resumed but the system remained closed for 3 h, when final samples were taken. Fish were then removed and weighed. The water samples taken at the beginning and end of the 3-h period were frozen at –20°C for later analysis of nitrogen waste products (total nitrogen, ammonia and urea).

Analysis of different nitrogen products

Total nitrogen (total-N) was analysed on an Antek 7000V nitrogen analyser using ammonium sulfate as a standard. The efficiency with which the Antek 7000V nitrogen analyser detected N in various compounds was assessed using 50 μmol l^{-1} ammonia-N [25 μmol l^{-1} $(NH_4)_2SO_4$] as a reference standard, with the unknowns made up in the same concentration range. Similarly, the degrees of detection of the same compounds by the ninhydrin reagent used for total amino acid analysis and the salicylate/hypochlorite assay used for ammonia analysis were also evaluated.

Protein concentration in water samples was measured by the dye-binding method of Bradford (1976) using Sigma reagent and bovine serum albumin (Sigma) as standards. Special attention was devoted to protein because it was incompletely detected by the total nitrogen analyser (see Results; Table 1) and also because it may precipitate out of solution. To solve the analytical problem, all protein was removed from water samples prior to total-N analysis. This was achieved by acidification (12 μl of 5 mol l^{-1} HCl, to 3 ml water sample) followed by ultrafiltration at 5000 ***g*** at 4°C, using Microsep™ Centrifugal devices with a 1000 Da molecular mass cutoff, and finally neutralisation of the filtrate with 5 mol l^{-1} KOH prior to total-N analysis. Tests demonstrated that protein removal was >95%. The N concentration determined on the total nitrogen analyser was then added to protein-N measured by the Bradford assay to yield total-N.

To deal with the precipitation problem, several preliminary tests were performed. We found that protein was often stratified in the water within the experimental containers. Therefore, in the standard measurement protocol, all the water was collected and thoroughly stirred in a large beaker prior to sub-sampling and analysis. We were also concerned that some excreted proteins may adhere to the wall of the experimental container and therefore not be detected in the water samples. Containers were therefore rinsed with 1 mol l^{-1} KOH (20 ml) and then wiped with KOH-soaked filter paper to remove container-bound protein. This fraction was analysed separately for protein.

The ammonia content of the water was measured by the salicylate/hypochlorite method (Verdouw et al., 1978) using $(NH_4)_2SO_4$ standards, and the amino acid content of the water was measured by ninhydrin assay (Moore, 1968), using Sigma reagent and glycine as a standard. However, it was found that these methods partially detect both ammonia and amino acids (see Results; Table 1). To determine actual ammonia and amino acid levels, the following treatment was performed. KOH (5 mol l^{-1}, 25 μl) was added to a 5-ml water sample. This basic water sample (pH=12.3) was then aerated for 4 h to remove volatilised ammonia, to give an ammonia-free water sample. This sample was then neutralised by HCl and analysed for amino acid-N by the ninhydrin method. Ammonia-N content was calculated as the difference between untreated and ammonia-free samples by the salicylate-hypochlorite method.

Urea-N levels were measured with the diacetyl-monoxime method (Rahmatullah and Boyde, 1980). The sum of nitrite and nitrate levels was measured with the hydrazine sulfate method (Rand, 1975).

Creatinine, creatine, TMA, TMAO and uric acid levels in water samples were very low. To increase the resolution of these nitrogenous products, water samples (generally 50 ml) were concentrated using a rotary evaporator and then reconstituted in a small volume of distilled water (1.2 ml). As samples are concentrated by evaporation, the remaining solution becomes basic and may promote precipitation of nitrogenous waste products. Prior to evaporation, 1 mol l^{-1} HCl (100:l) was added to maintain an acidic solution and promote N solubilisation. Creatinine levels were measured using a Sigma Diagnostics kit (No. 555). Creatine was converted to creatinine by a modification of the method of Smith (1929). Creatine content was calculated as the difference between samples of newly converted creatinine and creatinine levels in unmodified samples. TMA and TMAO levels were measured by the ferrous sulfate and EDTA method (Wekell and Barnett, 1991). Uric acid was measured using the appropriate Sigma Diagnostics kit (No. 686).

Calculations

The concentration of the various nitrogen end products was converted to molar concentrations of N in the traditional manner. For example, urea excretion was multiplied by 2 to yield urea-N, creatinine and creatine were multiplied by 3 to yield creatinine-N and creatine-N, respectively. Based on recovery tests, a small correction factor was applied to the ninhydrin measurement of total amino acid-N, as explained in the Results. Protein standard in Sigma bovine serum albumin contains 0.155–0.165 g N g^{-1}. For the purposes of this study, protein-N content was regarded as 0.16 g N g^{-1} (i.e. ~731 moles N per mole BSA). Total-N was the value determined by the nitrogen analyser on deproteinised samples with the addition of separately measured protein-N, for the reason described above. Unknown nitrogen was total-N minus the sum of all forms of measured N.

For the respirometry experiments of series 2, $\dot{M}_{O_2}$ was calculated in the traditional manner from the change in molar concentration of oxygen in the water, factored by individual fish mass, time and respirometer volume. Nitrogen quotient (NQ) was calculated as the ratio of the nitrogen excretion rate

Table 1. *Detection efficiency (%)*

	Nitrogen	Concentration (μmol l^{-1})	Concentration (μmol l^{-1} N)	Ninhydrin method (%)	Salicylate/ hypochlorite method (%)	Total nitrogen analyser (%)
Glycine	1	50	50	100	20	97
Ammonia	2	25	50	82	100	100
Taurine	1	50	50	102	3	103
Asparagine	2	50	100	26	3	99
Glutamine	2	50	100	55	5	95
Lysine	2	50	100	52	3	84
Histidine	3	50	150	37	4	104
Tryptophan	2	50	100	48	7	96
Arginine	4	50	200	26	2	101
β-Alanine	1	50	50	52	8	93
π-Methyl-L-histidine	3	50	150	32	3	82
Carnosine	4	50	200	21	2	99
Anserine-HNO_3	5	50	250	8	0	99
Urea	2	50	100	1	2	101
Protein	1	0.16	114.3	7	1	75
Creatine	3	50	150	1	0	91
Creatinine	3	50	150	1	0	100
$NaNO_2$	1	50	50	1	1	94
$NaNO_3$	1	50	50	0	1	94

