The Dogfish Shark (*Squalus acanthias*) Increases both Hepatic and Extrahepatic Ornithine Urea Cycle Enzyme Activities for Nitrogen Conservation after Feeding

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ABSTRACT

Urea not only is utilized as a major osmolyte in marine elasmobranchs but also constitutes their main nitrogenous waste. This study investigated the effect of feeding, and thus elevated nitrogen intake, on nitrogen metabolism in the Pacific spiny dogfish *Squalus acanthias*. We determined the activities of ornithine urea cycle (O-UC) and related enzymes in liver and nonhepatic tissues. Carbamoyl phosphate synthetase III (the rate-limiting enzyme of the O-UC) activity in muscle is high compared with liver, and the activities in both tissues increased after feeding. The contribution of muscle to urea synthesis in the dogfish body appears to be much larger than that of liver when body mass is considered. Furthermore, enhanced activities of the O-UC and related enzymes (glutamine synthetase, ornithine transcarbamoylase, arginase) were seen after feeding in both liver and muscle and were accompanied by delayed increases in plasma urea, trimethylamine oxide, total free amino acids, alanine, and chloride concentrations, as well as in total osmolality. The O-UC and related enzymes also occurred in the intestine but showed little change after feeding. Feeding did not change the rate of urea excretion, indicating strong N retention after feeding. Ammonia excretion, which constituted only a small percentage of total N excretion, was raised in fed fish, while plasma ammonia did not change, suggesting that excess ammonia in plasma is quickly ushered into synthesis of urea or protein. In conclusion, we suggest that N conservation is a high priority in this elasmobranch and that feeding promotes ureogenesis and growth. Furthermore, exogenous nitrogen from food is converted into urea not only by the liver but also by the muscle and to a small extent by the intestine.

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Introduction

To achieve osmotic equilibrium with their surroundings, marine elasmobranchs utilize nitrogenous waste products as osmolytes. The Pacific spiny dogfish (*Squalus acanthias*), like many other marine elasmobranchs, retains urea as the predominant osmolyte in the body (Wood et al. 1995). Urea accounts for one-third to one-quarter of total osmolytes in plasma and muscle, with trimethylamine oxide (TMAO) and amino acids also contributing significantly to elevated osmotic status (Robertson 1975).

There are two primary pathways for urea synthesis, the ornithine urea cycle (O-UC) and purine metabolism. In *S. acanthias*, most of the urea is synthesized via the O-UC (Schooler et al. 1966), the pathway that predominates for urea synthesis in most elasmobranchs, ureogenic teleosts, and terrestrial vertebrates. Nevertheless, differences exist in the nature of the O-UC between elasmobranchs and terrestrial vertebrates. The major distinction is that the O-UC in elasmobranchs requires glutamine as the nitrogen-donating substrate, with the enzyme carbamoyl phosphate synthetase III (CPS III) catalyzing the entry of nitrogen into the cycle. In mammals, ammonia is the nitrogen donor, and CPS I is the enzyme initiating urea synthesis. Glutamine synthetase (GS) catalyzes the synthesis of glutamine from ammonia and glutamate and is considered to be a major point of regulation involved in promoting ureogenesis in some ureogenic teleostean fishes (Anderson and Walsh 1995; Walsh and Milligan 1995; Iwata et al. 2000; Kong et al. 2000). Subsequently, glutamine is a substrate for CPS III, the rate-limiting enzyme of the O-UC (Casey and Anderson 1985). Although, in general, the liver is the site of urea synthesis via the O-UC in mammals and fish, it has been reported that some ureogenic fish also express the O-UC suite of enzymes in nonhepatic tissues and that even some nonureogenic teleosts

Table 1: Mass of food recovered in fed *Squalus acanthias*

	6 h	20 h	30 h	48 h
Solid food in stomach	3.2 ± .4 (11)	2.2 ± .4 (11)	3.6 ± .6 (7)	1.8 ± .5 (9)
Minimum–maximum	1.2–4.9	.1–3.6	1.8–5.5	.7–5.5
Vomited solid food in tank	3.3 ± 1.1 (3)	2.9 ± 1.5 (2)	…	4.1 ± 1.4 (3)
Minimum–maximum	1.2–5.2	1.5–4.4	…	1.2–5.5
Vomited and stomach food	3.7 ± .6 (11)	2.5 ± .4 (11)	3.6 ± .6 (7)	3.2 ± 1.0 (9)

Note. Units are percent body mass. Values are means ± 1 SEM (*N*).

possess detectable levels of CPS III in muscle (for review, see Anderson 2001). Recently, Steele et al. (2005) reported that the O-UC was present in the skeletal muscle of the elasmobranch *Raja erinacea*, and they hypothesized that it was important in scavenging muscle-produced ammonia for urea production. Furthermore, Tam et al. (2003) reported that Asian freshwater stingray (*Himantura signifier*) has a full set of O-UC enzymes not only in the liver but also in the stomach.

While urea is of great importance as an osmolyte to marine elasmobranchs, it is also energetically expensive to produce. To synthesize 1 mol of urea via the O-UC, 5 mol of ATP need to be hydrolyzed to ADP (Anderson 2001). As a result, the excretory organs of elasmobranchs (kidney and gills) are specialized to reduce urea permeability and to actively retain urea (Schmidt-Nielsen et al. 1972; Pärt et al. 1998; Fines et al. 2001; Morgan et al. 2003). Paradoxically, urea also constitutes the main nitrogenous waste of elasmobranchs. Wood et al. (1995) have reported that *S. acanthias* excretes most of its nitrogen waste as urea (>97%) after 1–3 wk starvation.

Despite the constant slow "leak" of urea from the animal as nitrogen waste, *S. acanthias* is able to survive at least 41 d without feeding (Cohen et al. 1958; Leech et al. 1979). During this period, plasma urea levels and osmolality slowly fall, with losses of the former ranging from 8% over 19–25 d in *S. acanthias* (Leech et al. 1979) to about 18% over 31 d in the pajama shark *Poroderma africanum* (Haywood 1973), followed by a rapid rise on refeeding. However, it remains unknown what happens to nitrogen excretion after feeding. On the one hand, Mommsen and Walsh (1991) have speculated that elasmobranchs may excrete excess nitrogen mainly as ammonia at the time so as to minimize cost. On the other hand, Wood (2001) has argued that elasmobranchs may be nitrogen limited in nature and therefore would attempt to minimize ammonia excretion by converting as much "excess" nitrogen as possible to urea after a meal.

The feeding ecology of *S. acanthias* is relatively well studied (Jones and Geen 1977; Hanchet 1991; Tanasichuk et al. 1991; Cortés 1999; Laptikhovsky et al. 2001; Koen Alonso et al. 2002). It is clear that food supply is unpredictable for this carnivore, such that daily ration may vary greatly temporally and spatially, and feeding may occur only at irregular intervals. With this background in mind, we investigated the effect of feeding, and thus elevated nitrogen intake, on nitrogen metabolism in *S. acanthias*. We determined (i) urea and ammonia excretion rates, (ii) concentrations of nitrogen compounds in plasma, and (iii) enzyme activities related to urea synthesis in both liver and extrahepatic tissues in starved and naturally fed animals at various times after feeding.

