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INFLUENCE OF FEEDING, EXERCISE, AND TEMPERATURE ON NITROGEN METABOLISM AND EXCRETION

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I. INTRODUCTION

The literature on nitrogen metabolism is unbalanced. The vast majority of fish swim actively, eat regularly, grow continually, live in nonextreme environments, and, at least if they are teleosts, excrete mainly ammonia by way of the gills. Their major environmental variables are temperature and food availability. However, as illustrated by other chapters in this volume, and a host of recent reviews (Mommsen and Walsh, 1992; Wood, 1993, 1995; Jobling, 1994; Korsgaard *et al.*, 1995; Wright, 1995; Walsh, 1997a; Wilkie, 1997), most experimental studies have concentrated on fish that are confined in small chambers and therefore not swimming, feeding, or growing; the fish are often subjected to severe environmental challenges, but the temperature is usually held constant. The species studied are often

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selected because they are suspected to have unusual end products or pathways of nitrogenous waste excretion, rather than because they are representative of the great majority of teleosts. The primary aim of the present review is to redress this imbalance by examining the separate and interactive effects of feeding, exercise, and temperature on nitrogen metabolism in representative teleost fish. Two additional areas highlighted are the possibility of additional nitrogen products that may be excreted, and the mechanism(s) of ammonia excretion, both in actively feeding fish. Finally, the meager information available on these topics for elasmobranchs is summarized, in the hope of stimulating further research on this most interesting but poorly studied group.

II. FEEDING AND NITROGEN METABOLISM

When a fish eats another fish, or a commercial diet made from fish meal, it is eating about 40% protein on a dry weight basis. Assuming a daily ration of 4% for an intensively feeding fish and 0.16 gram of nitrogen per gram of protein (Cho, 1990), then the fish is eating 2.56 g/kg or 183,000 μmol of nitrogen per day. If this were all excreted, then the average excretion rate would be 7625 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. Typically, measured nitrogen excretion rates for nonfed fish confined in boxes are less than 500 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. Of course, because fish grow all their lives, a substantial portion of this ingested nitrogen is not excreted but rather converted to protein growth. Nevertheless, the comparison serves to illustrate the widespread misconception that the examination of nitrogen metabolism in nonfed fish (which by definition cannot be growing) provides a picture of their normal physiology. Instead, it provides a picture of their physiology during starvation, a special circumstance that can be considered a component of normal physiology in only a few circumstances (e.g., anorexia during migration, overwintering at cold temperature, or nest guarding). The critical influence of feeding on physiologic function has been routinely ignored, often for the sake of experimental convenience.

When experiments are performed on quiescent fish that are not being fed (a common procedure in many laboratories), nitrogen excretion rates tend to drop markedly, stabilizing after about a week of starvation (Fromm, 1963; Iwata, 1970; Smith and Thorpe, 1976; Rychly and Marina, 1977; Kaushik and Teles, 1985). These abnormally low rates of nitrogen excretion represent the so-called *endogenous* fraction, the minimum unavoidable loss that results from the normal protein, nucleic acid, purine, and pyrimidine base turnover required for body maintenance. The difference between this "baseline" rate seen in a starved fish and the much higher rates occurring in fed fish approximates the *exogenous* fraction, the portion not retained as growth from the diet.

Because confinement in small volumes is necessary to measure nitrogen waste

excretion in individual fish, and it is often inconvenient or impossible to have the fish feed or grow under such conditions, a viable alternative is to work with fish in groups, living in their normal feeding and holding tanks. Theoretically, there is absolutely nothing wrong with this approach, because both naturally and in aquaculture, fish tend to live and feed in groups. However, statisticians have an unfortunate habit of demanding that the number of tanks of fish be replicated to the same degree as the number of fish in individual experiments, a factor that has discouraged this approach. Fortunately, a number of researchers have disregarded this criticism and, thereby, produced some very useful data.

A landmark review is that of Handy and Poxton (1993), who gathered together a large amount of data derived from this "bulk in tank" approach, from the aquaculture of mainly marine species, much of it from literature that is difficult to access for most researchers. This synthesis demonstrated that nitrogen excretion rates depend critically on feeding, and may often rise above 3000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. Wood (1995) similarly reviewed nitrogen waste excretion data for salmonids in aquaculture, and concluded that rates in actively feeding fish ranged as high as 2500 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, and probably even higher in salmon feeding in the wild. Subsequent studies have confirmed these very high rates in actively feeding salmonids and the great sensitivity of absolute rates to ration (e.g., Dockray *et al.*, 1996, 1998; Linton *et al.*, 1997, 1998a,b). Leung *et al.* (1999) have summarized similar recent data for a variety of nonsalmonid marine fishes. Figure 1, from the data of Alsop and Wood (1997), illustrates the marked effects on nitrogen waste excretion and metabolic rate of switching rainbow trout from a maintenance ration (1% per day) to either starvation or twice daily satiation feeding (3% per day); a difference of over 6-fold in nitrogen excretion rate resulted, but only 1.7-fold in metabolic rate (O_2 consumption).

Three classic studies from the earlier literature are particularly noteworthy. Despite the difficulties of motivating individually confined fish to eat, a heroic study by Beamish and Thomas (1984) on adult rainbow trout accomplished just this feat. Not only were individual fish trained to take a fixed ration from forceps inserted through a feeding port, they were also fitted with a chronic bladder catheter to collect urinary nitrogen excretion and housed in a chamber that allowed collection of their fecal nitrogen excretion. Rations tested were low (0.5 and 1.0% per day), but, nevertheless, both total nitrogen excretion and nitrogen retention increased in response to increased ration, and in response to increased nitrogen content in the ration. Fecal nitrogen losses were small, reflecting high nitrogen absorption efficiency (>93%) through the digestive tract. Overall, nitrogen retention was about 50%, and the great majority of nitrogen excretion occurred by way of the gills in the form of ammonia-N, with urea-N as a minor but significant component; urinary nitrogen excretion was less than 4% of the total. There have been no more thorough studies on the nitrogen budgets of individual fish since the work of Beamish and Thomas (1984), and their conclusions remain valid today.

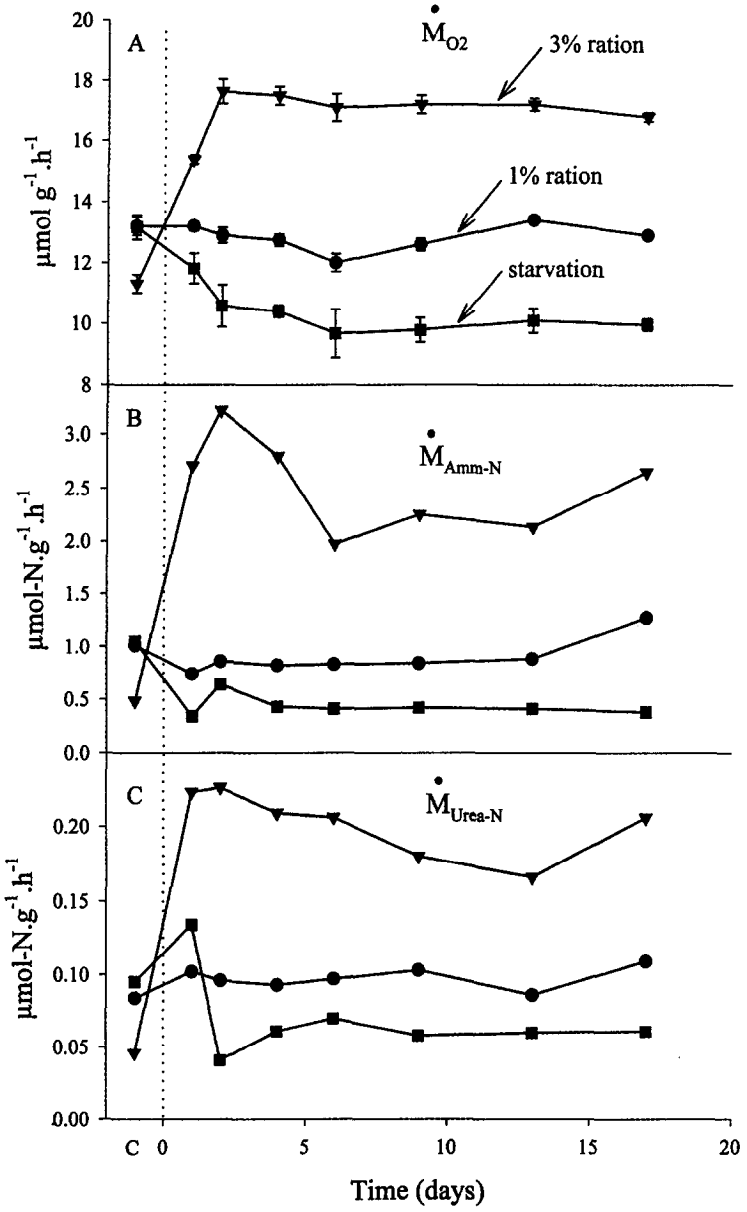

Fig. 1. Changes in the rates of (A) O_2 consumption (M_{O2}); (B) ammonia-N excretion (M_{Amm-N}); and (C) urea-N excretion (M_{Urea-N}) at 15°C in groups of about 120 juvenile rainbow trout (~8 g) over a 17-day period following the switch to daily rations of 3%, 1%, or starvation at day 0. Prior to day 0, all fish were fed a 1% ration. Note the much larger relative changes in M_{Amm-N} and M_{Urea-N} than in M_{O2}. Mean data for duplicate tanks; standard error bars in (A) are for the measurements, not the tanks. (Recalculated from Alsop and Wood, 1997. Reproduced with permission of the Company of Biologists Ltd.)