Ammonia (salicylate/hypochlorite and nitrogen analyser assays) and glycine (ninhydrin assays) were used as standard solutions for each analysis. All solutions were analysed at a concentration of 50 μmol l^{-1}, with the exception of the following: ammonia [$(NH_4)_2SO_4$] solution, which was analysed at 25 μmol l^{-1} (50 μmol l^{-1} N) and protein standard (bovine serum albumin: 0.16 μmol l^{-1}; 114.3 μmol N l^{-1}).

($\dot{M}_N$) to $\dot{M}_{O_2}$. This was calculated using either total-N excretion rate, or ammonia-N + urea-N excretion rate, or the excretion rate of the (estimated) sum of all oxidized nitrogen compounds (all N-compounds, including unknown-N but specifically excluding protein-N and amino acid-N) as the nitrogen products of interest. The percentage contribution of protein as an aerobic fuel source was then calculated using the following formula (van den Thillart and Kesbeke, 1978):

$$P = NQ/0.27 ,$$

where P is the fraction supplied by protein of the total fuels supporting $\dot{M}_{O_2}$, and 0.27 is the theoretical maximum for NQ.

Statistics

All values are expressed as means ± S.E.M. In series 1, an independent *t*-test was used to determine significance in nitrogen excretion between fasting and feeding and between sutured and unsutured groups. A one-way analysis of variance (ANOVA) followed by the LSD test was used to test significant differences in oxygen consumption and nitrogen excretion between groups of fish in series 2. For all analyses, $P<0.05$ was considered significant.

Results

Specificity and recovery of analytical techniques

The Antek 7000 total nitrogen analyser gave good recovery, close to 100%, for a selection of amino acids, dipeptides, urea, creatine and creatinine in the μmol l^{-1} N range (Table 1). Lysine and π-methyl-L-histidine recoveries were 80–85%, but the most notable exception was protein-N, for which recovery was only 75%. As this was suspected to potentially be an important component of the excreted N, protein was removed from samples prior to total-N analysis and was measured separately. An additional potential error could be failure to measure proteins that lost solubility and adhered to the surfaces of the container. As shown in Table 2, the protein-N levels that adhered to the surface were very small in all the groups, accounting for only 0.1–0.3% of the total protein-N excretion rate. This fraction was therefore not considered separately in this study, and hence the protein value in Table 3 represents the sum of protein both in water and adhered to container walls.

The salicylate/hypochlorite assay (Verdouw et al., 1978)

Table 2. *Distribution of protein within holding containers in Series 1, calculated as N-excretion rates (nmol N g^{-1} h^{-1})*

		Water	Container walls	%	*N*
Sutured fish	Fasted	29.08±4.23	0.04±0.01	0.14	16
	Fed	83.76±9.40	0.08±0.02	0.10	16
Unsutured fish	Fasted	15.63±1.69	0.05±0.01	0.32	12
	Fed	41.90±5.53	0.03±0.01	0.07	12

% represents the proportion of total protein-N bound to container walls, under each experimental condition.

Table 3. *A comparison of nitrogen excretion rates in Series 1 between trout that were sutured or unsutured and between fasted and fed treatments (nmol N g^{-1} h^{-1})*

		Sutured (*N*=16)	Unsutured (*N*=12)
Fasted	Total-N	529.7±35.1	499.2±35.3
	Ammonia-N	360.0±25.5	331.3±37.3
	Urea-N	45.7±4.5	51.2±5.7
	Amino acid-N	26.4±3.6	20.7±4.5
	Protein-N	29.1±4.2	15.7±1.7**
	Creatinine-N	1.9±0.1	2.1±0.2
	Creatine-N	2.8±0.6	1.2±0.3*
	Unknown-N	63.7±8.1	76.9±15.4
Fed	Total-N	751.3±48.3†††	1008.9±78.3**,†††
	Ammonia-N	396.9±34.3	628.0±56.5**,†††
	Urea-N	42.1±3.8	75.7±5.6***,††
	Amino acid-N	72.1±14.8††	72.4±11.6†††
	Protein-N	83.8±9.4†††	41.9±5.5***,†††
	Creatinine-N	5.3±0.8†††	11.1±1.8*,†††
	Creatine-N	3.2±0.5	3.2±1.2
	Unknown-N	147.7±22.1††	176.6±41.2†

Values represent means ± S.E.M. Significant differences at the *P*=0.05 (*,†), 0.01 (**,††), 0.001 (***,†††) were tested by *t*-test. *Significantly different from corresponding sutured groups. †Significantly different from corresponding feeding groups.

used for ammonia-N analysis proved to be relatively specific, the only cross-reaction of any note being with glycine-N (20% recovery; Table 1). Nevertheless, to ensure the greatest accuracy, ammonia-N was measured as the difference between untreated and ammonia-purged samples, using the Verdouw et al. (1978) assay. The ninhydrin assay used for amino acid analysis, on the other hand, proved to be relatively non-specific, detecting ammonia-N with 82% efficiency (Table 1). Therefore, this assay was run only on water samples that had been purged of ammonia. In addition, the ninhydrin reaction yielded widely varying recoveries for the N-content in different amino acids (Table 1). In general, the assay appeared to detect only the α-amino group, in accord with theory, and therefore underestimated the N-content for amino acids that contain 2–4 N per molecule. Furthermore, the efficiency of detection of N in β-amino acids was only ~50%. To correct for this, the assumption was made (see Discussion) that the profile (percentage composition) of excreted amino acids would reflect the profile of amino acids in the blood plasma of resting trout, as reported by Wood et al. (1999). By applying the ratio of the detection efficiency for each class of amino acid by the total nitrogen analyser (essentially 100%) to the corresponding detection efficiency by the ninhydrin assay (variable %) and weighting the outcome according to the relative contribution of each amino acid to the profile, a correction factor (×1.226) was derived and applied to the ninhydrin measurement of total amino acid-N. The adjustment is relatively small because of the quantitative predominance of amino acids bearing single α-amino groups in trout plasma.