Material and Methods

Experimental Animals

Spiny dogfish (*Squalus acanthias* L., 1.9–4.0 kg) were obtained by trawl in Barkley Sound, British Columbia, Canada, in July and August 2003. Dogfish were held at the Bamfield Marine Sciences Centre for 1–3 wk before experimentation in a 200,000-L circular tank served with running sea water at the experimental temperature (11° ± 1°C), salinity (30‰ ± 2‰), and pH (7.90 ± 0.15). We found that dogfish would not feed when held in smaller tanks or when isolated, but when held in a large group (approximately 100 fish) in this very large tank, a few dogfish would start feeding after about 1 wk in captivity; thereafter, the others would quickly learn to do so. Twice weekly, the animals were fed a ration equivalent to 2% of the estimated total biomass of all the dogfish in the circular tank, most of which was quickly devoured (fed treatment). Food consisted of whole dead trawl fish (mainly flatfish, herring, and eelpout). Samples of food were taken, minced with a food processor, and frozen at −20°C for later content analysis, as reported by Wood et al. (2005). One hour after feeding in the large circular tank, fed fish were then transferred to individual 40-L polyurethane-coated wooden boxes (seawater flow = 1 L min^{-1}), as described by Wood et al. (1995, 2005). Some fish (starved treatment) were removed from the circular tank to a separate 1,500-L tank 1 wk before the start of an experiment, not fed during this separation period, and then transferred to the 40-L boxes.

Experimental Design

Nitrogen Excretion. This series examined the effects of feeding on nitrogen excretion over 48 h. Dogfish ($N = 8$) were fed and after 1 h transferred to the 40-L boxes. A 1-wk starved group ($N = 12$) was also transferred. Measurement started after an

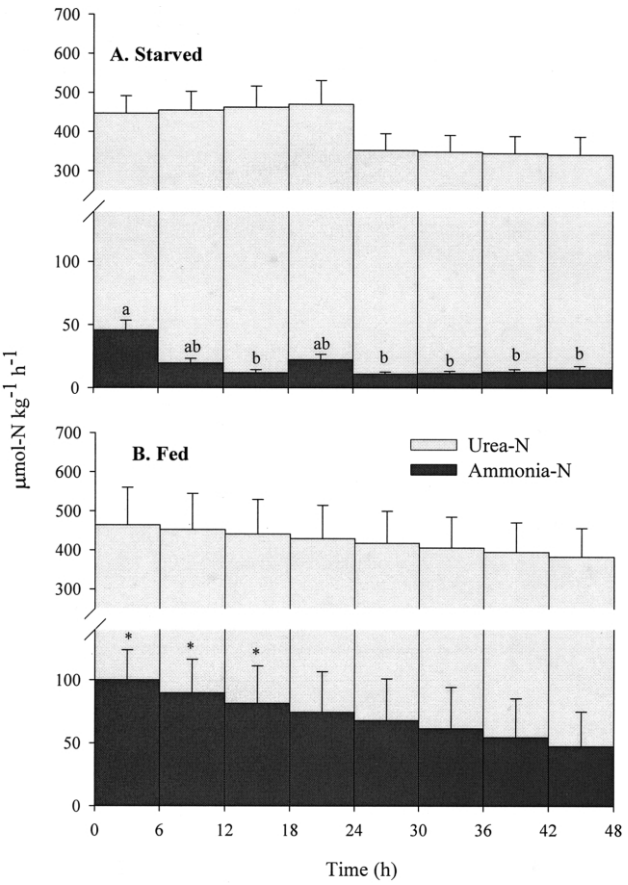

Figure 1. Nitrogen excretion rates of starved (*A*) and fed (*B*) *Squalus acanthias* (μmol N kg^{-1} h^{-1}). Time 0 h represents 2 h after the start of feeding and 1 h after transfer to the experimental boxes. Data are presented as means ± SEM. Asterisks indicate significantly different from starved treatment (Mann-Whitney test, $P < 0.05$). Fed: $N = 8$. Starved: $N = 12$. Within each treatment, means not sharing the same letter are significantly different (Friedman's test followed by Scheffé's post hoc test, $P < 0.05$). The absence of any letters implies that there were no significant changes observed in the parameter presented.

additional 1-h settling period in the boxes. Therefore, time 0 h represents 2 h after the start of feeding and 1 h after transfer to the experimental boxes. Water samples (15 mL) were drawn from the boxes at approximately 6-h intervals for ammonia and urea-N measurements. In this report, ammonia refers to the sum of NH_3 and NH_4^+. The box was also thoroughly flushed every 6–12 h by filling and partially emptying (to 8 L) three times over a 15-min period, without exposing the fish to air. Water samples were analyzed immediately or frozen at −20°C for later determination of ammonia and urea-N concentrations. After 48 h, fish were anesthetized with MS-222 (0.2 g L^{-1}) and weighed.

Blood and Tissue Sampling. This series examined the effect of feeding on blood chemistry and key enzymes of nitrogen metabolism in the tissues. Dogfish were fed and then transferred to the 40-L boxes as described above. Separate groups were killed at 6 h (4–8 h, $N = 15$), 20 h (19–22 h, $N = 13$), 30 h (29–31 h, $N = 8$), and 48 h (46–50 h, $N = 10$) after feeding. A starved group ($N = 14$) was also examined. Fish were anesthetized with MS-222, weighed, and placed on an operating table, and blood samples (by caudal puncture with a heparinized 10-mL syringe) and tissue samples were taken. Ten tissues were dissected: liver, muscle, gills, intestine, esophagus, anterior stomach (stomach 1), posterior stomach (stomach 2), brain, kidney, and rectal gland, the latter being part of a separate study. Kidney samples were taken only from starved fish and at 48 h postfeeding. Plasma samples, obtained by centrifugation at 9,000 *g* for 2 min, were frozen at −80°C for later analyses. Tissue samples were frozen immediately with liquid nitrogen and then stored at −80°C. The fed treatment received food amounting to 2% of the estimated body mass for the entire population of the 200,000-L tank. Food remaining in the stomach and intestine was collected into a dish and weighed. Most of the solid food was in the stomach. After removal of the food, stomach and intestine were washed with a cold saline solution and frozen at −80°C. Some fish had both empty stomach and empty intestine contents and were excluded from the analysis, since we could not be sure that they had fed. These fish comprised four out of 15 fish at 6 h, two out of 13 fish at 20 h, one out of eight fish at 30 h, and one out of 10 fish at 48 h after feeding. In addition, any solid food that was vomited into the boxes during the experiments was collected and weighed.