In another landmark study, Brett and Zala (1975) used an "in-tank" monitoring system with high temporal resolution to monitor rates of ammonia-N and urea-N excretion and O_2 consumption in a group of 30 juvenile sockeye salmon held together in a single tank. Following a once-daily feeding of 3% ration, ammonia-N excretion increased 3.5-fold to reach a peak 5 h later, and thereafter declined (Fig. 2). Notably, the O_2 consumption rate showed a very different pattern, rising *prior* to feeding, peaking coincident with feeding, and declining prior to the ammonia-N peak, presumably reflecting an entrainment of the fishes' activity level ("excitement") to the time when food was provided. The importance

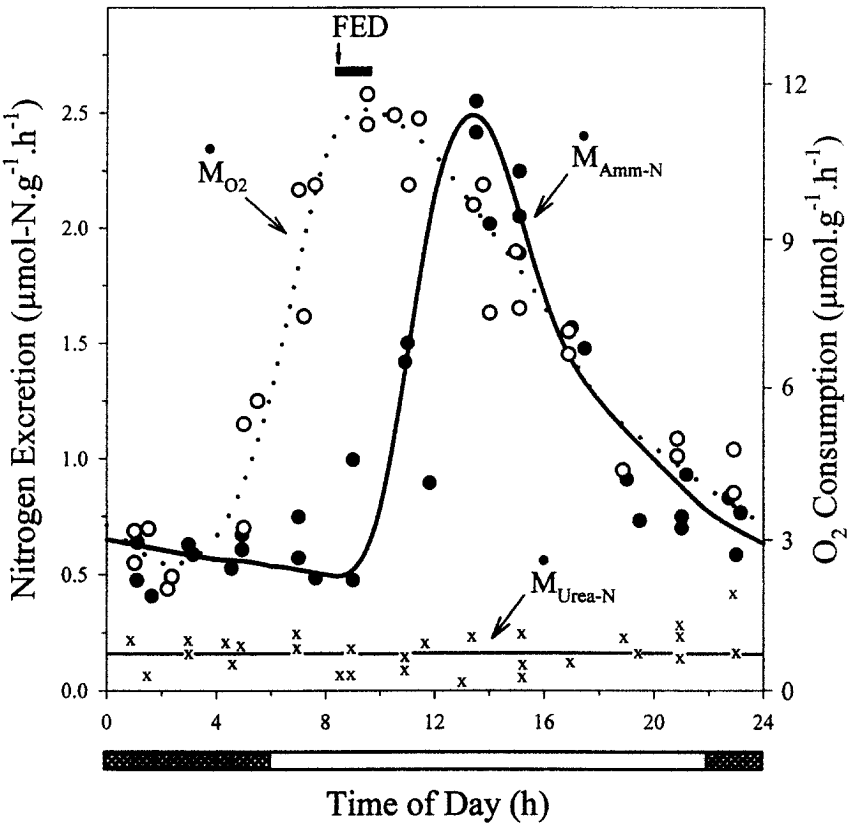

Fig. 2. Diurnal variation in the rates of O_2 consumption (M_{O2}, broken line, open circles), ammonia-N excretion (M_{Amm-N}, closed circles, thick solid line) and urea-N excretion (M_{Urea-N}, crosses, thin solid line) at 15°C in a group of about 30 freshwater-adapted juvenile sockeye salmon (~30 g) fed a 3% ration once daily between 8:30 and 9:30 A.M. Ammonia-N excretion and O_2 consumption rates have been scaled to the same peak value. Note that the O_2 consumption rates (right axis) rise prior to feeding and are about 5 times greater than the nitrogen excretion rates (left axis), which peak 4 h after feeding. (Recalculated from Brett and Zala, 1975. Reproduced with the permission of Her Majesty the Queen in Right of Canada, 2000, Fisheries and Oceans Canada.)

of this work is that it was the first to illustrate the marked and rapid variation in nitrogen excretion that occurs with feeding, and to dissociate it from changes in metabolic rate. The work was also important because urea-N excretion remained unchanged over the daily cycle, at about the same level seen in fish subjected to prolonged starvation, suggesting that urea-N was entirely part of the *endogenous* fraction. The latter was later replicated in Atlantic salmon (Wiggs *et al.*, 1989; Fivelstad *et al.*, 1990), Arctic charr (Fivelstad *et al.*, 1990) and sturgeon (Gershanovich and Pototskij, 1992) but disputed by experiments on seabass (Guerin-Ancey, 1976), carp (Kaushik, 1980), rainbow trout (Kaushik, 1980; Beamish and Thomas, 1984; Alsop and Wood, 1997; see Fig. 1C), and Nile tilapia (Wright, 1993) where urea-N excretion was responsive to alterations in ration, dietary composition, and starvation. The discrepancy likely reflects differences in diets and/or different metabolic pathways among species. For example, if excreted urea-N is produced from dietary arginine it may contribute to the *exogenous* fraction; if produced from uricolysis of nucleic acid and purine breakdown products associated with normal turnover, it will be mainly *endogenous*. However if uricolysis is activated in the face of environmental or metabolic disturbance (pH challenge, for example; see Section VI.A), this distinction may no longer be valid.

An ingenious study by Brown and Cameron (1991a,b) clarified the relationship between nitrogen metabolism and postprandial metabolic rate in fish. It is well known that in fish, as in other vertebrates, feeding is associated with an elevation of O_2 consumption—that is, metabolic rate—commonly called specific dynamic action (SDA) (Jobling, 1981a; Beamish and Trippel, 1990). Part of this is due to an increase in activity and general excitement associated with the presentation of food, as seen by Brett and Zala (1975) (see Fig. 2). However, even when this is corrected for by forcing the fish to swim continually while feeding (LeGrow and Beamish, 1986), the stimulation remains substantial—typically up to a doubling of metabolic rate. The cost of mechanical processing of the food appears to be relatively small (Tandler and Beamish, 1979; Jobling and Spencer Davies, 1980). In mammals, a protein meal is the most potent stimulator of SDA, reflecting the oxidative costs of deamination, transamination, and urea synthesis. However, it is the excretion of ammonia-N, rather than of urea-N, which is mostly stimulated by feeding in fish. Nevertheless, by analogy, it was originally assumed that the situation was similar in fish (e.g., Beamish, 1974; Knights, 1985); that is, SDA reflected the cost of ammonia production, and that was why SDA increased with the protein content of the meal (Jobling and Spencer Davies, 1980; LeGrow and Beamish, 1986). The innovative investigation by Brown and Cameron (1991a,b) on confined channel catfish put to rest any causative relationship between elevated ammonia-N excretion after feeding and elevated metabolic costs. Instead, their results provided confirmation of the original suggestion of Jobling (1981a)—that the cost of stimulated protein synthesis (i.e., growth) is the major cause of SDA. In brief, using direct intravascular infusions of essential amino

acids through indwelling catheters (to avoid mechanical costs), these workers demonstrated that the net elevation in ammonia-N excretion accounted for only 21% of the amino-N load, whereas the amino acids were completely cleared from the bloodstream in a temporal pattern that paralleled SDA. By using the [^{3}H]-phenylalanine flooding dose technique, they demonstrated that the protein synthesis rate was massively stimulated in both muscle and liver at this time, explaining the disappearance of the amino acids, and that the elevated rate could explain at least 80% of the SDA. Furthermore, when fish were pretreated with the protein synthesis inhibitor cyclohexamide, ammonia-N excretion was unaffected, but SDA was completely eliminated.

These findings are in accord with others showing that protein synthesis rates are greatly stimulated by feeding in fish (Lied *et al.*, 1983; McMillan and Houlihan, 1988, 1989; Fauconneau *et al.*, 1989; Lyndon *et al.*, 1992; Houlihan *et al.*, 1995c). Plasma and intracellular free amino acid levels rise following feeding (Kaushik and Teles, 1985; Ash *et al.*, 1989; Brown and Cameron, 1991a; Espe *et al.*, 1993; Walsh and Milligan, 1995). This rise, together with accompanying hormonal signals, is a likely driver of the synthetic machinery. Estimates of exact costs vary, but there is general agreement that protein synthesis is a very expensive process—at least 4 ATP hydrolyzed per peptide bond from basic theory, more if amino acid transport processes and simultaneous breakdown ("futile cycling") are taken into account. This being the case, and considering the fact that fish normally grow continually throughout their lives, it is likely that the protein synthetic cost of growth is the largest component of their energy budget. This topic has been reviewed in detail by Houlihan *et al.* (1995a,b) and Carter and Houlihan (Chapter 2). Suffice it to note here that since structural protein is so costly to manufacture, it would seem to make sense to minimize its use as a metabolic fuel.

III. METABOLIC FUEL USAGE AND NITROGEN METABOLISM

Despite the preceding argument, historically it has been believed that protein is a major metabolic fuel in fish (e.g., Driedzic and Hochachka, 1978; van Waarde, 1983; Jobling, 1994), especially during exercise (Krueger *et al.*, 1968; van den Thillart, 1986; Davison, 1989; Weber and Haman, 1996). The exact origin of this idea is unclear—certainly the marked depletion of protein that occurs during the spawning migration of anorexic salmon (Idler and Clemens, 1959; Mommsen *et al.*, 1980) is one cogent piece of evidence, but this is an extreme situation, and indeed lipid is used up before protein. The dominance of protein as a fuel has been backed up by some *compositional* studies (e.g., Krueger *et al.*, 1968; Beamish *et al.*, 1989), but not others (e.g., Brett, 1973; Christiansen *et al.*, 1989). Brett (1995) has pointed out the many potential errors to the compositional approach;

additional ones are that it assumes that fuels depleted in fish sacrificed at various times during an exercise regime represent fuels burned, and not fuels interconverted or excreted (Bever *et al.*, 1981; Lauff and Wood, 1996a). Other lines of evidence come from measurements of a relatively high respiratory quotient (RQ) (e.g., Kutty, 1968; van den Thillart, 1986), combined with an often-stated but poorly founded assumption that since fish burn very little carbohydrate, a high RQ must indicate substantial protein oxidation (e.g., van den Thillart, 1986).

Is this true? Lauff and Wood (1996a,b, 1997) have developed an extension of the respirometric approach, termed the *instantaneous* method, which casts light on this question. The instantaneous approach adapts standard mammalian metabolic theory (Kleiber, 1992) to fish metabolism in order to monitor fuel oxidation nondestructively from simultaneous measurements of O_2 consumption, CO_2 production, and most importantly nitrogen waste excretion in the external water. The method assumes that metabolism is completely aerobic, and that steady-state conditions apply (i.e., waste products are not held back), assumptions that appear reasonable for fish at rest or swimming at submaximal velocities.