The excretion rates of certain compounds by the fish were

Table 4. *Detection limits*

	μmol l^{-1}	nmol N g^{-1} h^{-1}
TMA	1.2	2.5
TMAO	1.2	2.5
Uric acid	1.1	0.6
Nitrate + nitrite	0.7	1.6

These nmol N g^{-1} h^{-1} values were calculated on the following basis: 24-h immersion of 30-g rainbow trout in 1500 ml holding water.

TMA (trimethylamine), TMAO (trimethylamine oxide) and uric acid detection limits were calculated on samples that were concentrated 41.67 times. Nitrate + nitrite detection limits (minimum increase detectable above background) were high due to high background levels (3.6 μmol l^{-1}).

below the limits of detection, despite the fact that pre-concentration was employed. These included TMA, TMAO, uric acid, and nitrate + nitrite. In the nitrate + nitrite case, while the background concentration (~3.6 μmol l^{-1} N) was certainly detectable in our water, the increment above this relatively high background was undetectable. Table 4 gives the practical detection limits and demonstrates that the total contribution of each of these compounds to total-N excretion could have been no greater than a few nmol N g^{-1} h^{-1}, trivial relative to measured total-N excretion rates, which routinely exceeded 500 nmol N g^{-1} h^{-1}.

Series 1 – nitrogen excretion rates, feeding and fasting

Based on these tests, unknown-N excretion in series 1 was calculated as:

$$\text{Total-N} - [\text{ammonia-N} + \text{urea-N} + \text{amino acid-N} + \text{creatine-N} + \text{protein-N} + \text{creatinine-N}] .$$

Trout in this experiment had been either fasted for 10 days or fed a 2% ration every other day for the same period.

The excretion rates for total-N, ammonia-N, urea-N, protein-N, amino acid-N, creatine-N, creatinine-N and unknown-N for both fed and fasted trout, with or without anal suturing to prevent fecal N-excretion, are shown in Table 3, while Fig. 1 portrays the relative contributions of each component on a percentage basis. As in most teleosts, the majority of nitrogen waste was excreted as ammonia-N in all the groups (53–68%), and urea-N was the next most important product (6–10%), except in the fed, sutured group where it was surpassed by both protein-N (11%) and amino acid-N (10%). In general, the contributions of protein-N and amino acid-N excretion were approximately equal (3–11% range), while creatinine-N and creatine-N made up <1% each. Thus, unknown-N amounted to 12–20% of the total, while the two products measured traditionally (ammonia-N and urea-N) accounted for ~58–77% of total-N excretion in the various treatments (Fig. 1). The relative contributions of unknown-N, amino-acid-N, protein-N and creatinine-N all tended to increase with feeding, with corresponding decreases in the relative contributions of ammonia-N and urea-N (Fig. 1).

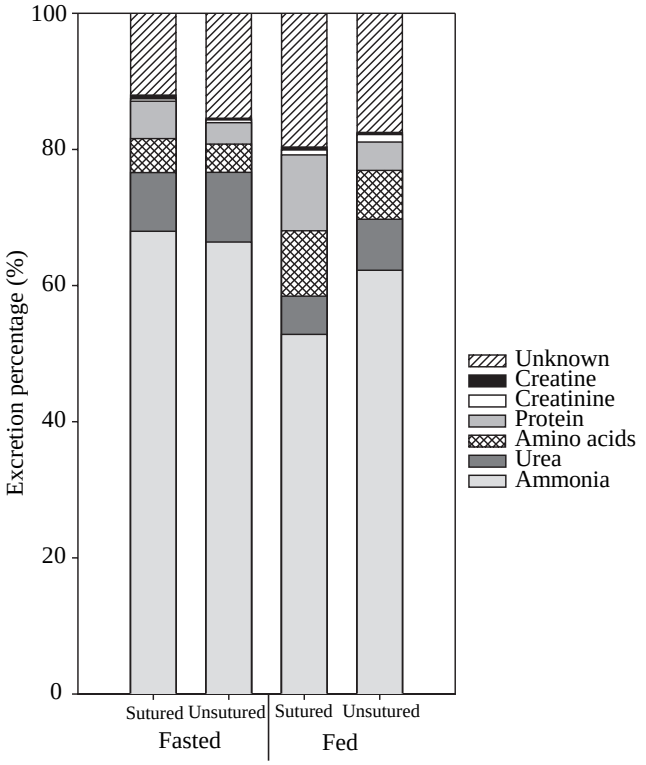

Fig. 1. The relative contributions of different compounds, expressed as a percentage of total nitrogen excretion, in fed or fasted trout of series 1 with or without anal suturing.

In fasted fish, anal suturing had little effect on the pattern or rate of nitrogen excretion, although protein-N excretion and creatine-N excretion were both significantly higher in the sutured group (Table 3). In fed fish, the rates of total-N, ammonia-N, urea-N and creatinine-N excretion were all significantly higher in unsutured *versus* sutured animals, while the reverse was true for protein-N excretion. There was no difference in either amino acid-N or unknown-N excretion between sutured and unsutured fish in either fasting or fed groups.