Analytical Techniques

Samples of food for protein, ammonia, total free amino acids (FAA), urea-N, TMAO, and creatinine analysis were prepared according to the methods used by Kajimura et al. (2002) and analyzed as described by Wood et al. (2005). Ammonia in water samples was determined by a salicylate-hypochlorite method (Verdouw et al. 1978). Plasma ammonia was measured enzymatically on the first thaw of nondeproteinized plasma using glutamate dehydrogenase (Raichem, ammonia reagent, product 85446). Urea-N concentrations in water and plasma were measured with a diacetyl-monoxime method (Rahmatullah and Boyde 1980). Osmolality was determined using vapor pressure osmometry (Wescor 5100C). FAA levels were measured by ninhydrin assay (Moore 1968), with subtraction of previously measured ammonia, owing to the partial detection of ammonia by the ninhydrin method (Kajimura et al. 2004). Plasma contained only a small proportion of ammonia (approximately 1% relative to FAA). TMAO levels were assayed by the ferrous sulfate and EDTA method (Wekell and Barnett 1991). Protein concentrations in plasma were measured by the dye-binding method of Bradford (1976) using Sigma reagent and bovine serum albumin (Sigma) as standards. Since the standard contains 0.155–0.165 g N g^{-1}, an N content of 0.16 g N g^{-1} protein

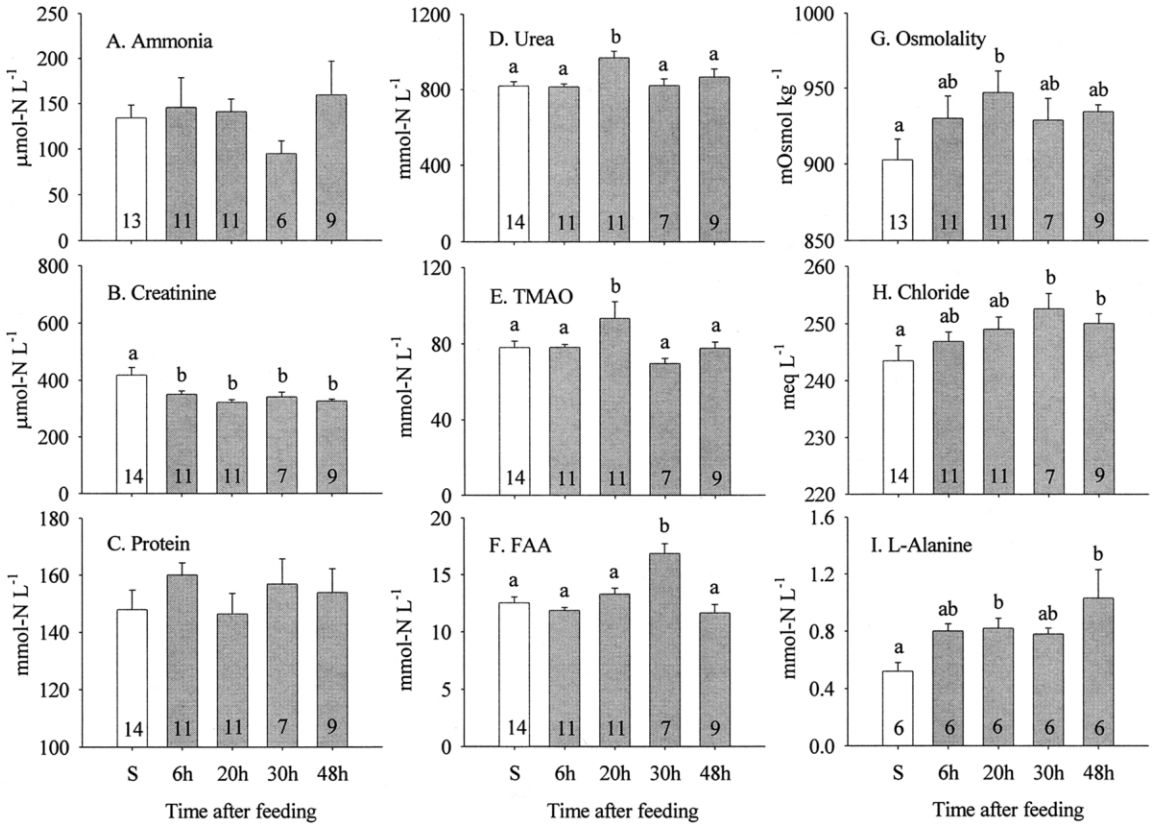

Figure 2. Plasma parameters of ammonia-N (*A*), creatinine-N (*B*), protein-N (*C*), urea-N (*D*), TMAO-N (*E*), FAA-N (*F*), osmolality (*G*), chloride (*H*), and L-alanine-N (*I*) in starved and fed dogfish *Squalus acanthias*. Values are means ± 1 SEM. *S* = starved. Units are mmol N L^{-1} for urea, TMAO, FAA, L-alanine, and protein; µmol N L^{-1} for ammonia and creatinine; mOsmol kg^{-1} for osmolality; and meq L^{-1} for chloride. Means sharing the same letter within the same compound are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$). The absence of any letters implies that there were no significant changes observed in the parameter presented.

was assumed in this study. L-alanine in plasma samples were determined enzymatically on deproteinized plasma, using L-alanine dehydrogenase (Williamson 1983). Creatinine concentration was measured using a Sigma Diagnostics kit (no. 555). Chloride concentration was analyzed using a CMT 10 chloride titrator (Radiometer, Copenhagen).

For analysis of enzyme activity, tissues were homogenized on ice in 5–10 volumes of homogenization buffer (20 mmol L^{-1} K_2HPO_4, 10 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol, 50% glycerol adjusted with NaOH to pH 7.5 at 24°C) using a Brinkman Polytron. Homogenates were centrifuged at 8,000 *g* for 20 min at 4°C. The supernatant, a 1 : 9 or a 1 : 99 dilution in homogenization buffer, was used directly for assaying the activity of GS (assayed via the formation of γ-glutamyl-hydroxamate; EC 6.3.1.2), CPS III (EC 6.3.5.5), ornithine-citrulline transcarbamoylase (OCT; EC 2.1.3.3), arginase (ARG; EC 3.5.3.1), GDH (EC 1.4.1.3), alanine aminotransferase (AlaAT; EC 2.6.1.2), and aspartate aminotransferase (AspAT; EC 2.6.1.1) by previously described methods (Mommsen and Walsh 1989; Barber and Walsh 1993; Walsh et al. 1994). For the CPS III assay, we employed the colorimetric production of citrulline CPSase assay (Mommsen and Walsh 1989) using 1.7 mM UTP, 20 mM ATP, 25 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM N-acetylglutamate, 2 mM dithiothreitol, 5 mM ornithine, 20 mM glutamine, 25 mM phosphoenolpyruvate, 6 U mL^{-1} pyruvate kinase, 1 U mL^{-1} ornithine-citrulline transcarbamoylase, and 50 mM HEPES (pH 8.0). Not all tissues were assayed for all enzymes.