In brief, the ratio of measured nitrogen waste excretion (M_N) to O_2 consumption (M_{O2}) yields the nitrogen quotient (NQ) (M_N/M_{O2}); from knowledge of the typical metabolism of fish protein, an NQ = 0.27 represents the condition when aerobic respiration is fueled 100% by protein (van den Thillart and Kesbeke, 1978). Therefore, under any steady-state condition, the percent of metabolism fueled by protein is calculated as 100% × NQ/0.27. The measured partitioning of M_N between ammonia-N and urea-N can then be used to predict the RQ of this protein component from standard theory (usually around 0.94–0.97 in teleosts, rather than the 0.83 of mammals; van den Thillart and Kesbeke, 1978; Kleiber, 1992). Once the relative contribution of protein to total fuel use is known, the contribution of the protein RQ to the overall RQ can be subtracted. The known RQs for carbohydrate (1.00) and lipid (0.71) can then be used to factor out the remaining contributions of these two fuel sources, respectively. Note that since the true RQ of protein in fish is quite close to that of carbohydrate, measurements of NQ are essential if RQ is to be correctly interpreted (c.f. van den Thillart, 1986).

The results of the instantaneous analysis have been informative. First, in resting nonfed fish, lipid is the dominant fuel (35–68%) and the contribution of protein oxidation to overall aerobic metabolic rate is low: 14–30% in four studies on rainbow trout (Lauff and Wood, 1996a,b; Alsop and Wood, 1997; Kieffer *et al.*, 1998), and 16–30% in the Nile tilapia (Alsop *et al.*, 1999). Recalculation of data from four earlier studies indicates similarly low protein oxidation rates in sockeye salmon (19–36%; Brett and Zala, 1975), Atlantic salmon (26%; Wiggs *et al.*, 1989), plaice (27%; Jobling, 1980), and freshwater mullet (34%; Kutty and Peer Mohamed, 1975). The low protein contribution in salmonids tends to increase during progressive starvation, but protein never becomes the dominant fuel (Brett

and Zala, 1975; Lauff and Wood, 1996a). Surprisingly, and contrary to popular belief, carbohydrate is the second most important fuel after lipid, and its contribution increases to a much greater extent during starvation (Lauff and Wood, 1996a). Note that reanalysis of the data of Kutty (1972) indicating >85% protein usage in the Mozambique tilapia, and Sukumaran and Kutty (1977) indicating >44% protein usage in the Madurai catfish portrays a very different pattern. Potential reasons for this discrepancy may be because only a short period of food deprivation (24–36 h) was used, so that the fish were still burning amino acids directly from the diet (see below) and/or because metabolism was partially anaerobic (c.f. van den Thillart and Kesbeke, 1978) under the conditions of the experiments as concluded by Kutty (1972).

Second, when fish are fed, the oxidation of protein clearly increases. For example, Kutty (1978) reanalyzed the data (Fig. 2) of Brett and Zala (1975) to illustrate the marked variation in protein oxidation over the feeding cycle. Translating to the assumptions of the instantaneous approach of Lauff and Wood (1996a,b), protein oxidation ranged from a low of 19% during the period of excitement prior to feeding to more than 90% during the peak of ammonia-N excretion 5 h after feeding, with an overnight plateau around 36%. Applying the same analysis to the data of Alsop and Wood (1997) on rainbow trout (Fig. 1), protein fueled 50–70% of metabolism in the satiation-fed fish, 25% in the fish on maintenance ration, and only 15% in the starved fish. Long-term feeding studies with rainbow trout similarly yielded values in the 50–85% range for satiation feeding regimes (Dockray *et al.*, 1996; Linton *et al.*, 1997, 1998a,b; D'Cruz *et al.*, 1998) and 25–50% for restricted ration regimes (Dockray *et al.*, 1996; D'Cruz *et al.*, 1998; Linton *et al.*, 1999). When fish eat a protein-rich meal, amino acids flood into the bloodstream, reaching a peak after a few hours (Brown and Cameron, 1991a; Espe *et al.*, 1993). Whatever is not needed for protein synthesis can be deaminated and oxidized in the citric acid cycle for the immediate provision of energy.

IV. EXERCISE AND NITROGEN METABOLISM

Does it make sense for an exercising fish to oxidize the very machinery (muscle structural protein) that is powering the swimming? In addressing this question, two types of exercise must be considered.

A. Sustainable Aerobic Swimming

This is the type of exercise that fish *normally* perform most of the time during their everyday lives. The instantaneous approach shows that when fish are made to swim at sustainable, submaximal velocities, the contribution of protein oxidation to overall fuel use stays the same, or in most trials actually *decreases* with

increasing velocity. Measurements of M_N either remain unchanged or increase to a lesser extent than M_{O2} in both trout (Lauff and Wood, 1996b, 1997; Kieffer *et al.*, 1998) and tilapia (Alsop *et al.*, 1999) (Fig. 3). Similarly, reanalysis of published data suggests a fall in the contribution of protein oxidation during spontaneous swimming in both freshwater mullet (Kutty and Peer Mohamed, 1975) and Atlantic salmon (Wiggs *et al.*, 1989). As velocity is increased, lipid remains the dominant fuel, but the relative contribution of carbohydrate increases, just as during starvation (Lauff and Wood, 1996b; Kieffer *et al.*, 1998). In accord with these observations, swimming at low speeds has been shown to stimulate protein synthesis rates to a greater extent than protein degradation rates in muscle tissues of trout, so that net protein accretion rates are improved (Houlihan and Laurent, 1987). In turn, these observations agree with a large literature demonstrating that continuous low-speed exercise improves growth, and particularly growth in protein content, in most though not all studies (reviewed by Davison, 1989, 1997). In fasted Arctic char, short-term low-speed swimming elevated the plasma concentrations of selected amino acids (Barton *et al.*, 1995), a signal that could help activate protein synthesis. However, it is not known whether this also occurs in fed fish, such as the Arctic char, which grew faster when continuously exercised (Christiansen *et al.*, 1989; Christiansen and Jobling (1990).

An interesting feature of the response to aerobic exercise is the fact that urea-N excretion increases to a greater relative extent than ammonia-N excretion as velocity rises (Fig. 3) (Lauff and Wood, 1996b; Alsop and Wood, 1997; Alsop *et al.*, 1999). The cause of this phenomenon is unknown. Lauff and Wood (1996b) suggested that it could reflect greater recruitment of white muscle fibers at higher swimming speeds and accompanying adenylate turnover, a by-product of which is urea produced by uricolysis. An alternative proposal by Anderson (Chapter 7) is that increased urea-N is produced via an inter-organ ornithine–urea cycle that serves to detoxify ammonia-N resulting from adenylate breakdown in white muscle. Regardless, if either explanation is correct, then this portion of urea-N excretion should have been excluded from the fuel use calculations because it was derived from adenylates, not amino acids, meaning that true protein utilization during exercise is even lower.

Another consistent finding from the instantaneous approach is that the relative contribution of protein oxidation appears to increase with the duration of sustained swimming (Fig. 3D), manifested as either an absolute increase of ammonia-N excretion (Kutty, 1972; Sukumaran and Kutty, 1977; Alsop *et al.*, 1999) or its constancy in the face of declining M_{O2} with time (Lauff and Wood, 1996b, 1997). At least in part, this may reflect the effects of starvation during the exercise test (see above) as much as it does prolonged exercise. Thus, when trout were trained for 2 weeks (during which time they were fed daily), and subsequently swim-tested, they exhibited substantially lower values of M_N, much lower rates of protein oxidation, and higher rates of lipid utilization during exercise

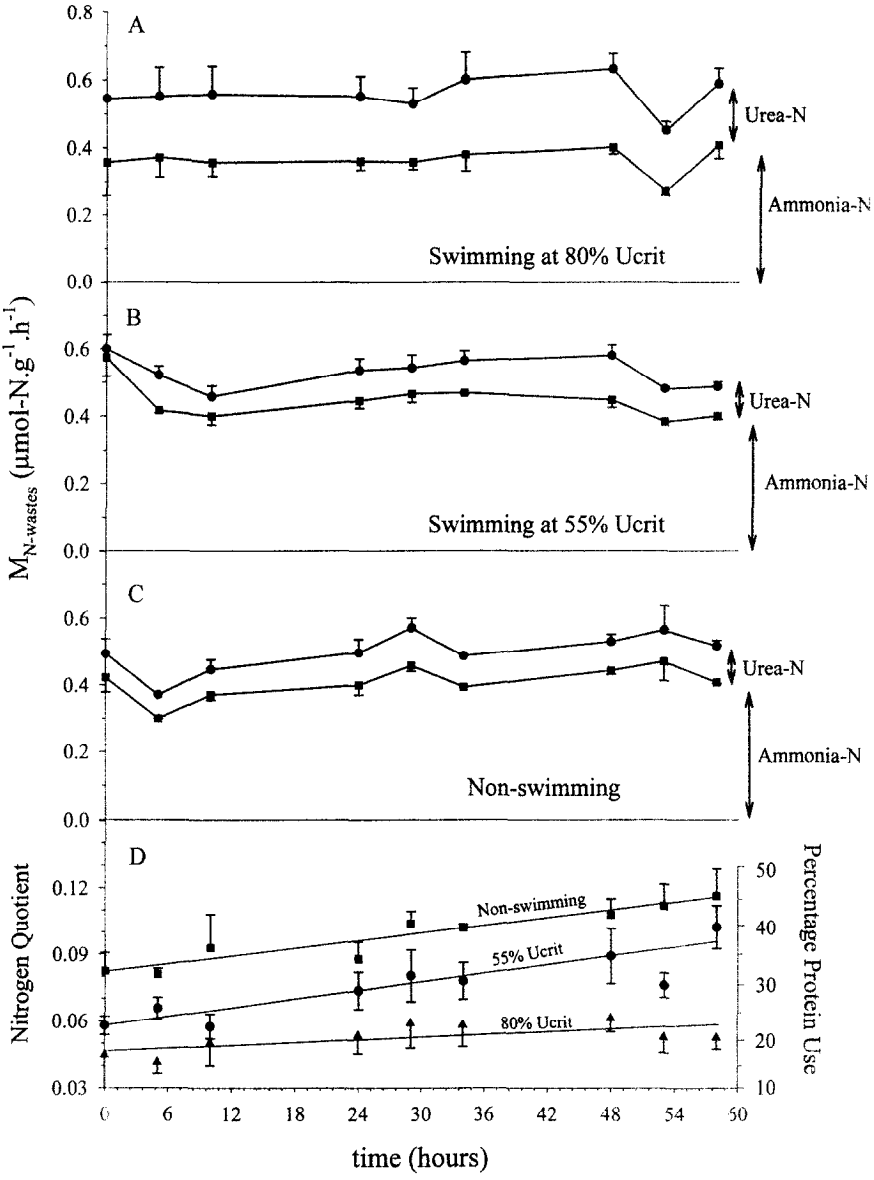

Fig. 3. The influence of aerobic, sustainable exercise on ammonia-N and urea-N excretion at 15°C in juvenile rainbow trout swimming in individual respirometers over a 58-h period. Fish (~ 17 g, 11 cm) were swum at speeds corresponding to (A) 80% or (B) 55% of their maximum sustainable velocity (U_{crit} = 3.84 body lengths·s^{-1}), or (C) were left in a mild current that would not induce swimming. Feeding was suspended 48 h prior to test. Panel (D) shows the percentage use of protein in oxidative metabolism, as calculated from the nitrogen quotient. Note the general lack of change of ammonia-N excretion with speed or time, but the higher urea-N excretion at the highest speed. Means ± SEM. (Data from Lauff and Wood, 1996b. Reproduced with the permission of Springer-Verlag GmBH & Co. Ltd.)