Feeding clearly increased total-N excretion rate, even when the anus was sutured. For example, in the sutured group, total-N excretion was 1.4 times higher in the fed group than in the fasted group. Amino acid-N, protein-N, creatinine-N and unknown-N excretion rates in sutured fed fish were 2–3 times higher than those in the fasted groups. There were significant differences in total-N, amino acid-N, protein-N, creatinine-N and unknown-N excretion between the fasted and fed sutured groups (Table 3).

In unsutured fish, the rate of total-N excretion in the fed group was two times that of fasted animals. The largest contributor was ammonia-N excretion, which almost doubled. Amino acid-N, protein-N and creatinine-N excretion rates in the fed group were 3–5 times higher than those in the fasted group. There were significant differences in total-N, ammonia-N, urea-N, amino acid-N, protein-N, creatinine-N and unknown-N excretion between the fasted and fed unsutured groups (Table 3).

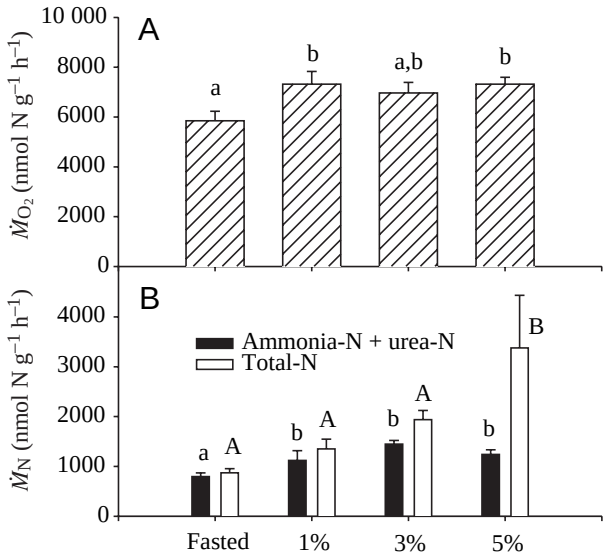

Fig. 2. The effect of food ration on (A) oxygen consumption and (B) nitrogen excretion in trout of series 2. Values are means ± S.E.M. [N=6, except fasted (N=12)]. Means sharing the same letter of the same case are not significantly different from one another (P<0.05).

Series 2 – ration and respirometry

In this series, ammonia-N, urea-N and total-N excretion rates were measured, in addition to oxygen consumption rates, in fish that had been either fasted for 13 days or fed 1%, 3% or 5% daily rations throughout the preceding week. Thus, unknown-N was total-N – [ammonia-N + urea-N].

Fed groups had higher $\dot{M}_{O_2}$ values than the fasted group, a difference that was significant for the 1% and 5% ration groups (Fig. 2). However, $\dot{M}_{O_2}$ did not increase with increasing ration, as there were no significant differences detected among the fed groups. $\dot{M}_N$, when expressed as the sum of ammonia-N + urea-N, followed a very similar pattern to $\dot{M}_{O_2}$, being higher in all fed groups than in the fasted fish, although there were no significant differences among the 1%, 3% or 5% ration treatments. However, when expressed as total-N excretion, $\dot{M}_N$ exhibited a somewhat different pattern, increasing steadily with ration, although only the value at 5% ration was significantly different from the fasted group. Clearly, the proportion attributable to unknown-N excretion products increased with food ration. In fasted fish, the percentage of unknown nitrogen (i.e. not ammonia-N or urea-N) was 9%, and this increased to 17%, 25% and 63%, respectively, in trout fed 1%, 3% and 5% daily rations. The impact on calculated NQ is illustrated in Fig. 3 (see Discussion).

Discussion

In series 1, the profile of nitrogenous excretory products in rainbow trout, under both fasting and feeding regimes, was examined. As with many previous investigations (see Table 5), ammonia-N and urea-N were confirmed as the major forms of nitrogen excretion. In addition, however, the measurement of alternative nitrogen waste products delineated some interesting, previously unreported, patterns.

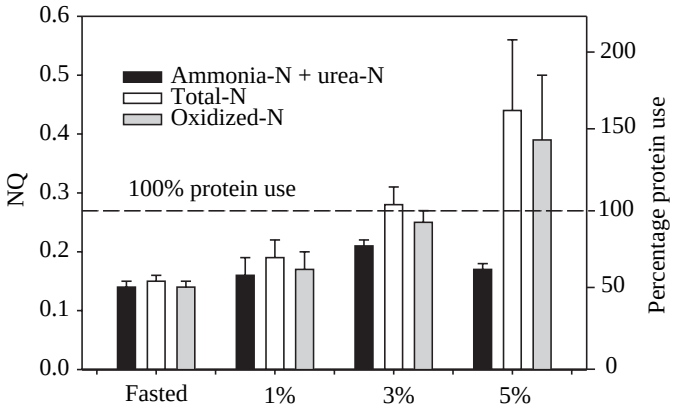

Fig. 3. Nitrogen quotient (NQ; left *y*-axis) and percentage protein use (%; right *y*-axis) for fasted and fed fish of series 2, calculated on the basis of three different assumptions (see Discussion for details). Values are means ± S.E.M. [*N*=6, except fasted (*N*=12)].