Data Analysis and Statistical Treatments

All data are reported as means ± SE (*N* = number of animals). Since the intervals for taking water samples were not constant in each fish, the rate of nitrogen excretion was estimated by fitting a polynomial curve for the time course of urea and ammonia excretion for each fish and back-calculating the excretion rate at each 6-h interval. For multiple comparisons for differences in ammonia and urea excretion within treatment groups, values were tested for significance using a Friedman's test followed by a Scheffé's post hoc test because the data were

Table 2: Glutamine synthetase activities in tissues of starved or fed *Squalus acanthias*

	Starved	6 h	20 h	30 h	48 h
Liver	9.87 ± .87 (8)^{A}	14.18 ± .93 (12)^{B}	15.25 ± 1.05 (4)^{B}	10.40 ± 1.03 (7)^{AC}	13.38 ± 1.39 (9)^{BC}
Muscle	.36 ± .07 (8)	.25 ± .03 (12)	.28 ± .05 (5)	.25 ± .04 (7)	.28 ± .06 (6)
Gills	.70 ± .04 (8)^{AB}	.70 ± .06 (12)^{AB}	.85 ± .07 (5)^{A}	.57 ± .07 (7)^{B}	.54 ± .07 (6)^{B}
Intestine	16.61 ± 1.73 (8)^{AB}	16.23 ± 1.66 (7)^{AB}	19.57 ± .57 (5)^{A}	13.35 ± 1.88 (7)^{B}	15.62 ± 2.08 (3)^{AB}
Esophagus	1.30 ± .05 (5)^{A}	.73 ± .08 (7)^{B}	1.16 ± .12 (5)^{A}	.76 ± .15 (7)^{B}	.69 ± .10 (3)^{B}
Stomach 1[a]	3.36 ± .36 (8)^{AC}	4.34 ± .66 (12)^{A}	6.88 ± .69 (5)^{B}	2.40 ± .16 (7)^{C}	2.82 ± .12 (9)^{C}
Stomach 2[a]	2.76 ± .33 (8)^{A}	2.66 ± .17 (7)^{A}	4.42 ± .78 (5)^{B}	1.87 ± .19 (7)^{AC}	.98 ± .38 (3)^{C}
Brain	14.41 ± .63 (8)	14.22 ± .79 (12)	15.16 ± .88 (5)	13.59 ± .88 (7)	14.12 ± .87 (6)
Kidney	10.31 ± .84 (5)	…	…	…	9.01 ± 2.48 (3)

Note. Values are means ± 1 SEM (*N*). Units are μmol min^{-1} g^{-1}. Means sharing the same letter in the same tissue are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$).

[a] Stomachs 1 and 2 indicate anterior stomach and posterior stomach, respectively.

not normally distributed. For comparison of ammonia-N and urea-N excretion rates at the same times between fed and starved fish, values were tested for significance using a Mann-Whitney test. All other data were normally distributed, and multiple comparisons for differences in metabolite concentrations and enzyme activities at different sampling times were evaluated by a one-way ANOVA followed by an LSD post hoc test. Significance was accepted at $P < 0.05$.

Results

Nitrogen Content of Food and Feeding Rates

The total nitrogen content of food as reported by Wood et al. (2005) was 1,625 μmol N g^{-1}, composed of 94.5% protein-N, 2.8% FAA-N, 0.4% ammonia-N, 1.4% TMAO-N, 0.8% urea-N, and 0.1% creatinine-N. Most of the nitrogen in the food exists as protein.

The amount of solid food in the stomach averaged about 2.6% of body mass using data points from all fish killed up to 48 h postfeeding (excluding those fish that had no food in the stomach) and did not vary significantly with time after feeding, indicating slow digestion in these elasmobranchs (Table 1). In addition, some dogfish vomited large amounts of solid food in the small tank during holding. This did not appear to be an artifact of confinement in boxes, because we commonly observed the same phenomenon in the 200,000-L tank. When the vomited food was included in the total, the ration amounted to about 3.2% of body mass. Indeed, two fish took 8% of the body mass.

Pattern of Nitrogen Excretion after Feeding

The excretion rates of urea-N and ammonia-N for starved and fed treatments are shown in Figure 1. In the starved fish, the majority of nitrogen was excreted as urea-N (91%–98%) throughout the 48-h measurement period. During this time, urea-N excretion fell by 24%, and ammonia-N excretion fell by 69%. In the fed fish, the ratio of urea-N to ammonia-N excretion fish was slightly lower than in the starved fish but increased with time after feeding. Urea-N represented 82%–89% of total N excretion and again decreased gradually over time.

Urea-N excretion rates did not differ between starved and fed fish at any time. However, feeding increased the ammonia excretion rate significantly relative to starved values, up to 18 h; thereafter, the ammonia excretion rate gradually decreased and was about 50% less at 48 h. There was significantly elevated ammonia excretion in fed versus starved fish at 6, 12, and 18 h postfeeding.

Nitrogen and Osmotic Constituents in Plasma

There were significant increases in the concentration of most measured nitrogen constituents in plasma at either 20 or 30 h postfeeding (Fig. 2). The exceptions were ammonia (Fig. 2*A*), creatinine (Fig. 2*B*), and protein (Fig. 2*C*); ammonia remained constant at a low level of about 134 μmol N L^{-1}, while creatinine was also low in starved animals (418 μmol N L^{-1}) and decreased significantly after feeding. Plasma protein concentration remained constant at about 146 mmol N L^{-1} (which represents approximately 13 g protein L^{-1} in traditional units) throughout the 48 h after feeding. Plasma urea (Fig. 2*D*) was about 819 mmol N L^{-1} in starved animals and at 6 h postfeeding, peaking to about 969 mmol N L^{-1} after 20 h before returning to starved values by 30 h postfeeding. Both plasma TMAO (Fig. 2*E*) and plasma FAA (Fig. 2*F*; about 78 and 12 mmol N L^{-1}, respectively, in starvation) followed trends similar to plasma urea, though with slightly differing time courses. Plasma osmolality (Fig. 2*G*) was about 903 mOsmol kg^{-1} in starved fish, became elevated after feeding (947 mOsmol kg^{-1} at 20 h), and then decreased slightly. Plasma chloride (Fig. 2*H*) was 243 meq L^{-1} in starved dogfish and significantly increased to about 250 meq L^{-1} at 30–48 h postfeeding. L-alanine (Fig. 2*I*) was 0.52 mmol N L^{-1} in

Table 3: Alanine aminotransferase activities in tissues of starved or fed *Squalus acanthias*

	Starved	6 h	20 h	30 h	48 h
Liver	7.03 ± .60 (8)	7.66 ± .42 (12)	8.28 ± .48 (4)	6.86 ± .97 (7)	8.30 ± .67 (9)
Muscle	.55 ± .07 (8)^{A}	.30 ± .03 (12)^{B}	.37 ± .11 (5)^{AB}	.39 ± .08 (7)^{AB}	.54 ± .09 (6)^{A}
Gills	.55 ± .07 (8)^{A}	.96 ± .08 (12)^{B}	1.00 ± .13 (5)^{BC}	.73 ± .06 (7)^{AC}	.90 ± .05 (6)^{AB}
Intestine	12.64 ± 1.37 (8)^{AB}	10.73 ± .57 (7)^{A}	15.19 ± 1.27 (5)^{B}	11.92 ± 1.76 (7)^{AB}	13.65 ± 1.06 (3)^{AB}
Esophagus	.56 ± .08 (5)^{A}	.58 ± .06 (7)^{A}	2.39 ± .30 (5)^{B}	.53 ± .11 (7)^{A}	1.62 ± .71 (3)^{C}
Stomach 1[a]	3.56 ± .40 (8)^{A}	2.71 ± .25 (12)^{B}	3.72 ± .35 (5)^{A}	2.07 ± .20 (7)^{B}	2.67 ± .13 (9)^{B}
Stomach 2[a]	2.99 ± .36 (8)^{A}	2.72 ± .26 (7)^{AB}	2.93 ± .33 (5)^{AB}	2.13 ± .24 (7)^{B}	1.83 ± .13 (3)^{B}
Brain	.81 ± .08 (8)	.84 ± .09 (12)	.95 ± .06 (5)	.89 ± .16 (7)	.63 ± .05 (6)
Kidney	1.80 ± .18 (5)	…	…	…	1.71 ± .22 (3)

Note. Values are means ± 1 SEM (*N*). Units are μmol min^{-1} g^{-1}. Means sharing the same letter in the same tissue are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$).

