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Decreasing Urea : Trimethylamine N-Oxide Ratios with Depth in Chondrichthyes: A Physiological Depth Limit?

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ABSTRACT

In marine osmoconformers, cells use organic osmolytes to maintain osmotic balance with seawater. High levels of urea are utilized in chondrichthyans (sharks, rays, skates, and chimaeras) for this purpose. Because of urea's perturbing nature, cells also accumulate counteracting methylamines, such as trimethylamine N-oxide (TMAO), at about a 2 : 1 urea : methylamine ratio, the most thermodynamically favorable mixture for protein stabilization, in shallow species. However, previous work on deep-sea teleosts (15 species) and chondrichthyans (three species) found an increase in muscle TMAO content and a decrease in urea content in chondrichthyans with depth. We hypothesized that TMAO counteracts protein destabilization resulting from hydrostatic pressure, as is demonstrated in vitro. Chondrichthyans are almost absent below 3,000 m, and we hypothesized that a limitation in urea excretion and/or TMAO retention might play a role. To test this, we measured the content of major organic osmolytes in white muscle of 13 chondrichthyan species caught with along-contour trawls at depths of 50–3,000 m; the deepest species caught was from 2,165 m. Urea and TMAO contents changed significantly with depth, with urea : TMAO declining from 2.96 in the shallowest (50–90 m) groups to 0.67 in the deepest (1,911–2,165 m) groups. Urea content was 291–371 mmol/kg in the shallowest group and 170–189 mmol/kg in the deepest group, declining linearly with depth and showing no plateau. TMAO content was 85–168 mmol/kg in the shallowest group and 250–289 mmol/kg in the deepest groups. With data from a previous study for a skate at 2,850 m included, a second-order polynomial fit suggested a plateau at the greatest depths. When data for skates (Rajidae) were analyzed separately, a sigmoidal fit was suggested. Thus, the deepest chondrichthyans may be unable to accumulate sufficient TMAO to counteract pressure; however, deeper-living specimens are needed to fully test this hypothesis.

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Introduction

Maintaining cell volume is one of the most fundamental challenges of homeostasis for all living organisms. In marine organisms, two main systems have evolved to deal with the osmotic challenges of a highly saline environment: osmoregulation and osmoconformation. Bony fish (Actinopterygii) utilize osmoregulation, actively maintaining a consistent internal milieu hypotonic to the surrounding seawater by pumping out excess ions. Cartilaginous fish (Chondrichthyes: elasmobranchs and holocephalans) and the coelacanth (Sarcopterygii) rely on osmoconformation. They maintain an internal solute concentration approximately equal to that of their environment or slightly hyperosmotic, at approximately 1,050 mOsm/L (compared to seawater, at about 1,000 mOsm/L), in part by accumulating and retaining small molecules known as organic osmolytes. Chondrichthyes and the coelacanth have been termed "ureosmotic" (Smith 1931) because they retain high quantities of urea in order to maintain osmoconformation. Urea may be well suited for use in osmoconformation because it is a metabolic waste product energetically cheap to synthesize and retain. At the same time, urea is a perturbant, inhibiting protein folding and binding when present in high concentrations. In order to overcome this problem, these fish must synthesize or ingest other organic osmolytes, principally methylamine compounds, including trimethylamine N-oxide (TMAO) and glycine betaine (GB; N-trimethylglycine). These organic osmolytes accumulate to a greater degree in cells than in extracellular fluids and have been shown to counteract the perturbing effects of urea on proteins (Yancey et al. 1982; Yancey 2005).

In light of recent studies showing that TMAO may additionally be used in fishes to counteract the inhibitory effects of hydrostatic pressure on protein function (Gillett et al. 1997; Yancey and Siebenaller 1999; Yancey et al. 2004), this study examines differences in urea and methylamine content between deep- and shallow-dwelling Chondrichthyes. In shallow-dwelling chondrichthyans, the urea-to-methylamine ratio has been consistently documented as approximately 2 : 1 (Yancey and Somero 1979, 1980; Yancey et al. 1982; Treberg et al. 2006). This ratio represents the most thermodynamically favorable mixture for general protein stabilization and typically maintains normal protein conformation in vitro (Yancey et al. 1982; Wang

and Bolen 1997). Moreover, TMAO is a stronger stabilizer in this regard than the other common osmolytes, which rank TMAO > GB > sucrose ~ trehalose ~ sarcosine (N-methylglycine) > sorbitol ~ proline > glycerol (Street et al. 2006).