One significant discovery was the considerable excretion of nitrogen in the form of amino acids (Table 3). We employed the ninhydrin method of Moore (1968) to detect amino acids *en masse*, and the detection efficiencies that we recorded at quite low levels (50 μmol l^{-1}) were very comparable with those reported by other authors (Moore and Stein, 1948, 1954; Fisher et al., 2001). The assay detects only α-amino nitrogen groups with full efficiency; consequently, the ninhydrin assay will underestimate the nitrogen content of those amino acids with single (i.e. tryptophan, asparagine, glutamine, lysine) and multiple side-chain nitrogen groups [i.e. histidine (two), arginine (three), as well as β-amino acids]. By contrast, the total nitrogen analyser detects the N-content of such amino acids with close to 100% efficiency. Thus, the occurrence of these amino acids in the excreted amino acid pool would create an artificial discrepancy between these two methods and thus promote a higher value for the unknown portion of nitrogenous waste. To correct for this, a correction factor (×1.226) was applied, based on the assumption that the profile (percentage composition) of excreted amino acids would reflect the profile of amino acids in the blood plasma of resting trout, as reported by Wood et al. (1999). Nevertheless, if this assumption is in gross error, a significant discrepancy may still exist, especially in the case of fed fish, where amino acid-N excretion reached 7.2–9.6% of total-N, nearly twice that of fasting animals (Fig. 1). However, when we compared this correction factor (×1.226) calculated from the plasma amino acid profile of fasted trout (Wood et al., 1999) with calculations based on the data of Espe et al. (1993) for Atlantic salmon (*Salmo salar*) at fast (×1.346) or at various times (6 h, 12 h, 24 h) after feeding (×1.314, 1.413, 1.395), it seems probable that the error would be quite small.

Espe et al. (1993) reported that plasma amino acid levels were significantly higher at 6-h and 12-h post-prandium in Atlantic salmon, while in the channel catfish (*Ictalurus punctatus*) it has further been proposed that this surge may facilitate the loss of amino acids by leakage across the branchial membrane (Brown and Cameron, 1991). This suggests that a significant amount of amino acid-N excretion noted here probably passes though the gills, which would constitute a significant energetic loss. Presumably, this is an unavoidable feature of gill design and does not have direct adaptive value. We are aware of no data on renal amino acid-N losses, but total urinary N-losses are small (see below). The lack of difference between amino acid-N excretion rates in sutured and unsutured fish, regardless of feeding or fasting (Table 3), indicates that the amino acid-N excretion was not sourced from the gastrointestinal tract.

Protein-N excretion was also surprisingly high (Table 3) and, like amino acid-N excretion, was also greater in fed fish than in fasted fish, reaching 11.2% in the fed sutured treatment (Fig. 1). This is probably related to the increased nitrogen intake associated with eating. Like amino acid-N, it does not appear to be excreted *via* the gastrointestinal tract because protein-N excretion rates were actually higher in sutured than in unsutured fish, regardless of feeding or fasting (Table 3). Again, the 'purpose' of excreting a valuable energy resource may seem counterintuitive. However, in the kelp bass (*Paralabrax* sp.), Bever et al. (1981) found that a significant portion of the [^{14}C] radioactivity from labeled amino acids injected into the bloodstream quickly appeared in body mucus. It is possible that most protein-N excretion in rainbow trout is that detected from the shedding of mucus and may be performing a role secondary to the excretion of nitrogen (e.g. protection; Shephard, 1994). Such a hypothesis is supported by the finding that the rate of protein-N excretion in sutured fish was higher than that in unsutured fish, suggesting that stress caused by handling, suturing and/or anesthesia stimulated mucus production by the skin. The ability of the Bradford assay to detect different protein is known to vary with protein composition (Sapan et al., 1999) but, to our knowledge, the capacity of the Bradford assay to detect mucus glycoproteins has not been specifically investigated. However, given that the mechanism of dye interaction with protein appears to depend mainly on interaction with protonated amino groups (Sapan et al., 1999), there is no mechanistic reason to believe that our results either under- or overestimate the contribution of protein to overall nitrogen excretion, although this possibility cannot be dismissed.

The use of anal sutures permitted an investigation of the importance of the gut as a route of excretion. In fasting fish, there were only minor differences in nitrogenous waste products between sutured and unsutured groups (Table 3). It is unlikely that the gastrointestinal tract is an important route for nitrogen excretion in rainbow trout under fasting conditions, where the gills (and/or the skin) predominate. Although nitrogen in the urine was not measured in the present study, it is unlikely to contribute significantly to nitrogen excretion based on salmonid literature reviewed by Wood (1995). For example, Fromm (1963) reported that total nitrogen excretion rate in the urine was only 3.3% of the branchial excretion rate in starved rainbow trout.

Nitrogen excretion increased with feeding, even when the anus was sutured. In fed fish, the difference between nitrogen excretion in sutured and unsutured fish indicates nitrogen excretion *via* the gut and/or nitrogen that was not absorbed

Table 5. *Relative contributions (% of total) of various N-products to nitrogen excretion rates reported for different fish species*

		°C	Ammonia	Urea	Creatinine + creatine	TMAO + TMA	Uric acid	Amine	Nitrite + nitrate	Amino acids	Protein	Unknown	Ref.
Carp (*Cyprinus carpio*)		18.5	56.0–63.5	5.7–6.7	4.4–6.5		0.2	3.2–9.5				15.9–28.1	1
Sculpin (*Leptocottus armatus*)		12	52.6–72.4	16.9–24.7	0.0–1.4	1.3–5.2						4.1–22.3	2
Starry flounder (*Platichthys stellatus*)		12	81.4–86.2	11.5–12.1	0.1–0.3	0.2–1.6						0.4–6.2	2
Blue sea-perch (*Taeniotoca lateralis*)		12	48.0	38.1	0.0	0.7						13.2	2
Peruvian anchovy (*Engraulis ringens*)		15	40.1–49.4	12.7–16.5	15.9–30.1							4.0–31.3	3
White steenbras (*Lithognathus lithognathus*)	Fasted	15–25	83.0	6.0					<1	11			4
	Fed		79.0	8.0					<1	13			4
Atlantic Salmon (*Salmo salar*)		12	64.7	10.0								25.3	5
		10	70.6	10.6								18.8	5
		4	64.5	9.7								25.8	5
Arctic Char (*Salvelinus alpinus*)		6	66.8	7.7								25.5	5
Midshipman (*Porichthys notatus*)	Fasted	11–13	69.2	3.7								27.1	6
	Fed		63.6	2.3								34.1	6
Rainbow trout (*Oncorhynchus mykiss*)	Fasted	13	27.0–51.9	18.7–33.3							21.9–33.0	1.2–10	7
Rainbow trout (*Oncorhynchus mykiss*)		10	52.4	36.4								11.2	8
Rainbow trout (*Oncorhynchus mykiss*)		14	32.1	4.3								63.6	9
Rainbow trout (*Oncorhynchus mykiss*)	Fasted	11	66.4	10.3	0.6	ND	ND		ND	4.1	3.1	15.5	10
	Fed		62.2	7.5	1.4					7.2	4.2	17.5	10
Rainbow trout (*Oncorhynchus mykiss*)	Fasted	11	68.0	8.6	0.9	ND	ND		ND	5.0	5.5	12.0	11
	Fed		52.8	5.6	1.1					9.6	11.2	19.7	11