[a] Stomachs 1 and 2 indicate anterior stomach and posterior stomach, respectively.

starved animals, became elevated after feeding, and reached 1.03 mmol L^{-1} at 48 h postfeeding.

Clearly, urea-N was the dominant N metabolite in the plasma. Because plasma protein-N is probably not part of the direct N metabolite pool, TMAO-N and FAA-N were next in order of importance, and plasma ammonia-N was negligible relative to these compounds. Plasma TMAO-N accounted for about 8%–9% of the total N metabolites in the plasma.

Enzyme Activities in Tissues

GS activities in intestine, liver, brain, and kidney were substantially higher than in the other tissues (Table 2). In general, compared with the starved treatment, the activity following feeding was elevated.

Liver GS activity increased about 1.4-fold immediately after feeding, remaining elevated at 48 h, although a drop in activity was noted at 30 h postfeeding. GS activities in stomach 1 and stomach 2 followed a trend similar to that in the liver and increased 2.0- and 1.6-fold, respectively, at the 20 h postfeeding sampling. Muscle GS activity was about 0.3 μmol min^{-1} g^{-1}, the lowest in all the measured tissues, and did not change significantly after feeding. Brain GS activity was very high, but feeding had no effect on its activity.

In general, AlaAT, AspAT, and GDH did not incur any increases following feeding in most of the tissues (Tables 3–5). Indeed, there was a general tendency for the activity levels of these enzymes in the gastrointestinal tract to decline after feeding, especially in the stomach tissues (Tables 3–5). However, in gills and esophagus, AlaAT activity increased at 20 h postfeeding.

Ornithine Urea Cycle Enzymes

Three key enzymes of the O-UC, CPS III, OCT, and ARG activities were assayed in the liver, muscle, and intestine (Table 6). CPS III activities in muscle and intestine were higher than those in liver, with the activity in the muscle 1.6–4.7-fold higher than in liver (Table 6). Intestinal CPS III activity did not change, while liver CPS activity fell below the level in starved dogfish at 6, 30, and 48 h postfeeding. However, feeding led to significant increases in muscle CPS III activity. Activity had increased 1.6-fold by 20 h after feeding, remaining elevated until 30 h. CPS III activities were below detection limits in dogfish gill and kidney.

In contrast to CPS III activities, OCT activity in the liver was much higher than in the muscle and intestine. Hepatic OCT activities were 13–32-fold greater than those in the muscle in the starved animals and throughout postfeeding sampling. In liver and muscle, OCT activities significantly increased 1.5-fold at 6 h and 2.1-fold at 20 h postfeeding, respectively. OCT was also assayed in all other tissues. OCT proved undetectable in gills, kidney, and esophagus and averaged 3.38 ± 0.50 (8) in stomach 1, 2.74 ± 0.53 (5) in stomach 2, and 0.48 ± 0.24 (4) μmol min^{-1} g^{-1} in brain. Values increased in the brain but decreased in both stomach sections at various times after feeding (data not shown).

The highest ARG activities were noted in the liver, followed by the muscle and intestine. Liver ARG activity was about 22 μmol min^{-1} g^{-1} in starved animals and increased significantly at 20 h postfeeding. Muscle ARG activities were very variable but showed a significant 1.8-fold increase 48 h following feeding. ARG was assayed in all other tissues except brain. ARG was undetectable in the esophagus and both sections of the stomach but averaged 0.55 ± 0.07 (8) μmol min^{-1} g^{-1} in the gills during starvation, and it increased about twofold at most times after feeding (data not shown). Kidney ARG activity was 1.80 ± 0.18 (5) μmol min^{-1} g^{-1} in starved dogfish and remained almost identical at 48 h postfeeding.

Discussion

A notable finding of this study is that *Squalus acanthias* possesses high CPS III activity in muscle, 1.6–4.7-fold greater than

Table 4: Aspartate aminotransferase activities in tissues of starved or fed *Squalus acanthias*

	Starved	6 h	20 h	30 h	48 h
Liver	14.57 ± 1.04 (8)	14.71 ± .61 (12)	15.90 ± .98 (4)	13.66 ± 1.24 (7)	15.15 ± 1.06 (9)
Muscle	7.03 ± .31 (8)	7.12 ± .41 (12)	7.63 ± .66 (5)	6.95 ± .52 (7)	7.13 ± .63 (6)
Gills	7.58 ± .29 (8)	7.56 ± .36 (12)	7.00 ± .28 (5)	6.74 ± .42 (7)	7.24 ± .44 (6)
Intestine	1.79 ± .10 (8)^{A}	1.20 ± .07 (7)^{B}	1.66 ± .13 (5)^{AC}	1.47 ± .08 (7)^{CD}	1.30 ± .04 (3)^{BD}
Esophagus	7.66 ± .33 (5)^{AB}	8.09 ± .31 (7)^{A}	7.69 ± .35 (5)^{AB}	7.20 ± .25 (7)^{B}	7.46 ± .29 (3)^{AB}
Stomach 1[a]	30.27 ± 1.87 (8)^{A}	22.17 ± 2.89 (12)^{BC}	28.79 ± .90 (5)^{AB}	15.84 ± 2.00 (7)^{C}	18.62 ± .99 (9)^{C}
Stomach 2[a]	15.80 ± 1.54 (8)^{A}	13.50 ± 1.34 (7)^{AB}	13.66 ± 1.83 (5)^{AB}	10.10 ± .94 (7)^{BC}	7.92 ± .24 (3)^{C}
Brain	19.46 ± .74 (8)^{A}	19.34 ± .56 (12)^{A}	20.44 ± .64 (5)^{A}	18.08 ± .92 (7)^{A}	16.27 ± 1.01 (6)^{B}
Kidney	19.11 ± .71 (5)	…	…	…	16.07 ± 1.35 (3)

Note. Values are means ± 1 SEM (*N*). Units are μmol min^{-1} g^{-1}. Means sharing the same letter in the same tissue are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$).