Previous work on numerous deep-sea teleosts (Actinopterygii) and a few elasmobranchs found that TMAO content in muscle tissue increased with depth. In teleosts, this resulted in increasing internal osmotic pressure with depth. TMAO content of teleost muscle samples rose in a sigmoidal pattern both inter- and intraspecifically from about 40 mmol/kg in shallow species to 261 mmol/kg in an abyssal species from 4,800 m (Gillett et al. 1997; Kelly and Yancey 1999; Yancey et al. 2004; Samerotte et al. 2007). A similar increase in TMAO was also found in some crustaceans and squid (Kelly and Yancey 1999). However, in previously analyzed elasmobranchs, urea content declined as TMAO content increased, so that osmoconformation was probably maintained. Muscle TMAO and urea contents in shallow species had ranges of 80–180 and 300–400 mmol/kg, respectively, while one deep skate species from 2,850 m had TMAO and urea values of 244 and 136 mmol/kg, respectively (a species from 1,900 m had intermediate values; Kelly and Yancey 1999). The skate from 2,850 m had a 1 : 1.8 urea : TMAO (U : T) ratio and so may be better termed "TMAO-osmotic" rather than ureosmotic. Similarly, a deep shark specimen was also reported to have higher TMAO and lower urea content than shallower sharks (Treberg and Driedzic 2002).

The consistent increase in TMAO with depth among all species studied was hypothesized to counteract protein destabilization induced by hydrostatic pressure. Many proteins of deep-sea organisms are more resistant to pressure than are those of shallower homologues, but such resistance is rarely complete; often, some "residual" pressure sensitivity remains (e.g., Gibbs and Somero 1989). Several in vitro experiments on enzyme kinetics and protein stability have shown that TMAO can counteract this residual sensitivity (Gillett et al. 1997; Yancey and Siebenaller 1999; Yancey et al. 2001, 2004). Thus, TMAO may be a "piezolyte" (pressure-adaptive solute; Martin et al. 2002) as well as an osmolyte. Adaptations for proper protein function in the deep sea may therefore involve two levels: an intrinsic change in amino acid structure and an extrinsic change involving the solute (osmolyte) environment. In experiments that tested other osmolytes with proteins, TMAO was found to be the best pressure counteractant, in the order TMAO > GB ≫ myo-inositol ~ glycine (Yancey and Siebenaller 1999; Yancey et al. 2004). The concomitant decline in urea content in elasmobranchs was proposed not only to maintain osmoconformation with rising TMAO content but also to reduce the compounding of urea's perturbing effects by increasing hydrostatic pressure (Kelly and Yancey 1999). However, since only three deep-sea Chondrichthyes (two skates and one shark) had been examined before our current study, the data for deep-sea elasmobranchs were not robust enough to test this hypothesis.

Overcoming the problems associated with hydrostatic pressure has allowed many organisms to inhabit the deep sea. Among fish, Actinopterygii have been documented living to depths of about 8,400 m. In comparison, Priede et al. (2006) have documented that occurrences of Chondrichthyes decline precipitously below 3,000 m, with relatively few species reported between 3,000 and 4,156 m (and none deeper than that). The authors hypothesized that this absence of chondrichthyans in the abyssal ocean is due to their high metabolic needs, in part for the maintenance of enlarged, lipid-rich livers.