[1]Smith (1929), [2]Wood (1958), [3]McCarthy and Whitledge (1972), [4]Cockcroft and Du Preez (1989), [5]Fivelstad et al. (1990), [6]Walsh et al. (2001), [7]Olson and Fromm (1971), [8]Beamish and Thomas (1984), [9]De Boeck et al. (2001), [10]present study (unsutured group), [11]present study (sutured group).

ND, not detected.

from the ingested food and therefore passed out in the feces. The contamination by any N that was present as particulate matter in the feces was minimised by sieving the water with cheesecloth, but clearly this would not eliminate any soluble forms. Wood (1995) reviewed the aquacultural literature on fecal-N excretion in salmonids and concluded that the true or 'metabolic component' was probably less than 50 μmol N kg^{-1} h^{-1}, with the remainder, which is generally a larger fraction in fed animals, originating from non-absorbed N-compounds. However, this conclusion was based on experiments using largely indirect measurement techniques. In the present study, we cannot eliminate the possibility that significant amounts of ammonia-N and urea-N (totaling far more than 50 μmol N kg^{-1} h^{-1}; cf. Table 3) may have been excreted across the gut wall in fed fish (i.e. true 'metabolic-N excretion'), perhaps as a result of local metabolism in the tissues of the gastrointestinal tract. Indeed, it seems unlikely that this gastrointestinal N-output originated directly from non-absorbed N-compounds, because it was not present in the form of protein-N or amino acid-N. More likely, if of 'non-absorbed' origin, it was produced by bacterial conversion to ammonia-N, urea-N and creatinine-N.

Regardless, the finding that 47% of total ammonia-N output, 32% of total urea-N output and 52% of total creatinine-N output were sourced from the gut implies an important role for this pathway in the total nitrogen budget of fed fish. This subject is worthy of future study. Notably, there was no difference in these parameters (except creatinine-N) between sutured fed fish and sutured fasted fish. This suggests that the rates of ammonia-N and urea-N excretion from the gill (and skin) were not changed with feeding, in contrast to the surge in these products from the gastrointestinal tract.

In series 1, despite investigating the presence of a wide range of potential nitrogenous excretory products, a significant proportion (12–20%) of total nitrogen remained unaccounted for. Table 5 summarises previous studies investigating the nature of nitrogen waste products in fish and highlights the ubiquity of the unknown nitrogen fraction.

In many studies, a possible explanation for the discrepancy between total nitrogen and the sum of individually measured nitrogen components may lie with methodology used, unless the efficiency of N-detection by the various assay methods has been checked and taken into account. In the present study, the only important error occurred with the ninhydrin method

(Table 1), which tends to underestimate the N-content of certain amino acids, and this was taken into account by the use of a correction factor. However, Doi et al. (1981) and Fisher et al. (2001) have reported that the ninhydrin assay detects not only amino acids but also small peptides (di- and tripeptides) to a limited extent. Thus, some of the detected amino acid-N may actually have represented peptide-N. On the other hand, if there is substantial excretion of small peptides, then this could account for part of the discrepancy, as di- or tripeptides will have additional undetectable N groups. For example, tests in the present study demonstrated that the N content in two ubiquitous dipeptides (the 'buffer peptides', carnosine and anserine) were detected with only 8–21% efficiency (Table 1). If these compounds were excreted to a significant extent, they would elevate the discrepancy between 'amino acids/peptides' as analysed by the total nitrogen analyser and as measured *via* the ninhydrin method.

The proposition that some of this unknown nitrogen component may exist in the form of small peptides is not without merit. For example, some fish species contain large amounts of carnosine, while others, such as salmonids, are known to contain large amounts of anserine (histidine-related dipeptide; β-alanyl-π-methyl-L-histidine) in their tissues (Abe, 1983, 1991; Van Waarde, 1988). For example, Espe et al. (1993) reported that anserine levels in the epaxial muscle of the Atlantic salmon were 6–300-fold higher than any amino acid. In fact, anserine concentrations in white muscle of rainbow trout have been measured at levels approaching 20 μmol g^{-1} and are even higher in other salmonids (Van Waarde, 1988). Anserine levels in the kidney (a possible excretion route) were at least 2 μmol g^{-1} wet mass (Abe, 1991). Interestingly, Abe (1991) found that the anserine levels in the white muscle of rainbow trout were considerably lower than reported by Van Waarde (1988), potentially underestimating the potential importance of renal anserine. A further important peptide may be π-methyl-L-histidine, which is also found in high levels in trout tissues (Abe, 1991). This entity is of particular interest given that the demethylating enzyme responsible for converting π-methyl-L-histidine to L-histidine is considered not to exist in trout, suggesting that π-methyl-L-histidine may be excreted without reutilisation (Abe, 1991). The importance of these nitrogen-containing compounds lies not only in their relative abundance but also in the fact that they contain multiple nitrogen groups. Anserine contains four, while π-methyl-L-histidine has three nitrogen groups. It is reasonable to speculate that some of the unknown-N excreted by rainbow trout may exist undetected in these forms. Other forms of nitrogen, for example 'rotting compounds' such as putrescine, may also contribute to the unknown component. Examination of unknown nitrogen using HPLC is likely to provide insight in future investigations.