[a] Stomachs 1 and 2 indicate anterior stomach and posterior stomach, respectively.

in the liver (Table 6). The liver levels we report are similar to values from previous reports for this species (0.24 μmol min^{-1} g^{-1}; Anderson 1980, 1981, 2001). The contribution of muscle as a potential source of urea synthesis via CPS III appears to be much larger than that of liver when body mass is considered. Although the relative contributions of liver and muscle to total body mass were not measured in this study, it has been reported that the liver accounts for approximately 10% of body mass in *S. acanthias* (Leech et al. 1979) and that the white muscle accounts for 50% of the body mass in salmon shark *Lamna ditropis* (Bernal et al. 2003). The values in Figure 3 were calculated assuming an average body mass of 2.2 kg, a liver mass of 10% (220 g), and a muscle mass of 50% (1,100 g). Using these parameters, we can estimate the contribution of CPS III in muscle as 878 μmol carbamoyl phosphate (urea) min^{-1} in starved animals, increasing up to 1,377 μmol min^{-1} at 20 h after feeding. By way of contrast, the liver in starved dogfish could provide 109 μmol carbamoyl phosphate (urea) min^{-1}, rising to only 149 μmol urea min^{-1} at 20 h after feeding and declining at most other times. Therefore, the CPS III capacity in muscle is approximately 8–23-fold greater than that in liver. The urea excretion rate in *S. acanthias* is around 400 μmol N kg^{-1} h^{-1}, that is, 200 μmol kg^{-1} h^{-1} (Fig. 1), which corresponds to about 7.3 μmol urea min^{-1} for a 2.2-kg fish. Consequently, the values of estimated CPS III capacity for urea production in muscle and liver (Fig. 3*A*) are much greater than the measured urea excretion rate. *Squalus acanthias* thus possesses more than enough CPS III activity to account for urea production in muscle or liver alone. Similarly, considering the mass of muscle, the enzymes both preceding (GS) and following (OCT) CPS III in the O-UC seem to have high activities. The GS assay was directly compared with the transferase assay on purified *S. acanthias* GS by Shankar and Anderson (1985): the ratio is 15 units of transferase : 1 unit of synthetase. Therefore, GS is >18 μmol glutamine min^{-1} in muscle and >145 μmol glutamine min^{-1} in liver (on the basis of on Table 2). OCT is >410 μmol citrulline min^{-1} in muscle and >2,137 μmol citrulline min^{-1} in liver (on the basis of Table 6). In either tissue alone, the enzymes have sufficient activities to generate urea at levels well in excess of the measured net urea production level. However, because these measurements are made under ideal conditions in a test tube, actual enzyme flux rates in vivo may be lower than these results, although it is likely that the proportions of activity in the various tissues may be relatively the same in vivo. If some of the potential for urea synthesis is realized in vivo, such that synthesis rates exceed excretion rates, one possibility may be that the O-UC replaces a significant quantity of urea that is hydrolyzed by bacterial urease shown to be present in elasmobranchs (Knight et al. 1988).

The level of CPS III (Table 6) in the muscle of *S. acanthias* was many-fold higher than reported by Steele et al. (2005) in the muscle of the little skate *Raja erinacea*, the only other elasmobranch where muscle O-UC activities have been detected; indeed, rates for dogfish muscle were comparable to those in the muscle of the ureotelic Lake Magadi tilapia *Alcolapia grahami* reported by Lindley et al. (1999). Surprisingly, muscle OCT activities (Table 6) in *S. acanthias* were about 10-fold lower than those reported in the skate and the Lake Magadi tilapia. The reason for this difference is unclear, but it may not be functionally important because CPS III is likely the key rate-limiting enzyme of the O-UC (Casey and Anderson 1985), and there is already excess capacity in muscle OCT as outlined above.

High CPS III activity was present not only in muscle but also in the intestine of *S. acanthias* (Table 6). Considering intestinal mass (likely no more than 2% of total body mass by our estimation), the contribution of CPS III in intestine is small relative to the other tissues. However, GS, the preceding enzyme to CPS III, is quite high (Table 2), while the O-UC enzymes, OCT and ARG, also exist in the intestine (Table 6). Moreover, the intestine absorbs large quantities of amino acids and possesses aminotransferases (AlaAT and AspAT) and GDH that can supply nitrogen to the cycle (Tables 3–5). Because the intestine has direct access to dietary nitrogen and possesses the

Table 5: Glutamate dehydrogenase activities in tissues of starved or fed *Squalus acanthias*

	Starved	6 h	20 h	30 h	48 h
Liver	8.68 ± .91 (8)	9.28 ± .86 (12)	10.09 ± .75 (4)	8.49 ± 1.28 (7)	11.58 ± 1.23 (9)
Muscle	.69 ± .07 (8)	.73 ± .06 (11)	.78 ± .07 (5)	.67 ± .08 (7)	.70 ± .07 (6)
Gills	2.57 ± .28 (8)	2.32 ± .15 (12)	2.62 ± .13 (5)	2.17 ± .16 (7)	2.23 ± .21 (6)
Intestine	14.26 ± 1.03 (8)^{A}	11.28 ± .87 (7)^{B}	14.71 ± 1.02 (5)^{A}	12.26 ± 1.37 (7)^{AB}	13.96 ± .46 (3)^{AB}
Esophagus	1.58 ± .17 (5)^{AB}	1.55 ± .08 (7)^{AB}	1.58 ± .07 (5)^{AB}	1.32 ± .06 (7)^{A}	1.92 ± .26 (3)^{B}
Stomach 1[a]	4.21 ± .19 (8)^{A}	3.08 ± .32 (12)^{BC}	3.70 ± .19 (5)^{AB}	2.75 ± .28 (7)^{C}	2.76 ± .18 (9)^{C}
Stomach 2[a]	2.12 ± .13 (8)^{A}	1.81 ± .17 (7)^{AB}	1.89 ± .24 (5)^{AB}	1.60 ± .11 (7)^{B}	1.83 ± .22 (3)^{AB}
Brain	4.60 ± .32 (8)	4.35 ± .22 (12)	4.67 ± .37 (5)	4.28 ± .38 (7)	3.74 ± .35 (6)
Kidney	18.66 ± .75 (5)	…	…	…	19.40 ± 2.38 (3)

Note. Values are means ± 1 SEM (*N*). Units are μmol min^{-1} g^{-1}. Means sharing the same letter in the same tissue are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$).

[a] Stomachs 1 and 2 indicate anterior stomach and posterior stomach, respectively.

key enzymes needed to produce urea, it is reasonable to assume that this tissue may be important in N trapping for amino acid synthesis and may also produce urea. We suggest that intestine contributes to urea synthesis in the body of *S. acanthias*, although the contribution is not large compared with the liver and muscle.