Our work attempts to test another possibility: that these fishes' unique osmolyte system limits their depth distribution. We hypothesized two ways by which this might occur. First, the need for osmolytes in chondrichthyan fish, coupled with the oligotrophic nature of the deep sea, might result in the inability of these fish to accumulate high enough levels of TMAO to counteract both urea and hydrostatic pressure. The source of TMAO (endogenous synthesis and/or exogenous diet) is not well known for most Chondrichthyes, but in one study, a deep-sea shark had no detectable levels of the enzyme that produces TMAO (Treberg et al. 2006). Second, it may be difficult for these fishes to reduce their urea content beyond a certain level, perhaps because of structural adaptations in the gill and kidney that have evolved to retain urea. While euryhaline migratory stingrays and sharks moving into freshwater do reduce their urea and TMAO contents, they still maintain a relatively high urea concentration (about 170–200 mM, compared to ~300–400 mM in seawater; Thorson et al. 1973; Piermarini and Evans 1998). In terms of osmotic balance, there seems to be no reason for urea content to remain so high. In contrast, permanently freshwater stingrays (*Potamotrygon*) retain virtually no urea (their kidneys having lost the ability for urea reabsorption) but also cannot accumulate urea if placed in higher-salinity water (Griffith et al. 1973). Together, these observations suggest that if deep-sea chondrichthyans have a need for urea retention, they might also not be able to reduce urea content below a certain level. Retention may have remained in deep-sea species (as in euryhaline shallow species) so that urea content can be readily increased in the event of vertical migration.

To test these osmolyte-centered hypotheses, we analyzed the pattern of U : T ratios in a suite of chondrichthyans across a broad depth range in order to determine whether previously limited observations of osmolyte-depth relationships might indicate that TMAO accumulation (or urea retention) acts as a limiting factor in the depth distribution of these fish.

Material and Methods

Specimen Collection

Fish specimens were caught in along-contour otter and beam trawls during two research cruises on the *R/V Point Sur* to Monterey Bay, California, in April and October of 2009 (S. Wilson and J. C. Drazen, unpublished data). Along-contour trawls were targeted to depths of 100, 250, 500, 750, 1,000, 2,000, and 3,000 m. Median depths of the trawls while on bottom are reported in Table 1 for each specimen collected. Species caught at 50–87 m and 1,911–2,165 m are hereafter referred to as the shallowest and deepest groups, respectively. No Chondrichthyes were caught in the 3,000-m trawls. Specimens were placed on ice immediately upon trawl sorting.

Table 1: Urea, trimethylamine N-oxide (TMAO), and water contents of white muscle of Chondrichthyes from various depths

Depth, Species	Subclass or Division	Median Depth (m)	*n*	Water %	Urea (mmol/kg wet mass)	TMAO (mmol/kg wet mass)	Urea : TMAO
50–87 m:							
Raja stellulata	Skate	50	1	77.8	369	168	2.20
Hydrolagus colliei	Holocephalan	83	1	79.4	371	161	2.31
Raja rhina	Skate	87	1	78.7	360	85.3	4.23
Squalus acanthias	Shark	87	1	80.9	288	111	2.59
Torpedo californica	Ray	87	1	81.3	302	79	3.84
253–263 m:							
Bathyraja kincaidii	Skate	253	1	77.9	292	168	1.74
H. colliei	Holocephalan	263	4	79.3 ± .5	320 ± 21.3	132 ± 25.2	2.50 ± .60
500–543 m:							
B. kincaidii	Skate	500	2	78.0 ± .6	317 ± .62	169 ± .8	1.87 ± .01
Parmaturus xaniurus	Shark	533	2	79.1 ± 1.0	268 ± 3.7	222 ± 14.5	1.21 ± .09
R. rhina	Skate	533	3	78.9 ± .2	328 ± 12.7	144 ± 47.2	2.28 ± .89
Somniosus pacificus	Shark	533	2	83.6 ± 1.6	339 ± 16.9	207 ± 7.0	1.64 ± .03
H. colliei	Holocephalan	543	3	79.1 ± 1.5	317 ± 8.58	152 ± 20.7	2.08 ± .26
793 m:							
Apristurus brunneus	Shark	793	1	87.3	235	203	1.16
1,205–1,323 m:							
Bathyraja abyssicola	Skate	1,205	3	80.2 ± .3	287 ± 14.8	220 ± 16.2	1.31 ± .08
Amblyraja badia	Skate	1,280	3	82.1 ± 1.2	237 ± 8.37	247 ± 16.1	.96 ± .05
Bathyraja trachura	Skate	1,323	2	80.1 ± .1	285 ± 22.5	208 ± 13.4	1.37 ± .02
1,911–2,165 m:							
Apristurus profundorum	Shark	1,911	1	...	170	275	.62
Bathyraja microtrachys	Skate	2,107	5	79.0 ± 2.9	189 ± 17.7	289 ± 14.7	.65 ± .08
A. badia	Skate	2,165	2	82.2 ± .7	183 ± 5.1	250 ± 12.9	.73 ± .06