In series 2 of the present study, both oxygen consumption (Fig. 2A) and nitrogen excretion (Fig. 2B) increased with feeding. Furthermore, unknown-N production also increased with feeding, with unknown-N in fish fed 5% body mass day^{-1} accounting for 63% of total-N (Fig. 2B), a much higher value than in the series 1 results for trout on much lower daily ration (Fig. 1; Tables 3, 5) or those generally reported in other studies (Table 5; although see De Boeck et al., 2001).

Fig. 3 presents NQ, and associated protein utilisation values, calculated three different ways, and illustrates the marked effect that using various assumptions can have on the results. When NQ is calculated in the traditional manner based on the sum of measured ammonia-N + urea-N excretion, the value increases from ~0.14 to ~0.16–0.21 in fed fish. Thus, the estimated use of protein in aerobic metabolism increases from ~50% to ~60–80%. The former is somewhat higher than generally reported for fasted trout, but the latter values are very representative of values for fed salmonids calculated in earlier studies on the same basis (see Introduction). When total-N excretion is used in the calculation, there is little change in fasted fish, but the discrepancy progressively increases with ration such that the theoretical maximum value (NQ=0.27, representing 100% protein utilisation) is reached at 3% ration, and this value is greatly exceeded at 5% ration. This strongly suggests that the contribution of non-oxidized N-products increases greatly at high ration. Thus, NQ based on ammonia-N + urea-N excretion underestimates protein oxidation, while NQ based on total-N excretion overestimates it.

The third, and presumably best, way would be to calculate NQ from the sum of all oxidized N-products. Unfortunately, the alternative N-products were not measured in the experiments of series 2. However, an estimate of the sum of all oxidized N-products can be obtained by applying the percentage contribution data from series 1 (Fig. 1) for the sum of ammonia-N, urea-N, creatinine-N, creatine-N and unknown-N (i.e. specifically excluding protein-N and amino acid-N) to the measured total-N data of series 2 (from Fig. 2B). This should be most accurate for fasted fish and those fed a 1% daily ration, because the former treatment was duplicated in series 1 while the latter was approximated by the 2% ration fed every other day in series 1. The results of these model calculations (Fig. 3) indicate only modest changes in the estimation of protein utilisation (relative to calculations based on ammonia-N + urea-N) for these two treatments but much larger differences at the higher rations. Most notably, the NQ still stays above the theoretical maximum value at the 5% ration level. Multiple explanations are possible, but the most likely is that the contribution of non-oxidized N-products is increasing greatly at high ration level, such that percentage estimates based on data taken at low ration level are not applicable. This is an important area for future investigation. Despite many years of intensive research on the nitrogen metabolism of teleost fish, it is clear that a more comprehensive understanding of protein and amino acid excretion, of the nature of minor nitrogen end products and of how their output varies with feeding is required.

This study was supported by an NSERC Discovery grant to C.M.W. The authors would like to thank Shelby Steele for assistance with TMA and TMAO protocols. Members of the Wood laboratory are thanked for their various expertise in making this study possible.

References

Abe, H. (1983). Distribution of free L-histidine and related dipeptides in the muscle of fresh-water fishes. *Comp. Biochem. Physiol. B* **76**, 35-39.

Abe, H. (1991). Interorgan transport and catabolism of carnosine and anserine in rainbow trout. *Comp. Biochem. Physiol. B* **100**, 717-720.

Alsop, D. H. and Wood, C. M. (1997). The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout. *J. Biol. Chem.* **200**, 2337-2346.

Alsop, D. H., Kieffer, J. D. and Wood, C. M. (1999). The effect of temperature and swimming speed on instantaneous fuel use and nitrogenous waste excretion of Nile tilapia. *Physiol. Biochem. Zool.* **72**, 474-483.

Beamish, F. W. H. and Thomas, E. (1984). Effects of dietary protein and lipid on nitrogen losses in rainbow trout (*Salmo gairdneri*). *Aquaculture* **41**, 359-371.

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

Boutilier, R. G., Hening, T. A. and Iwama, G. K. (1984). Appendix: physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. 10A (ed. W. S. Hoar and D. J. Randall), pp. 403-430. Orlando: Academic Press.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brett, J. R. and Zala, C. A. (1975). Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. *J. Fish. Res. Bd. Canada* **32**, 2479-2486.

Brown, C. R. and Cameron, J. N. (1991). The induction of specific dynamic action in channel catfish by infusion of essential amino acids. *Physiol. Zool.* **64**, 276-297.

Cockcroft, A. C. and Du Preez, H. H. (1989). Nitrogen and energy loss via nonfaecal and faecal excretion in the marine teleost *Lithognathus lithognathus*. *Mar. Biol.* **101**, 419-425.

De Boeck, G., Alsop, D. and Wood, C. M. (2001). Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol. Biochem. Zool.* **74**, 858-868.

Doi, E., Shibata, D. and Matoba, T. (1981). Modified colorimetric ninhydrin methods for peptidase assay. *Anal. Biochem.* **118**, 173-184.

Espe, M., Lied, E. and Torriessen (1993). Changes in plasma and muscle free amino acids in Atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolyzed cod muscle protein. *Comp. Biochem. Physiol. A* **105**, 555-562.

Fisher, G. H., Arias, I., Quesada, I., D'Aniello, S., Errico, F., Di Fiore, M. M. and D'Aniello, A. (2001). A fast and sensitive method for measuring picomole levels of total free amino acids in very small amounts of biological tissues. *Amino Acids* **20**, 163-173.

Fromm, P. O. (1963). Studies on renal and extra-renal excretion in a freshwater teleost, *Salmo gairdneri*. *Comp. Biochem. Physiol.* **10**, 121-128.

Garrow, J. S. (1974). *Energy Balance and Obesity in Man.* Amsterdam, London: Elsevier Science.