In most tissues, the activities of the O-UC enzymes (CPS III, OCT, and ARG) and GS in postfeeding dogfish tended to be higher than in starved animals, indicating that the enzymes are effectively enhanced by feeding. Furthermore, in liver and muscle, the tissues that appear to be primarily responsible for urea synthesis, O-UC enzyme activities increased significantly at 4–30 h after feeding, suggesting that feeding resumption promotes urea synthesis. Moreover, plasma urea-N levels increased significantly at 20 h after feeding, presumably a result of this enzyme activation response. Thus, it seems that there is an increase in O-UC enzyme activity, leading to urea synthesis within 24 h. However, on average 3.2% of total body mass of solid food was present in the stomach at 6 h, and 1.8% (not significantly different) was present at 48 h postfeeding (Table 1). These values are similar to those reported by Tanasichuk et al. (1991), where *S. acanthias* in the wild took 0.8%–4.1% of body mass in daily ration. Tanasichuk et al. (1991) reported that digestive rates are very low (0.001%–0.25% body mass h^{-1}). Jones and Geen (1977) have suggested that mature males (2.15 kg) in *S. acanthias* digest 1.1% of body mass per day. According to these reports, it takes somewhere between 2 and 9 d to digest the typical meal of this study. It is thus assumed that urea production may continue well beyond the 48-h sampling time used herein.

In contrast to the enhancement of O-UC enzyme activities and stimulation of plasma urea-N, the rate of urea excretion did not change after feeding (Fig. 2). *Squalus acanthias* excreted 80%–90% of their total nitrogenous wastes as urea. This corresponds well to previous reports (Wood et al. 1995, 2005). Although spiny dogfish constantly lose about 400 μmol N kg^{-1} h^{-1} as urea, this loss rate does not increase after feeding, suggesting that *S. acanthias* has a strong N retention system. Furthermore, ammonia excretion, which constitutes only a few percent of total N excretion, was only moderately raised in fed fish, while plasma ammonia did not change. This baseline loss of urea-N is apparently irreducible despite the presence of conservation mechanisms in gills and kidney (Schmidt-Nielsen et al. 1972; Pärt et al. 1998; Fines et al. 2001; Morgan et al. 2003) and is presumably the unavoidable "cost" of employing a urea-based system for osmoregulation. The critical importance of the N conservation system after feeding to help offset these baseline urea-N losses can be illustrated by some simple calculations. The N content of a typical meal (3.2% of body mass) is equivalent to the summated baseline rate of N loss in a starved animal over only ~137 h (using the rates of Fig. 1*A*). The small "extra" loss associated with the small elevation of ammonia-N excretion after feeding (Fig. 1*B*) amounted to only about 6% of the ingested N load by 48 h, the measurement period of this study. By extrapolation beyond this time, the period of conservation would have been reduced from 137 to 112 h. Thus, in the wild, it is imperative that dogfish feed every 5–6 d just to maintain N balance, let alone grow.

In the little skate *R. erinacea*, Steele et al. (2005) suggested that a low level of O-UC activity in muscle served to scavenge muscle-produced ammonia. Given the high levels of O-UC activity in the muscle and its enhancement after feeding in the muscle of *S. acanthias*, this argument may be broadened to overall scavenging of ammonia and retention of dietary nitrogen. We suggest that enzymes such as GDH and GS ensure that "excess" ammonia is taken into nitrogen metabolism for both urea synthesis and protein growth. In future studies, it will be of interest to measure protein synthesis rates in elasmobranch muscle to see whether they increase markedly after feeding, as they do in teleosts (cf. Wood 2001).

Urea-N in plasma was highest 20 h after feeding (Fig. 2*D*). The activities of GS and ARG in liver and of CPS III and OCT in muscle showed the largest increases in activities at this time point (Tables 2, 6). This does not, however, suggest that the

Table 6: Carbamoyl phosphate synthetase III (CPS III), ornithine transcarbamoylase (OCT), and arginase (ARG) activities in the liver, muscle, and intestine of starved or fed *Squalus acanthias*

	Starved	6 h	20 h	30 h	48 h
CPS III:					
Liver	.49 ± .09 (8)^{AC}	.29 ± .05 (8)^{B}	.68 ± .11 (4)^{A}	.26 ± .04 (6)^{B}	.39 ± .08 (8)^{BC}
Muscle	.80 ± .15 (8)^{A}	1.04 ± .05 (9)^{AB}	1.25 ± .14 (5)^{B}	1.21 ± .10 (7)^{B}	.91 ± .06 (3)^{AB}
Intestine	1.50 ± .30 (8)	.82 ± .17 (7)	1.21 ± .37 (5)	.96 ± .18 (7)	.77 ± .15 (2)
OCT:					
Liver	9.71 ± 2.13 (8)^{A}	14.31 ± .97 (12)^{B}	14.81 ± 2.15 (4)^{AB}	12.44 ± 1.43 (7)^{AB}	11.69 ± 1.57 (8)^{AB}
Muscle	.57 ± .15 (8)^{A}	.94 ± .20 (7)^{AB}	1.17 ± .18 (5)^{B}	.63 ± .24 (6)^{AB}	.37 ± .07 (3)^{A}
Intestine	1.27 ± .60 (4)	.69 ± .50(5)	.86 ± .50 (2)	2.23 ± .72 (5)	.02 ± .01 (2)
ARG:					
Liver	22.11 ± 1.38 (8)^{A}	23.41 ± .96 (12)^{A}	27.55 ± .61 (4)^{B}	21.29 ± 1.18 (7)^{A}	23.55 ± 1.31 (9)^{AB}
Muscle	1.58 ± .36 (8)^{AB}	2.14 ± .40 (11)^{AC}	.95 ± .18 (4)^{AB}	.93 ± .29 (4)^{B}	2.81 ± .25 (6)^{C}
Intestine	.32 ± .12 (5)	.32 ± .07 (2)	.44 ± .06 (3)	.78 ± .16 (5)	.19 ± .09 (2)

Note. Values are means ± 1 SEM (*N*). Units are μmol min^{-1} g^{-1}. Means sharing the same letter in the same tissue are not significantly different from one another (one-way ANOVA followed by an LSD post hoc test, $P < 0.05$).

liver may provide substrate for the O-UC cycle in the muscle. Glutamine generated in the mitochondria is not transported out but is utilized in the mitochondria for carbamoyl phosphate and then citrulline synthesis in elasmobranchs (Anderson 1991). Glutamine production for urea synthesis likely occurs in the same tissues that produce the carbamoyl phosphate; indeed, muscle GS activity appears to be sufficient to account for whole body urea production (see above). Considering this and the other evidence presented here, it seems that muscle may be the main site for urea synthesis in *S. acanthias*, given that muscle is also the primary storage tissue for urea (Robertson 1975). The fall in plasma creatinine after feeding (Fig. 2*B*) may be related to a recharging of the muscle creatine pool at this time.

L-alanine concentrations in plasma increased after feeding (Fig. 2*I*), indicating that alanine in food is carried to those tissues that appear to be responsible for urea synthesis, presumably muscle. There, alanine could be used for protein synthesis or converted to glutamate and pyruvate by AlaAT (Table 3), then to glutamine by the GS-catalyzed addition of an amino group for urea synthesis in the O-UC. In this study, concentrations of plasma L-alanine for this species were somewhat higher (0.52–1.03 mmol-N L^{-1}) than in a previous report, which shows 0.53 mmol N L^{-1} at 0 d and 0.17–0.18 mmol N L^{-1} from 1 to 22 d after capture and then holding without food (Leech et al. 1979). Starvation for 1 wk may not be long enough to affect alanine concentration.