White-muscle tissue samples were taken dorsolaterally in sharks and chimaeras and dorsally from the thickest part of the wing muscle in rays and skates. These samples were frozen in liquid nitrogen on board and transported to the University of Hawai'i or Whitman College on dry ice, where they were stored at −80°C. White muscle from one shark (*Apristurus profundorum*) was provided by Tracey Sutton (from Cruise HB200906, Mid-Atlantic Ridge, Station 25, trawl depth range 1,872–1,950 m; June 2, 2009). The sample was frozen on board and stored at −80°C, then shipped on dry ice.

In preparation for osmolyte compositional analysis, frozen tissue samples were homogenized in 9 parts of ice-cold 7% perchloric acid and kept overnight at 4°C to precipitate proteins. The samples were then centrifuged at 15,000 *g* for 20 min at 4°C, and the supernatant was pipetted off for use in TMAO and high-performance liquid chromatography (HPLC) analysis.

TMAO Analysis

TMAO concentrations were determined with a scaled-down (0.5-mL reaction mixtures) modification of the picric acid–ferrous sulfate method of Wekell and Barnett (1991). Each supernatant was diluted to either a 1 : 10 or a 1.5 : 10 ratio with ultrapure water, depending on expected TMAO values based on previous data. Samples from 500 m or less were expected to have lower TMAO concentrations and were diluted only to 1.5 : 10 to improve the detection limit of the assay. One hundred microliters of diluted samples (or standards of 1, 2, and 3 mM) was added to 0.6-mL microcentrifuge tubes with a reaction mixture of 300 μL toluene and 100 μL of a fresh ferrous sulfate–EDTA reagent. The tubes were then incubated for 5 min at 50°C to reduce TMAO to TMA (trimethylamine). After the tubes cooled to room temperature, 0.1 mL of 45% KOH was added to precipitate out the iron-EDTA. The tubes were vortexed for 15 s three times, with 5-min intervals, allowing TMA to be extracted to the toluene phase. Immediately thereafter, the toluene-TMA layer (210 μL) was removed and added to 20–25 mg anhydrous Na_2SO_4 to remove any residual water. A 0.02% picric acid–toluene solution (630 μL) was added to react with TMA, and the colored product was measured spectrophotometrically at 410 nm in a glass cuvette (since tol-

uene dissolves plastic cuvettes). The TMA concentration determined is equivalent to that of TMAO in the original tissue; a few samples were tested for endogenous TMA concentrations, which were found, as in previous work (Kelly and Yancey 1999; Treberg and Driedzic 2002), to be very low (<1 mmol/kg).

HPLC Analysis

The concentrations of all other organic osmolytes (urea, GB, creatine, sarcosine, and free amino acids) were determined by HPLC with the procedure of Wolff et al. (1989). Samples were neutralized with 2M KOH to pH 6.5–7.5 and passed through a solid-phase C18 cartridge (Analytichem), to remove lipids, and a 0.45-μM filter. They were then run through a Waters Sugar-Pak1 column and a refractive index detector (0.5 mL/min, 80°C). All samples were compared to known osmolyte standards for identification and concentration calculations.

Water and Dry-Weight Analysis

Water content for the same fish specimens was determined as by Drazen (2002). Triplicate samples of muscle were dried at 60°C, and water content was determined by the difference between wet and dry weights. A previous study on teleosts (Samerotte et al. 2007) found that very watery species had TMAO content considerably lower than that of less watery species from similar depths. However, since TMAO concentrations are much greater in cells than in extracellular fluids, such watery species (which typically have fewer cells per unit tissue volume) could have intracellular TMAO levels similar to those of the other species. To test this, Samerotte et al. (2007) measured water content and divided TMAO content by dry mass, with the latter reflecting cell density, and found that the watery species fell in line with other species. Therefore, we performed the same analysis for water and TMAO. Urea has been found at equal concentrations both intra- and extracellularly in virtually all elasmobranchs studied and was not analyzed by dry mass, because water content of the tissue would have little effect on it.