Fivelstad, S., Thomassen, J. M., Smith, M. J., Kjartansson, H. and Sando, A.-B. (1990). Metabolite production rates from Atlantic salmon (*Salmo salar* L.) and Arctic char (*Salvelinus alpinus* L.) reared in single pass land-based brackish water and sea-water systems. *Aquacult. Eng.* **9**, 1-21.

Jobling, M. (1980). Effects of starvation on proximate chemical composition and energy utilization of plaice, *Pleuronectes platessa*. *J. Fish. Biol.* **17**, 325-334.

Kieffer, J. D., Alsop, D. and Wood, C. M. (1998). A respirometric analysis of fuel use during aerobic swimming at different temperatures in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **201**, 3123-3133.

Kleiber, M. (1961). *The Fire of Life: an Introduction to Animal Energetics.* New York, London: John Wiley & Sons.

Kleiber, M. (1992). Respiratory exchange and metabolic rate. In *Handbook of Physiology* (ed. S. R. Geiser), pp. 927-938. Bethesda: American Physiological Society.

Korsgaard, B., Mommsen, T. P. and Wright, P. A. (1995). Nitrogen excretion in teleostean fish: adaptive relationships to environment, ontogenesis, and viviparity. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. A. Wright), pp. 259-288. Boca Raton: CRC Press.

Kutty, M. N. and Peer Mohamed, M. (1975). Metabolic adaptations of mullet *Rhinomucil corsula* (Hamilton) with special reference to energy utilization. *Aquaculture* **5**, 253-270.

Lauff, R. F. and Wood, C. M. (1996a). Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during starvation in juvenile rainbow trout. *J. Comp. Physiol. B* **165**, 542-551.

Lauff, R. F. and Wood, C. M. (1996b). Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during aerobic swimming in juvenile rainbow trout. *J. Comp. Physiol. B* **166**, 501-509.

McCarthy, J. J. and Whitledge, T. E. (1972). Nitrogen excretion by anchovy (*Engraulis mordax*) and jack mackerel (*Trachurus symmetricus*). *Fish. Bull.* **70**, 395-401.

Mommsen, T. P. and Walsh, P. J. (1991). Urea synthesis in fishes: evolutionary and biochemical perspectives. In *Biochemistry and Molecular Biology of Fishes*, vol. 1 (ed. P. W. Hochachka and T. P. Mommsen), pp. 137-163. New York: Elsevier.

Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* **48**, 583-592.

Moore, S. (1968). Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. *J. Biol. Chem.* **243**, 6281-6283.

Moore, S. and Stein, W. H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* **176**, 367-388.

Moore, S. and Stein, W. H. (1954). A modified ninhydrin reagent for photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**, 907-913.

Olson, K. R. and Fromm, P. O. (1971). Excretion of urea by two teleosts exposed to different concentrations of ambient ammonia. *Comp. Biochem. Physiol. A* **40**, 999-1007.

Rahmatullah, M. and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta* **107**, 3-9.

Rand, M. C. (ed.) (1975). *Standard Methods for the Examination of Water and Wastewater*. 14th edition. pp. 423. Washington, DC: APHA-AWWA-WPCF.

Sapan, C. V., Lundblad, R. L. and Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnol. Appl. Biochem.* **29**, 99-108.

Shephard, K. L. (1994). Functions for fish mucus. *Rev. Fish Biol. Fish.* **4**, 401-429.

Smith, H. W. (1929). The excretion of ammonia and urea by the gills of fish. *J. Biol. Chem.* **81**, 727-742.

van den Thillart, G. and Kesbeke, F. (1978). Anaerobic production of carbon dioxide and ammonia by goldfish *Carassius auratus* (L.). *Comp. Biochem. Physiol. B* **156**, 511-520.

Van Waarde, A. (1988). Biochemistry of non-protein nitrogenous compounds in fish including the use of amino acids for anaerobic energy production. *Comp. Biochem. Physiol. B* **91**, 207-228.

Verdouw, H., Van Echted, C. J. A. and Dekkers, E. M. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399-402.

Walsh, P. J. (1997). Nitrogen metabolism and excretion. In *The Physiology of Fishes.* 2nd edition (ed. D. H. Evans), pp. 199-214. Boca Raton: CRC Press.

Walsh, P. J., Wang, Y., Campbell, C. E., De Boeck, G. and Wood, C. M. (2001). Patterns of nitrogenous waste excretion and gill urea transporter mRNA expression in several species of marine fish. *Mar. Biol.* **139**, 839-844.

Wekell, J. C. and Barnett, H. (1991). New method for analysis of trimethylamine oxide using ferrous sulfate and EDTA. *J. Food Sci.* **56**, 132-138.

Wiggs, A. J., Henderson, E. B., Saunders, R. L. and Kutty, M. N. (1989). Activity, respiration, and excretion of ammonia by Atlantic salmon (*Salmo salar*) smolt and postmolt. *Can. J. Fish. Aquat. Sci.* **46**, 790-795.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. Evans), pp. 379-425. Boca Raton: CRC Press.

Wood, C. M. (1995). Excretion. In *Physiological Ecology of the Pacific Salmon* (ed. C. Groot, L. Margolis and W.C. Clarke), pp. 381-438. Vancouver: Government of Canada Special Publications Branch; UBC Press.

Wood, C. M. (2001). The influence of feeding, exercise, and temperature on nitrogen metabolism and excretion. In *Fish Physiology*, vol. 20 (ed. P. A. Anderson and P. A. Wright), pp. 201-238. Orlando: Academic Press.

Wood, C. M., Milligan, C. L. and Walsh, P. J. (1999). Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. *Am. J. Physiol.* **277**, R482-R492.

Wood, J. D. (1958). Nitrogen excretion in some marine teleosts. *Can. J. Biochem. Physiol.* **36**, 1237-1242.