In the subtropical dogfish *Poroderma africanum*, osmolality and urea levels decreased gradually during 1 mo of starvation and rose in a day after feeding (Haywood 1973). In our study, osmolality and urea levels in plasma increased significantly at 20 h after feeding compared with those of starved fish. However, with the exception of the 20-h postfeeding group, osmolality and urea level in plasma did not change significantly. This could be related to the starvation protocol. The starved fish were deprived of food for only 1 wk. Starvation for 1 wk may not affect osmolality and urea levels in plasma in *S. acanthias*, although feeding increases them temporarily. Leech et al. (1979) has reported that plasma urea in *S. acanthias* decreases 4% during 9–14 d and 8% during 19–25 d of starvation.

Urea, TMAO, and inorganic ions are major osmolytes in marine elasmobranchs and therefore have considerably influence on osmolality. The osmotic coefficient of urea is 0.96, that of TMAO is 1.19, and that of inorganic ions in plasma is 0.916 (Robertson 1975, 1989). In our dogfish, plasma urea levels were 820–969 mmol N kg^{-1}, thereby accounting for 393–465 mOsmol kg^{-1} (Fig. 2*G*). Similarly, plasma TMAO concentrations accounted for 83–111 mOsmol kg^{-1} and plasma chloride for 223–231 mOsmol kg^{-1}. Therefore, urea comprised about 42%–49%, TMAO about 9%–12%, and chloride about 24%–25% of total plasma osmolality (903–947 mOsmol kg^{-1}). In *S. acanthias*, most of the inorganic ions in plasma (96%) consist of almost equimolar amounts of chloride and sodium (Robertson 1975). Although we did not measure sodium concentrations in plasma, it is probable that sodium plus chloride accounted for about half of total plasma osmolality. These values are similar to a previous report for *S. acanthias* (inorganic ions 52%, TMAO 9%), but there urea accounted for only 29% (Robertson 1975).

Plasma osmolality 20 h following feeding was 44 mOsmol kg^{-1} higher than in starved animals (Fig. 2*G*). In parallel, the discrepancy between starved and 20-h postfeeding groups was 72 mOsmol kg^{-1} for urea and 18 mOsmol kg^{-1} for TMAO. Furthermore, osmolality at 30 h postfeeding slightly decreased, while plasma urea and TMAO dropped to levels similar to those in starved fish. Consequently, this suggests that changes in urea and TMAO largely account for the postfeeding changes in plasma osmolality, though alterations in inorganic ions may

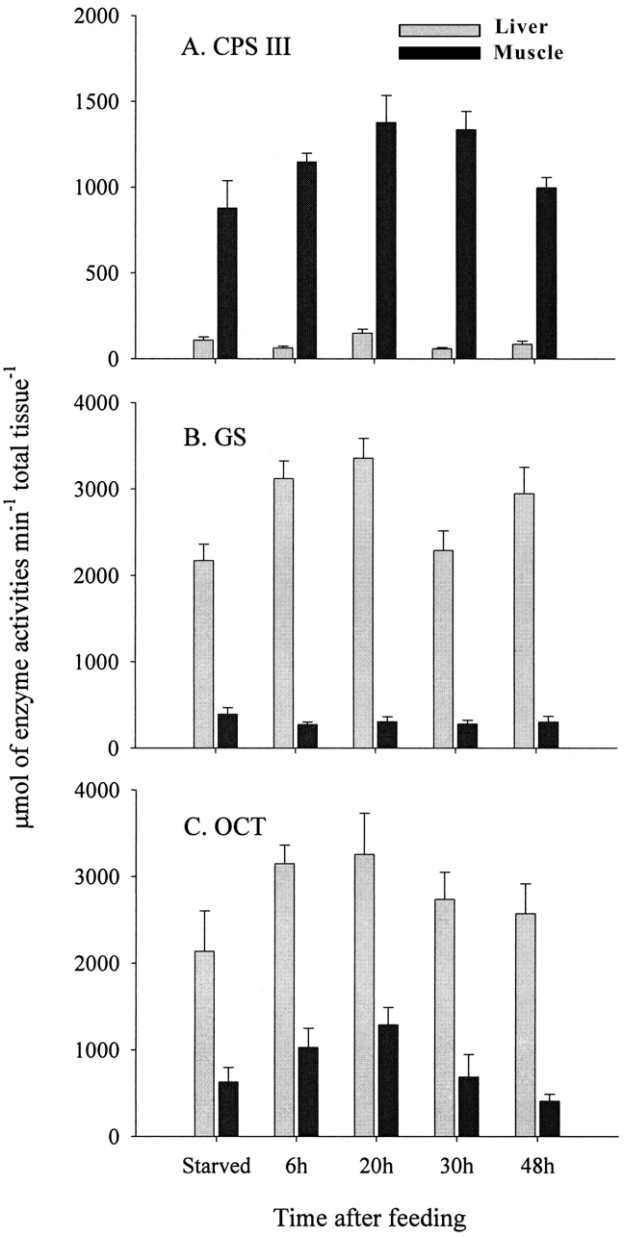

Figure 3. Estimated total enzymatic activities of carbamoyl phosphate synthetase (*A*), glutamine synthetase (*B*), and ornithine transcarbamoylase (*C*) in liver and muscle of a 2.2-kg specimen of *Squalus acanthias* after feeding. Data are presented as means ± SEM (see "Discussion").

also play a role. The rise in chloride at 30 h may be related to acid-base exchanges at the gills compensating postfeeding alkalosis (Wood et al. 2005).

In marine elasmobranchs, TMAO not only is an important osmolyte but also influences buoyancy and counteracts urea destabilization of various biological structures (Withers et al. 1994*a*, 1994*b*; Yancey 2001). It is generally believed that *S. acanthias* is not capable of de novo TMAO synthesis, because injected precursors (trimethylamine and choline) were not converted into TMAO either in intact animals (Goldstein et al. 1967) or in sliced or homogenized liver (Baker et al. 1963; Goldstein et al. 1967). However, the increase (~15 mmol L^{-1}) of plasma TMAO 20 h after feeding (Fig. 2*E*) seems somewhat larger than could be explained by direct absorption from the food, which contained only 23.1 μmol N g^{-1}, so the issue may be worthy of future study.

Amino acids (FAA) are also osmotic constituents, although their concentrations are low relative to urea, TMAO, and inorganic ions (Fig. 2*F*). The values of plasma FAA we report here (12–17 mmol L^{-1}) are similar to those described previously for *S. acanthias* (Robertson 1975). The small rise in plasma FAA at 30 h after feeding would have had little effect on osmolality and is commonly observed after feeding in many fish (Wood 2001).

In conclusion, we suggest that N conservation is a high priority in *S. acanthias* and that feeding promotes ureogenesis and thereby enhances osmoregulation. Furthermore, exogenous nitrogen from food appears to be converted into urea not only by the liver but also by extrahepatic tissues, most importantly, the large skeletal muscle mass.

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