(Lauff and Wood, 1997). It would appear that training actually reorganizes metabolism so as to spare the muscle, with the result that protein growth is promoted over lipid growth in fish that are both fed and continually swum (Christiansen *et al.,* 1989; Lauff and Wood, 1997).

This raises the question of key importance: What happens to nitrogen waste excretion when fish are feeding *during exercise,* which is of course the normal behavior that fish perform naturally? In other words, is the *exogenous* fraction of nitrogen excretion, the portion not retained as growth from the diet, larger, lower, or the same in fish that are swimming during feeding than in fish that are not swimming but are fed the same ration? To my knowledge, the definitive experiment has not been done, for the simple reason that fish tend to become spontaneously more active anyway during feeding, whereas fish that are forced to swim against a current appear to decrease their spontaneous or "nonspecific" activity (e.g., LeGrow and Beamish, 1986; Christiansen and Jobling, 1990), that is, the actual degree of exercise cannot be perfectly controlled. However, in view of the convincing literature that net protein accretion rates, protein conversion efficiency from the diet, and overall growth rates are all improved if fish are continually swimming (Houlihan and Laurent, 1987; Christiansen *et al.,* 1989; Davison, 1989, 1997), it seems likely that the *exogenous* fraction of nitrogen excretion will be lower if fish swim aerobically while feeding; that is, amino acids from the free pool will be funneled preferentially toward protein synthesis, rather than toward deamination and oxidation. Because feeding seems to preferentially elevate the concentrations of essential amino acids in the blood plasma (Brown and Cameron, 1991a; Espe *et al.,* 1993), whereas swimming preferentially elevates nonessential amino acids (Barton *et al.,* 1995), it may be that the combination is most effective in stimulating protein synthesis. Interestingly, the SDA effect of feeding continues unabated (Beamish, 1974; Alsop and Wood, 1997) or may even increase (Muir and Niimi, 1972; Blaikie and Kerr, 1996) during submaximal exercise. Because SDA mainly represents the cost of elevated protein synthesis (see above), this indicates that carbohydrate or lipid are used to a greater extent, not only to power exercise itself, but to power protein synthesis during exercise.

B. Anaerobic Burst Swimming

This type of exercise, while occasionally critical to survival, probably represents only a small portion of the daily energy budget of most fish. However, exhaustive exercise (usually by chasing) has become a favorite model for metabolic and acid-base studies; Wood and Wang (1999) note that more than 200 papers have been written on exhaustively exercised trout alone, and their review provides a guide to recent studies and earlier reviews. For nitrogen metabolism, the key event is a massive and rapid generation of ammonia-N by the white muscle. Almost all of this results from the deamination of adenylates through one arm of the

purine nucleotide cycle; less than 2% is attributable to the depletion of aspartate (Mommsen and Hochachka, 1988). ATP stores are degraded through ADP and the adenylate kinase reaction to AMP, which in turn is deaminated via AMP deaminase, resulting in stoichiometrically equivalent increases in inosine monophosphate (IMP) and ammonia in the intracellular compartment of the muscle (Fig. 4A). Because the intracellular compartment of the white muscle accounts for about 35% of the fish's body mass, and typical increases in intracellular concentrations of ammonia are in the range of 5–10 mmol·liter^{-1} (Wood, 1988; Wright *et al.*, 1988; Mommsen and Hochachka, 1988; Wang *et al.*, 1994, 1996), the load is considerable, around 2500 μmol·kg^{-1} of total body mass. Plasma ammonia levels do increase by as much as fivefold, but on an absolute basis this is a negligible elevation (<200 μmol·liter^{-1}) relative to intracellular levels (Fig. 4A). At least in adult trout, only a modest increase occurs in ammonia-N excretion to the water through the gills amounting to about an extra 200 μmol·kg^{-1}·h^{-1} during the next 4 h (Fig. 4B), and this probably helps clear excess H^+ ions from the blood (Wood, 1988). The time of elevated branchial excretion corresponds to the period during which most of the ammonia is cleared from the muscle, but quantitatively there is a large discrepancy (Fig. 4). Experiments with a perfused tail–trunk preparation have shown that ammonia release rates from muscle to plasma really are low (Wang *et al.*, 1996, 1998) and correspond to the measured excretion rates to the water. The obvious conclusion is that the majority of the ammonia generated is held back inside the muscle cells and removed *in situ*, rather than excreted.

This retention has several obvious advantages: It provides an effective base (NH_3) to buffer H^+ ions produced (with lactate and pyruvate) by glycolysis (Dobson and Hochachka, 1987), it serves to activate phosphofructokinase (Su and Storey, 1994), thereby maintaining glycolytic flux (Mommsen and Hochachka, 1988), and, most importantly, it provides a ready source of ammonia-N for resynthesis of ATP. During recovery, AMP deaminase is shut down, while the other arm of the purine nucleotide cycle (adenylsuccinate synthetase and adenylsuccinate lyase) effectively removes IMP and ammonia, and replenishes the adenylate pool (Mommsen and Hochachka, 1988). Note the inverse symmetry between the removal of IMP and ammonia, and the regeneration of ATP during recovery (Wang *et al.*, 1994; Fig. 4A). Ammonia is actually removed by resynthesis of glutamate from α-ketoglutarate. Glutamate in turn reacts with oxaloacetate (from malate and fumarate) to furnish the required aspartate as the nitrogen donor for adenylate resynthesis, thereby regenerating α-ketoglutarate. It would be interesting to know if there was also excess urea-N production at this time of increased adenylate turnover in white muscle (perhaps via uricolysis, or an interorgan ornithine–urea cycle; see above), but the measurements do not appear to have been made.

Given that ammonia is often considered a highly diffusive molecule, how is it retained in muscle tissue? The answer is discussed in detail by Wood and Wang

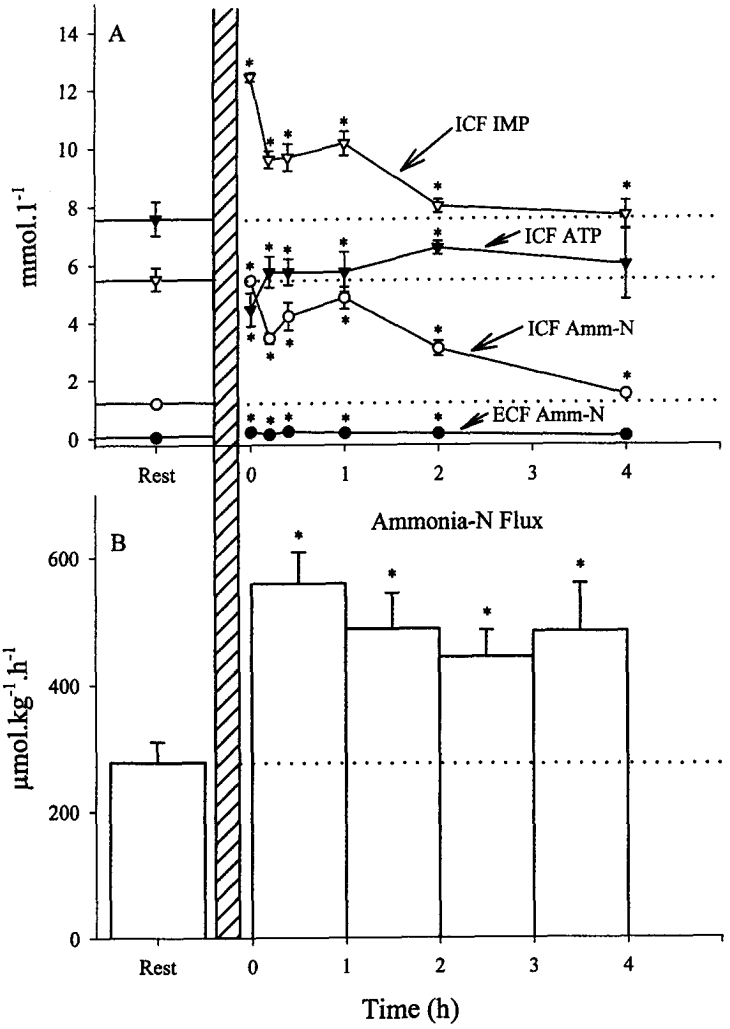

Fig. 4. Changes in (A) inosine monophosphate (IMP), adenosine triphosphate (ATP), and ammonia-N in the intracellular fluid (ICF) of white muscle and ammonia-N in extracellular fluid (ECF = blood plasma); and (B) branchial ammonia-N excretion (M_{Amm-N}) at 15°C in adult rainbow trout (~250 g) following 6 min of exhaustive anaerobic burst exercise. Note the reciprocal changes in ICF ammonia-N and IMP versus ATP, and the much larger changes in ICF ammonia-N than ECF ammonia-N. Note also the small absolute rates of branchial ammonia-N excretion relative to ICF ammonia-N. Means ± SEM. Asterisks indicate significant difference from value at rest. [Data from (A) Wang *et al.* 1994, and (B) Wood, 1988. Both reproduced with the permission of the Company of Biologists Ltd.]