Data and Statistical Analysis

Data plotted versus depth were fitted with linear and/or polynomial curve fits (Kaleidograph, Synergy Software). Regression statistics were performed with Statistica 7.1 software (Statsoft). Polynomial fits were chosen because, in a previous study on teleosts (Samerotte et al. 2007), there was a clear sigmoidal pattern for TMAO versus depth that has been shown to be a common pattern with increasing pressure effects on protein stability and function (see "Discussion"). In some analyses, skate (Rajidae) samples were analyzed separately to avoid possible phylogenetic biases due to the preponderance of skate samples at greater depths, compared to the single species of holocephalan (all from relatively shallow depths) and the small sample sizes of shark species (see "Results").

Results

Thirteen species of chondrichthyan fish were caught at depths between 50 and 2,165 m (Table 1). In total, one holocephalan, five sharks, and eight skates and rays were examined. Species caught were as follows (with common names, plus depth ranges from Ebert 2003):

Holocephali: *Hydrolagus colliei* (spotted ratfish; intertidal to 971 m);

Selachii (sharks): *Squalus acanthias* (dogfish shark, surface to 1,236 m), *Somniosus pacificus* (Pacific sleeper shark, surface to >2,000 m), *Parmaturus xaniurus* (filetail catshark, 91–1,251 m), *Apristurus brunneus* (brown catshark, 33–1,298 m), and, from the middle North Atlantic (see "Material and Methods"), *Apristurus profundorum* (deepwater catshark, 1,100–2,000 m);

Batoidea (skates and rays): *Torpedo californica* (Pacific electric ray, 3–200 m), *Raja stellulata* (starry skate, <100–732 m), *Raja rhina* (longnose skate, nearshore to 1,000 m), *Bathyraja kincaidii* (sandpaper skate, 200–500 m), *Bathyraja abyssicola* (deep-sea skate, 362–2,906 m), *Bathyraja trachura* (roughtail skate, 400–2,550 m), *Bathyraja microtrachys* (1,995–2,900 m), and *Amblyraja badia* (broad skate, 846–2,324 m).

Also included in data plots were our 1999 data (Kelly and Yancey 1999) for *Bathyraja spinosissima* (spiny skate) and *Bathyraja* spp. (2,850 m) caught off the coast of Oregon; urea and TMAO analyses for these were conducted in the same laboratory with the same instrumentation as those for the current study, and water content was measured by the procedure described in "Material and Methods."

Four species were captured at multiple depths: *H. colliei*, *R. rhina*, *B. kincaidii*, and *A. badia*. Despite several trawls, no Chondrichthyes were caught between 2,800 and 3,200 m. However, *A. badia* was observed at a concurrently deployed baited camera at 2,869 m (Yeh and Drazen 2011).

Species water and major organic solute contents are reported in Tables 1 and 2. Species are grouped by closely related depths of capture, with median trawl depth indicated. Osmolytes include TMAO, urea, and GB, as well as β-alanine (a common osmolyte in some Chondrichthyes). Creatine content (high in vertebrate white-muscle tissue as part of the creatine-phosphate energy system) is also reported because creatine, though not considered an osmolyte, is a major contributor to osmotic pressure.

Urea and TMAO

Between 50 and 543 m, urea was a greater contributor to osmolality than the methylamines, while TMAO dominated in the deepest group (Fig. 1). Urea content was 291–371 mmol/kg in the shallowest group and 170–189 mmol/kg in the deepest group (Table 1). Urea content declined with depth linearly, with no indication of a leveling off or plateau, with $r^2 = 0.82$ and $P < 0.0001$ (Fig. 1). A second-order polynomial fit gave essentially a straight line.