(1999). Very simply, the retention can be explained by passive phenomena, as originally suggested by Wright *et al.* (1988) and Wright and Wood (1988), a theory that was initially quite controversial (c.f. Heisler, 1990). There is no evidence for active retention. The theory derives from physicochemical principles first articulated by Boron and Roos (1976) for the distribution of weak bases where there is significant permeability to the charged form. Ammonia (as NH_4^+) thereby distributes as an ion via the Nernst relationship according to the muscle membrane potential (−85 to −102 mV), rather than as a weak base via the Jacobs-Stewart relationship (Jacobs and Stewart, 1936) according to the pH gradient (0.4–0.8 units), because the NH_4^+ to NH_3 permeability ratio (pNH_4^+/pNH_3) of the muscle cell membrane is relatively high (Wood and Wang, 1999). This means that at passive equilibrium, intracellular total ammonia concentration will be about 35 times higher than extracellular ammonia, rather than about 5 times higher if only the pH gradient were involved. The theory has been confirmed by direct measurements of ammonia distribution ratios and pH gradients *in vivo* in exercised fish (Wright *et al.*, 1988; Wright and Wood, 1988; Tang *et al.*, 1992; Wang *et al.*, 1994), together with determinations of muscle membrane potential *in situ* (Beaumont *et al.*, 2000), and experimental manipulations of both it and transmembrane pH gradients in a perfused tail–trunk preparation (Wang *et al.*, 1996). Indeed, pNH_4^+/pNH_3 appears to actually increase after exhaustive exercise, thereby favoring passive retention (Wang *et al.*, 1994, 1996). This ability to store and tolerate high concentrations of ammonia in the muscle mass may also be of survival value in situations where branchial ammonia excretion is inhibited such as high external pH (Wilkie and Wood, 1995) and copper intoxication (Beaumont *et al.*, 2000), though contractility and swimming performance appear to be negatively impacted (Randall and Brauner, 1991; Beaumont *et al.*, 2000).

Although the above scenario attributes the retained ammonia plus the small excess ammonia-N excretion after exhaustive exercise to adenylate deamination, note that in at least one study on very small trout, excess ammonia-N excretion was much greater, and exceeded ATP depletion by several-fold (Scarabello *et al.*, 1992). In that experiment, electrical stimulation was employed, which may have elevated the stress component and, therefore, cortisol mobilization, as well as possibly depolarizing the membrane potential of muscle so as to favor ammonia washout. Milligan (1997) has shown that even in adult trout exercised to exhaustion without shocks, there is a substantial (up to threefold) but selective mobilization of free amino acids (only 6 of 22 individual amino acids increased) in the blood plasma, white muscle, and liver for up to 4 h after exercise. In the muscle, part of the elevation appeared to occur actually during the exercise itself (see also Storey, 1991). Pharmacological blockade of cortisol elevation completely eliminated this amino acid surge in all compartments, which Milligan (1997) attributed to stimulation of proteolysis in the liver by cortisol. It is conceivable that some of these mobilized amino acids may be deaminated and oxidized to help fuel glyco-

gen resynthesis (and thereby contribute to excess ammonia-N excretion after exercise), while others are directed toward protein synthesis for muscle repair.

V. TEMPERATURE AND NITROGEN METABOLISM

Apart from food availability, daily and seasonal variations in temperature are probably the most important *normal* influence of the environment on nitrogen metabolism in fish. However, in nature it is likely that feeding and temperature are dependent rather than independent variables. Nevertheless, we will start with the simpler situation, those few studies where nitrogen waste excretion has been measured at different temperatures in the absence of feeding.

A. Nonfed Fish

In general, such studies have demonstrated that nitrogen waste excretion is highly sensitive to temperature, much more so than is O_2 consumption, which generally has a Q_{10} less than 2.0. Maetz (1972) reported a Q_{10} of 4.0 for ammonia-N excretion in goldfish abruptly transferred from 16°C to 6°C, but the Q_{10} dropped to 1.9 when the fish were loaded with a great excess of ammonia. Maetz interpreted this to mean that the majority of the acute temperature sensitivity was in the metabolic production mechanism, rather than in the branchial excretion mechanism. In long-term acclimated rainbow trout (Kieffer *et al.*, 1998), the Q_{10} values for ammonia-N excretion between 5°C and 15°C were lower (1.4 to 2.9 in trout at different sustainable exercise levels), but still above those for M_{O2} (1.3–1.5). The Q_{10} remained high even after exhaustive exercise (Kieffer and Tufts, 1996). Similarly, in long-term acclimated Nile tilapia between 15 and 30°C, the Q_{10} for ammonia-N excretion was 2.8 versus 1.7 for O_2 consumption (Alsop *et al.*, 1999). Q_{10} values for urea-N excretion were comparable to those for ammonia-N excretion. Comparably high sensitivities of ammonia-N excretion to temperature have been reported in larval carp (Kaushik *et al.*, 1982), various sturgeon species (Gershanovich and Pototskij, 1996), the plaice (Jobling, 1981b), the surf steenbras (Cockcroft and Du Preez, 1989), and in the mangrove snapper and areolated grouper (Leung *et al.*, 1999). The overall conclusion is that as temperature increases, an increasing percentage of aerobic metabolism in nonfed fish is fueled by the oxidation of protein.

B. Actively Feeding Fish

Here, the situation is much more complex because net protein accretion and nitrogen storage are also occurring. Protein synthesis increases with temperature

(e.g., Fauconneau and Arnal, 1985; Loughna and Goldspink, 1985; Watt *et al.*, 1988). A recent meta-analysis of the literature by McCarthy and Houlihan (1997) indicated that white muscle and whole-body protein synthesis rates actually rise in an exponential fashion as temperature increases, and this conclusion has now been reinforced by an experimental study on a single species, the marine wolffish, fed to satiation at four different acclimation temperatures (McCarthy *et al.*, 1999). Nevertheless, it is likely that this overall relationship disguises some important variation. For example, protein synthesis rates exhibit considerable thermal acclimation, such that the acute temperature-dependent relationship is displaced upward by cold acclimation and vice versa (Loughna and Goldspink, 1985; Watt *et al.*, 1988). Furthermore when food is restricted or withheld entirely, such as in studies by Foster *et al.* (1992) on adult cod and Mathers *et al.* (1993) on rainbow trout fry, the increase in protein synthesis with temperature is either greatly attenuated or does not occur.

The interaction of temperature and ration is well illustrated by our recent work on the long-term responses of rainbow trout to global warming, where climate change has been simulated by adding 2°C to the natural annual thermal cycle for inshore Lake Ontario, Canada. Protein turnover rates proved to be surprisingly sensitive to this small (2°C) temperature difference, increasing on average by 10–30% in gill and liver in four different 3-month seasonal exposures during which trout were fed to satiation (Reid *et al.*, 1995, 1997, 1998; Morgan *et al.*, 1998). Absolute rates were generally lower in the cold winter months (4–10°C) than in the hot summer months (13–24°C), but the stimulation caused by +2°C was much greater in winter. Overall, these increases in protein synthesis, degradation, and net accretion correlated well with similar increases in food consumption. When ration was restricted in one summer study, protein turnover and net accretion rates decreased, rather than increased, in response to a chronic 2°C elevation (Morgan *et al.*, 1999). An additional complexity is that even in the presence of unlimited ration, once a certain critical optimum temperature for protein synthesis is exceeded, then protein accretion rates drop precipitously, largely through an increase in protein degradation rates (Reid *et al.*, 1995, 1997, 1998). The optimal temperature for net protein accretion may be higher than the optimal temperature for net growth because of differing optima for food consumption, protein synthesis, and protein degradation rates (McCarthy *et al.*, 1999).

These global warming simulation studies with rainbow trout appear to be the only temperature investigations in which M_N and M_{O2} measurements have been performed in parallel to protein turnover and feeding measurements. In general, they indicate that nitrogenous waste excretion increases in concert with increases in feeding, protein synthesis, and overall metabolic rate as temperature rizes. The majority of these changes occur in the dominant ammonia-N fraction, rather than in the urea-N fraction. The stimulatory effect of slow seasonal temperature rise on all of these metabolic functions is much greater in the cold winter months than the

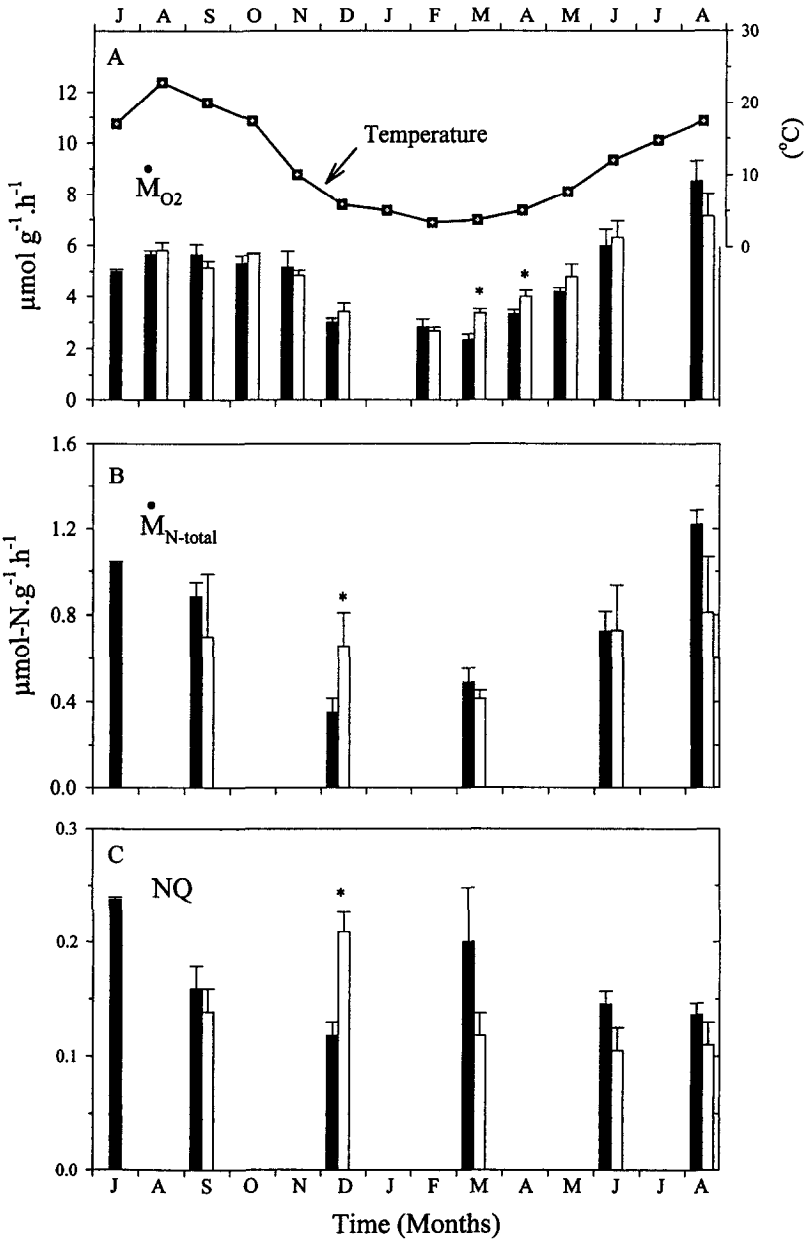

Fig. 5. (A) O_2 consumption (M_{O2}; left axis); (B) total nitrogen excretion ($M_{N\text{-}total}$) as measured with a nitrogen oxidizer; and (C) Nitrogen quotient (NQ) in rainbow trout over a 14-month period during which the fish were exposed to the natural thermal regime of inshore Lake Ontario (solid bars)

warm summer months (Dockray *et al.*, 1996; Linton *et al.*, 1997, 1998a,b). Similarly the stimulatory effect of +2°C on metabolic functions is much greater in the winter, and in both winter and summer it is eliminated when ration is restricted (Dockray *et al.*, 1998; D'Cruz *et al.*, 1998; Linton *et al.*, 1999). In winter, increases in M_N keep pace with or exceed increases in M_{O2}, so the percent of aerobic metabolism fueled by the oxidation of protein stays constant or increases as temperature rises (Linton *et al.*, 1998b; D'Cruz *et al.*, 1998), similar to the pattern discussed earlier for nonfed fish (Kieffer *et al.*, 1998; Alsop *et al.*, 1999). However, in summer, the contribution of protein to aerobic respiration tends to decrease as temperature rises in actively feeding trout (Linton *et al.*, 1997). When protein synthesis drops at critically high temperatures, both nitrogenous waste excretion and metabolic rate fall simultaneously, with the former effect being more marked, so protein oxidation decreases even further (Dockray *et al.*, 1996; Linton *et al.*, 1997). These patterns are well illustrated by periodic measurements of M_{O2} and M_N in the 14-month study of Linton *et al.* (1998a) with satiation-fed trout, a period during which the fish increased their original body mass by 35-fold (Fig. 5)! Note, however, that the "critical high temperature" was lower in the second year than the first, perhaps due to differences in age, size, or thermal history (Fig. 5).

VI. OTHER NITROGEN PRODUCTS?

A. Urea versus Ammonia

A handful of unusual teleosts, such as the Lake Magadi tilapia (Randall *et al.*, 1989; Wood *et al.*, 1989), the gulf toadfish (Mommsen and Walsh, 1989; Walsh, 1997b), and several species of catfish of the Indian subcontinent (Saha and Ratha, 1998), excrete urea as their major nitrogen waste either all the time or under certain environmental conditions. In these cases, urea-N production is attributable to a full expression of the enzymes of the ornithine–urea cycle (OUC) in their liver and sometimes other tissues; details are reviewed by Anderson (Chapter 7) and Ip *et al.* (Chapter 4). The Lahontan cutthroat trout and several other species endemic

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or this natural regime + 2°C (open bars). The natural thermal regime is shown on the right axis in panel (A). The exposure started in early July (J) 1995 and ended in late August (A) 1996. The fish were fed to satiation once per day. Over the experimental period, the fish grew from from ~11 to ~365 g, so the data are scaled to 1 kg using the mass exponent 0.824 determined by Cho (1990). Note the marked changes in absolute rates with the temperature, the stimulatory effect of + 2°C on $M_{N\text{-total}}$ and NQ in winter (December) and the inhibitory effect in summer, especially at peak water temperatures (August). Means ± SEM of 4 replicate tanks for each treatment, each containing approximately 80 fish at the start and 40 at the end of the experiment (reduction due to periodic sampling). Asterisks indicate a significant difference due to + 2°C. (Data from Linton *et al.*, 1998a. Reproduced with permission of the Minister of Public Works and Government Services Canada, 2001.)

to alkaline (pH 9.4) Pyramid Lake, Nevada, USA (Wright *et al.,* 1993; McGeer *et al.,* 1994), and a cyprinid endemic to alkaline (pH 9.8) Lake Van, Turkey (Danulat and Kempe, 1992), excrete urea-N at higher rates than most teleosts,, with corresponding reductions in ammonia-N excretion, but ammonia-N still predominates. Available evidence suggests that these fish produce urea via uricolysis or arginolysis for which the enzymes are present; one or more key enzymes of the OUC are absent.

The focus here, however, is on more representative teleosts living in nonextreme environments. There is general accord that the vast majority of such fish excrete predominantly ammonia-N plus lesser amounts of urea-N, the latter again formed by uricolysis or arginolysis. Wood (1993), Handy and Poxton (1993), and Jobling (1994), in quantitatively reviewing a large number of studies, all noted that there appears to be a general tendency for greater urea-N excretion rates in marine teleosts (up to 40% of ammonia-N) than in freshwater teleosts (5–35%). The reason is unknown; perhaps it allows marine fish to use urea as a minor osmolyte, as suggested by the results of Wright *et al.* (1995b). When challenged with environments that tend to inhibit ammonia-N excretion across the gills (e.g., high external ammonia, high pH), and/or internal ammonia accumulation and alkalosis, many "standard" teleosts (goldfish, various salmonids, killifish, Nile tilapia) exhibit an increase in urea-N excretion, which has usually been attributed to an activation of the enzymes of uricolysis as a mechanism for detoxification of ammonia (Olson and Fromm, 1971; Wood *et al.,* 1989; Wilkie and Wood, 1991, 1996; Wilkie *et al.,* 1993, 1994; Wright, 1993; McKenzie *et al.,* 1999; Patrick and Wood, 1999). However, such fish eventually reestablish a pattern where ammonia-N excretion predominates. Even in the adult largemouth bass, which is unusual in expressing a full complement of the OUC enzymes in its liver, ammonia-N excretion normally predominates, and exposure to high external ammonia evokes only a transient increase in urea-N excretion (Kong *et al.,* 1998).

Conflicting with this general pattern of ammoniotelism, it is now well established that at least two "standard" teleosts (the rainbow trout and the Atlantic cod) living in circumneutral environments produce urea-N during their embryonic stages, apparently via a full expression of the OUC enzymes at this time (Wright *et al.,* 1995a; Korte *et al.,* 1997; Wright and Land, 1998; Chadwick and Wright, 1999). The capacity appears to be lost at some point after hatch, and some of the OUC enzymes, particularly hepatic carbomyl phosphate synthetase III, are no longer present in juveniles and adults (see Chapter 5). However, it is not inconceivable that under certain conditions (e.g., continuous dietary nitrogen loading by intensive feeding), the OUC could continue to function and promote ureotelism in juvenile and adult life in "standard" teleosts such as salmonids. In this regard, there have been several surprising reports from hatchery studies where the effluents from large outdoor ponds containing a known biomass of intensively fed salmonids were analyzed diurnally and seasonally. In one study on chinook

salmon fingerlings (Burrows, 1964), the nitrogenous composition of the effluent changed from almost exclusively urea-N to largely ammonia-N as loading density and temperature increased over the summer. In another study on juvenile coho salmon (McLean and Fraser, 1974), urea-N contributed anywhere from 0 to 78% of the sum of ammonia-N plus urea-N excretion on different days, and at different times of individual days. Urea-N excretion predominated on days of high light intensity, whereas higher densities and temperatures were again associated with a predominance of ammonia-N excretion. These hatchery observations have never been replicated. They appear to have been very carefully performed, but controls for urea production by microbial activity were not carried out. Large-scale interconversion of ammonia to urea (or vice versa) by microbial activity in the ponds seems unlikely but not impossible.

Recently we had the opportunity to perform a similar study at a hatchery, monitoring the outflows from outdoor tanks that each contained ~10,000 juvenile lake char or lake char-brook char hybrids fed by "demand" feeders (M. P. Wilkie, Y. Wang, and C. M. Wood, unpublished results). Both ammonia-N and urea-N excretion underwent dramatic fluctuations during the day, but urea-N excretion was at maximum 35% of the total, and averaged less than 10% overall. However because of cool spring temperatures, daily ration was low (~0.7%·day^{-1}) and nitrogen excretion rates in these char were less than 20% of those reported by Burrows (1964) and McLean and Fraser (1974). Because so few studies have yet been performed on intensively feeding fish under "real-world" conditions, it is important to keep an open mind on this question of possible ureotelism in juvenile and adult teleosts.

B. Other Compounds

An equally important issue is the possibility that nitrogen products *other than* ammonia-N and urea-N might be excreted in amounts that are quantitatively significant. Most workers would probably wish to avoid this issue entirely, because simple spectrophotometric assays exist for ammonia-N and urea-N that are sensitive, rapid, and economical; the same is not true for the tedious, more expensive procedures for measuring total nitrogen and the variety of possible other compounds that could contribute to the total. Nevertheless, there are two reasons to suspect that other nitrogen products may be excreted. The first is the marked discrepancy observed between protein oxidation measured respirometrically by the instantaneous approach from the excretion of ammonia-N and urea-N (see Section III) and the much larger amount estimated from the compositional approach by protein depletion from the carcass (Lauff and Wood, 1996a). This discrepancy applies not only to the loss of protein nitrogen, but also to the total loss of calories from the carcass, which may greatly exceed that estimated from O_2 consumption integrated over the test period (Krueger *et al.*, 1968; Brett, 1995; Lauff and Wood,

1996a). The second line of evidence comes from those few studies (all in the older literature!) that have actually measured total nitrogen excretion (by Kjeldahl digestion or a nitrogen oxidizer) and compared it directly to the sum of ammonia-N plus urea-N excretion. In the Pacific staghorn sculpin (Wood, 1958) and two species of anchovies (McCarthy and Whitledge, 1972), 13%–19% of total nitrogen excretion remained unaccounted for, even after small measured excretions of creatine/creatinine-N and trimethylamine/ trimethylamine oxide-N were subtracted. Other reported discrepancies were 23–31% in two carp specimens (Smith, 1929), 12% in the surf steenbras (Cockcroft and Du Preez, 1989), 23–32% in Atlantic salmon and Arctic char (Fivelstad *et al.*, 1990), and 24% in the rainbow trout (Olson and Fromm, 1971). The latter study identified virtually all of this discrepancy (23%) as the excretion of protein nitrogen. Feeding was not controlled in these studies, but Beamish and Thomas (1984) reported data indicating that the discrepancy varied on different dietary regimes in rainbow trout.

Recently, we have started to measure total nitrogen excretion in our metabolic work with trout (G. DeBoeck, D. Alsop, and C. M. Wood, unpublished) and have invariably found that it exceeds the sum of ammonia-N plus urea-N excretion (Fig. 6). The discrepancy is highly variable, but it tends to be greater (sometimes

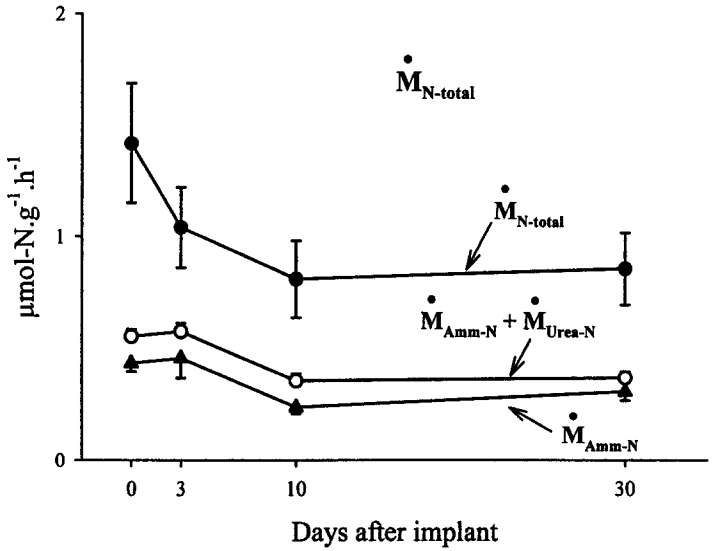

Fig. 6. A comparison of direct measurements of total nitrogen excretion ($M_{N\text{-}total}$) as determined with a nitrogen oxidizer versus the sum of ammonia-N excretion ($M_{Amm\text{-}N}$) and urea-N excretion ($M_{Urea\text{-}N}$) at 14°C in rainbow trout (10–20 g) over a 30-day treatment following placement of a coconut oil implant in the peritoneal cavity. This was a sham treatment in an endocrine study; the elevation in rates at days 0–3 was probably due to the disturbance of the initial surgery. The fish were fed a daily ration corresponding to approximately half of satiation. Means ± 1 SEM. (Unpublished data of G. DeBoeck, D. Alsop, and C. M. Wood.)

greater than twofold the sum of ammonia-N plus urea-N) in actively feeding fish, and declines with starvation. An identical pattern has also been seen in the Pacific midshipman (Walsh *et al.,* 2001). This finding, of course, helps reconcile the discrepancies in the instantaneous versus the compositional approaches to protein oxidation, but raises numerous important questions. What is the unmeasured nitrogen product? How is it excreted? Should it be considered a nitrogenous product of protein oxidation? The latter is perhaps the most important, because if it really is a product of aerobic respiration, then most of what is written about the generally low reliance on protein as a metabolic fuel in Section III may well be wrong! For example, in the data set of Fig. 6 (where O_2 consumption was measured simultaneously), protein oxidation would account for only about 22% of metabolism based on ammonia-N plus urea-N excretion, but around 50% based on total nitrogen excretion.

Our present working hypothesis is that the original findings of Olson and Fromm (1971) are correct, and that most of the discrepancy represents protein nitrogen (or amino acid nitrogen) excretion—that is, nonoxidized moieties. If this is the case, then we are correct in not using this "extra nitrogen" in fuel use calculations, but it nevertheless represents an important vehicle for both calorie and nitrogen losses. Amino acids could be lost by "leakage" across the gills as plasma levels surge after feeding (Brown and Cameron, 1991a; Espe *et al.,* 1993), together with metabolic rate, gill blood flow, and perhaps gill permeability to amino acids. Nevertheless, it makes little teleological sense to waste such a valuable energy source in this fashion. More likely, the major portion of the unidentified nitrogen reflects loss of mucoprotein, an unavoidable cost of an aquatic lifestyle. In the kelp bass, 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. In rainbow trout, Miller and MacKay (1982) reported net mucous excretion rates to the water under control conditions averaging an incredible 4 $g \cdot kg^{-1} \cdot h^{-1}$, which increased by about 60% after exposure to pH 3.6–4.0. Given any reasonable estimate of nitrogen content for mucus, these mucoprotein excretion rates would dwarf the sum of normal ammonia-N plus urea-N excretion rates. Clearly, more work is needed in this potentially important area.

VII. EXCRETION MECHANISMS

The majority of ammonia-N and urea-N is excreted across the gills rather than through the kidney (see Wood, 1993, for a quantitative summary of many studies); the skin may also play some small role, especially in marine teleosts. The "unmeasured nitrogen" excretion (see Section VI) also appears to be via the gills and/or skin, at least based on the original bladder catheterization experiments of Smith (1929). On a relative basis, ammonia-N excretion through the kidney may increase greatly in response to metabolic acidosis, largely by tubular secretion (Wood

et al., 1999), but this remains a small percentage of the overall total. Renal excretion of urea-N appears to be minimized by active tubular reabsorption, at least in the freshwater rainbow trout (McDonald and Wood, 1998). Branchial excretion of urea-N has traditionally been considered to occur by simple diffusion. However, in light of recent findings of a facilitated diffusion transporter for urea-N in the gills of the ureotelic gulf toadfish, it is possible that such transporters might also occur in the gills of a wide variety of "standard" ammoniotelic teleosts (see Chapter 8 and Walsh *et al.*, 2001). Certainly, urea-N excretion across the gills of rainbow trout can be greatly accelerated in response to exogenous loading, thereby maintaining homeostasis of plasma urea-N concentrations (McDonald and Wood, 1998). Interestingly, there is one report of urea-N concentration in the chloride cells of gills of the European eel in seawater (Masoni and Garcia-Romeu, 1972).

The area of greatest research focus and controversy has been the mechanism(s) of ammonia-N excretion across the gills. The literature and arguments surrounding this issue have been reviewed in detail by Walsh (1997a) and Wilkie (1997), and will not be revisited here. In seawater, the situation remains unclear: apical Na^+/NH_4^+ exchange, passive transcellular NH_3 diffusion and paracellular NH_4^+ diffusion, and substitution of NH_4^+ for K^+ on basolateral Na^+,K^+/ATPase, and $Na^+,K^+/2Cl^-$ cotransporters may all be important. However, in freshwater, it now appears that a consensus may be emerging. Wilkie (1997) concludes: "In freshwater, ammonia excretion likely takes place via passive NH_3 diffusion down favourable blood-gill water P_{NH3} gradients—facilitated by acidification of the expired gill water—by carbonic anhydrase-catalyzed hydration of CO_2. The likely absence of apical electroneutral Na^+/H^+ exchange probably rules out a role for (apical) Na^+/NH_4^+ exchange—and the low cationic permeability of freshwater gills makes significant diffusion of NH_4^+ unlikely." In light of most available evidence, this conclusion appears very reasonable. However, a note of caution must be sounded. Virtually all the data on which this is based comes from starved, quiescent fish, confined in boxes. Plasma total ammonia levels in such fish are usually very low, often less than 100 μmol·liter^{-1} when obtained by chronic cannulation (see Wood, 1993, for a critique of methods). Unfortunately, there appear to be no chronic cannulation data for actively feeding fish, but plasma ammonia concentrations obtained by caudal puncture rise dramatically after a meal (Kaushik and Teles, 1985), and may be over 500 μmol·liter^{-1} in satiation-fed trout (Linton *et al.*, 1997). Even in starved trout (cannulated), plasma ammonia levels may reach 200–400 μmol·liter^{-1} after intensive exercise (Wood, 1988; Wright and Wood, 1988; Wang *et al.*, 1994). Heisler (1990) reported that the excellent relationship between the blood-to-water P_{NH3} gradient and net branchial ammonia-N flux in cannulated trout broke down when plasma total ammonia levels exceeded 200 μmol·liter^{-1}, and suggested that a threshold (i.e., >200 μmol·liter^{-1}) was passed for activation of an outward transport mechanism, additional to the role of simple diffusion.

There are numerous reports in the literature of "apparent" Na^+/NH_4^+ exchange; for example, in one study (Wright and Wood, 1985; further analyzed by Wood, 1989), Na^+ uptake and ammonia excretion were linearly related once the diffusive movement of NH_3 was subtracted. It is also known that raising blood total ammonia levels by infusion of ammonium salts markedly stimulates Na^+ uptake in approximate proportion to the increase in net ammonia excretion (e.g., Wilson *et al.*, 1994). Although this was originally explained as a side effect of the accompanying acidosis (increased H^+-linked Na^+ uptake, and associated diffusion trapping of NH_3 in the gill water by elevated H^+ excretion; Wilson *et al.*, 1994), recent experiments have shown that the same phenomenon occurs even when NH_4HCO_3 is infused, which induces alkalosis rather than acidosis (Salama *et al.*, 1999). The Na^+ uptake/ammonia-N excretion linkage appears to be direct but loose, because pharmacological blockade of Na^+ uptake only slightly reduces ammonia-N excretion under these conditions (Wilson *et al.*, 1994). Similarly, experimentally varying Na^+ uptake rate by rapidly changing water Na^+ levels has only a minor influence on net ammonia-N flux (Salama *et al.*, 1999). In trout, Na^+ uptake is stimulated during sustained exercise (Postlethwaite and McDonald, 1995) and after exhaustive exercise (Wood, 1988). It would be interesting to know if the same phenomenon occurs after feeding. Some sort of mechanistic linkage between active Na^+ uptake and ammonia-N excretion across the gills remains a real possibility under natural conditions of ammonia loading from feeding and/or exercise.

VIII. ELASMOBRANCHS

The elasmobranchs are often viewed as a parallel evolutionary line to the teleosts, and there has been much theoretical interest in the comparison of their adaptive strategies relative to those of teleosts. For example, Kirschner (1993) concluded that the energetic efficiency of their evolutionary "choice" to retain urea for osmoregulation (Smith, 1936) so as to avoid drinking seawater was about equal to that of the teleost "choice" to drink seawater and excrete the excess salt at the gills. However, this theoretical interest has translated into remarkably few experimental studies, probably because elasmobranchs have little importance in aquaculture or recreational and commercial fishing. Earlier reviews have focused on their nitrogen metabolism (Perlman and Goldstein, 1988; Mommsen and Walsh, 1991; Wood, 1993; Wright, 1995; Goldstein and Perlman, 1995), and recently Ballantyne (1997) has summarized what is known about their overall metabolism in great detail. From these, it is clear that almost nothing is known about the effects on nitrogen metabolism of the three factors that form the focus of this chapter: feeding, exercise and temperature. The main purpose of this section is to draw attention to this deficit in the hope of stimulating new research.

Available information indicates that all marine elasmobranchs are strongly ureotelic, reflecting a full expression of the OUC in the liver (Chapter 7). The OUC is also present in the freshwater rays of the Amazon basin (Potomotrygonidae family), but these unusual fish are ammoniotelic, excreting large amounts of ammonia-N and small amounts of urea-N just like most teleosts (e.g., Goldstein and Forster, 1971; Barcellos *et al.*, 1997). Urea is not retained in the blood fluids of freshwater rays. In contrast, marine elasmobranchs in full-strength seawater retain 300–500 mmol·liter^{-1} of urea (600–1000 mmol·liter^{-1} of urea-N) in their blood plasma, and excrete large amounts of urea-N and very small amounts of ammonia-N, almost exclusively at the gills (e.g., Wood *et al.*, 1995; see also Chapter 8). Trimethylamine oxide excretion may also be important in nitrogen balance, amounting to 10–20% of urea-N excretion (Goldstein and Palatt, 1974). Renal nitrogen waste excretion is small because of apparent active reabsorption of urea-N at the kidney (Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen *et al.*, 1972). Given the massive blood-to-water urea gradient across the gills, it is remarkable that urea-N excretion is not greater. Wood *et al.* (1995) calculated that branchial urea-N permeability in the dogfish shark was only about 7% of that in a typical teleost, while branchial ammonia-N permeability was only 4%! Based only on circumstantial evidence, branchial urea-N impermeability has been variously attributed to an unusual lipid composition of gill membranes (Boylan, 1967), an active "back-transport" mechanism in gill (Wood *et al.*, 1995), or a combination (Pärt *et al.*, 1998). New direct evidence for the presence of an ATP-dependent Na-urea antiporter and an unusually high cholesterol:phospholipid ratio in the basolateral membrane of the shark gill indicates that both factors are important (Fines *et al.*, 2001). Branchial ammonia-N impermeability has been attributed to a "scavenging" by high-affinity glutamine synthetase in the gills (Wood *et al.*, 1995). It seems likely, therefore, that the system is primarily designed to retain nitrogen, rather than to excrete it!

All the above work has been performed on nonfed fish confined in chambers. It would be very interesting to know what happens when a marine elasmobranch feeds. Haywood (1973) found that plasma urea concentrations progressively declined and osmoregulation was impaired when pyjama sharks were starved, but both recovered quickly after refeeding (Fig. 7). Plasma urea similarly fell, and both alanine and ammonia were released into the blood by the caudal musculature during starvation in the spiny dogfish (Leech *et al.*, 1979). Armour *et al.* (1993) fed lesser spotted dogfish on high-protein and low-protein diets for 1 month. Plasma urea concentrations were unaffected in the low-protein diet animals, but [^{14}C]urea turnover studies indicated decreased metabolic production and clearance rates of urea-N, plus an inability to elevate plasma urea concentration in the face of an osmotic challenge. These data reinforce the idea that elasmobranchs may be nitrogen limited, with a resultant strategy aimed primarily at nitrogen retention, but unfortunately nitrogen excretion rates were not measured in any of

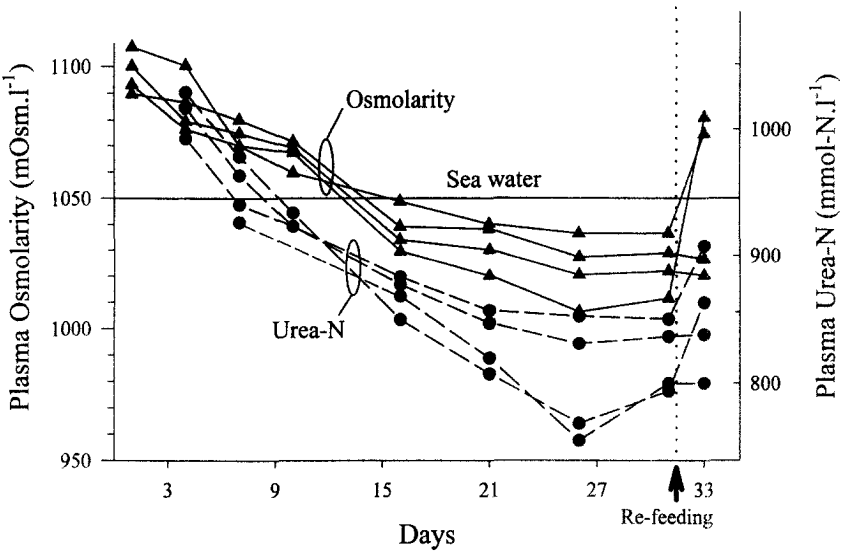

Fig. 7. The effect of progressive starvation for 30 days on plasma osmolarity (triangles; left axis) and plasma urea-N concentration (circles; right axis) at 13°C in four previously well-fed pyjama sharks (1–3 kg). On day 30, two of the sharks were fed (vertical dotted line), and their plasma osmolarity and urea-N levels immediately rose; the other two were not refed and exhibited no change. (Data recalculated from Haywood, 1973. Reproduced with the permission of Springer-Verlag GmBH & Co. Ltd.)

these studies. Mommsen and Walsh (1991) speculated that since urea-N is much more costly to make than ammonia-N, it would make sense for elasmobranchs to excrete extra *exogenous* nitrogen, over and above the needs of osmoregulation, in the form of ammonia-N rather than urea-N. The only information available to answer this speculation comes from a study in which dogfish shark were infused with ammonia-N (as neutralized NH_4Cl) at a rate of 1500 μmol·kg^{-1}·h^{-1} for 6 h (Wood *et al.*, 1995). Based on ration measurements in the wild for this species (Tanasichuk *et al.*, 1991), the dietary nitrogen load is normally about 1000 μmol·kg^{-1}·h^{-1}, so this nitrogen loading rate appears to be quite reasonable. Both ammonia-N and urea-N excretion rose to similar extents during infusion, though the former more rapidly, and the entire ammonia-N load (actually 132%) was excreted within 18 h. When urea-N was infused at a fourfold higher rate (6000 μmol·kg^{-1}·h^{-1} for 6 h), essentially none of it was excreted. Thus it appears likely that Mommsen and Walsh (1991) may have been at least partially correct, but NH_4Cl infusions are very different from natural feeding.

It would also be interesting to know what happens when a marine elasmobranch exercises, because glutamine appears to be a preferred oxidative fuel of muscle (Ballantyne, 1997), so nitrogen waste production might be expected to

increase. However, there appear to be no measurements of nitrogen excretion in elasmobranchs performing sustainable aerobic exercise. Following exhaustive anaerobic exercise, Holeton and Heisler (1983) reported that ammonia-N excretion remained unchanged at the very low pre-exercise rate for up to 30 h postexercise in the larger spotted dogfish; urea-N excretion was not measured.

Very little is known about temperature effects. Acute temperature changes in the range of 1–15°C had no effect on branchial urea-N excretion in an externally irrigated, otherwise intact preparation of the gills of the spiny dogfish, but above 15°C, urea-N efflux increased steeply with a Q_{10} of about 6.0 up to 30°C (Boylan, 1967). Because only the temperature of the water irrigating the gills was apparently changed, and not the temperature of the rest of the dogfish, it is unlikely that these effects reflected alterations in the rate of metabolic production by the liver. In light of recent studies (Pärt *et al.*, 1998; Fines *et al.*, 2001), this breakpoint phenomenon can be interpreted as the result of a "phase change" in the gill lipids and/or a breakdown in the back-transport mechanism in the gill at higher temperatures. Ammonia-N flux was not measured in this study, but Heisler (1978) reported that it was extremely low and did not change when larger spotted dogfish were subjected to step temperature changes up or down between 10 and 20°C.

IX. CONCLUDING REMARKS

There are more than 22,000 species of teleosts and 600–700 species of elasmobranchs in the world, many of them now threatened by overfishing, climate change, and habitat destruction. Elasmobranchs are particularly vulnerable; this ancient group is now being decimated by collection for cancer therapies, high seas drift nets, souvenirs, and "live-finning." With teleosts, metabolism has been examined in perhaps several hundred species, enough to establish "standard" and unusual patterns, but few of these studies have been performed under natural conditions where fish eat, swim, and undergo temperature changes. With elasmobranchs, so few species have been studied that we do not even know what a "standard" pattern is, and the influence of feeding, exercise, and temperature remain virtually unknown. It is hoped that all these trends will be reversed in the new millennium.

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