Conversely, TMAO content was 85–168 mmol/kg in the shallowest group and 250–289 mmol/kg in the deepest species. A

Table 2: Glycine betaine, creatine, and β-alanine contents of white muscle of Chondrichthyes from various depths

Depth, Species	Subclass or Division	Average Depth (m)	*n*	Glycine Betaine (mmol/kg wet mass)	Creatine (mmol/kg wet mass)	β-alanine (mmol/kg wet mass)
50–87 m:						
Raja stellulata	Skate	50	1	.10	32.0	8.28
Hydrolagus colliei	Holocephalan	83	1	80.3	30.7	30.3
Raja rhina	Skate	87	1	66.6	22.4	19.7
Squalus acanthias	Shark	87	1	69.9	23.9	0
Torpedo californica	Ray	87	1	25.1	19.2	0
253–263 m:						
Bathyraja kincaidii	Skate	253	1	1.48	31.7	9.15
H. colliei	Holocephalan	263	4	81.4 ± 9.6	14.6 ± 1.3	14.6 ± 1.2
500–543 m:						
B. kincaidii	Skate	500	2	8.54 ± 6.77	30.2 ± 1.9	26.1 ± 2.2
Parmaturus xaniurus	Shark	533	2	73.8 ± 1.6	24.7 ± .02	4.28 ± 2.2
R. rhina	Skate	533	3	18.8 ± 14.8	28.2 ± 1.5	29.6 ± .2
Somniosus pacificus	Shark	533	2	12.7 ± 6.8	11.3 ± .8	0
H. colliei	Holocephalan	543	3	66.1 ± 28.0	23.7 ± 5.9	15.5 ± 1.6
793 m:						
Apristurus brunneus	Shark	793	1	40.6	12.2	6.72
1,205–1,323 m:						
Bathyraja abyssicola	Skate	1,205	3	1.35 ± .62	19.2 ± 2.9	40.4 ± 3.2
Amblyraja badia	Skate	1,280	3	5.12 ± 3.17	12.2 ± .9	8.61 ± 3.7
Bathyraja trachura	Skate	1,323	2	4.52 ± 1.13	21.6 ± 4.3	32.4 ± 13.0
1,911–2,165 m:						
Apristurus profundorum	Shark	1,911	1	30.7	20.8	20.2
Bathyraja microtrachys	Skate	2,107	5	9.08 ± 5.82	20.2 ± 6.6	19.6 ± 11.8
A. badia	Skate	2,165	2	16.7 ± 5.9	11.8 ± 2.5	20.1 ± 1.3

second-order polynomial fit suggested a possible leveling off or plateau at the greatest depths, with $r^2 = 0.75$ and ANOVA $P < 0.0001$ (Fig. 1).

In a previous study on teleosts, TMAO content within a single species caught at more than one depth was higher in specimens caught at greater depths (Samerotte et al. 2007). In our current study, four species were caught at more than one depth. However, for three of these, the depths were too shallow and/or the *n* values too low to discern patterns. First, the three *R. rhina* from 533 m had higher TMAO (144 vs. 85 mmol/kg) and lower urea (328 vs. 360 mmol/kg) contents than the one individual from 87 m (Table 1), but clearly, conclusions cannot be drawn from an *n* of 1. Second, *H. colliei* caught at 83, 263, and 543 m had about the same TMAO content—161, 132, and 152 mmol/kg, respectively—while urea was highest in the shallowest specimen—371, 320, and 317 mmol/kg, respectively (Table 1). However, there was only one individual from 83 m. Third, *B. kincaidii* from 253 and 500 m had about the same urea and TMAO contents, with again only one individual from 253 m. Fourth, three *A. badia* were caught at 1,280 m and two at 2,165 m. Urea was lower in the deeper group—237 versus 183 mmol/kg ($P = 0.004$)—but TMAO was not different—247 versus 250 mmol/kg (Table 1).

Glycine Betaine, Creatine, and β-Alanine

GB content did not show a clear trend (Fig. 2*A*) and varied widely among taxa. GB averaged 45 mmol/kg in the shallowest group but with high variability, ranging from near 0 in the skate *R. stellulata* to 80 mmol/kg in the holocephalan *H. colliei*. The latter had the highest levels for animals in the two shallowest groups and the second-highest in the 500–543-m group (Table 2). Overall, while GB content appeared to decrease with depth (Fig. 2*A*), this trend was not quite significant ($r^2 = 0.16$, $P = 0.07$).

Other small organic solutes with significant osmotic levels were creatine and β-alanine (Table 2; Fig. 2*B*). Concentrations of β-alanine were highly variable and showed no significant depth trend (linear fit of $r^2 = 0.11$, $P = 0.16$). Like GB, β-alanine appeared to be taxon specific, ranging from very low

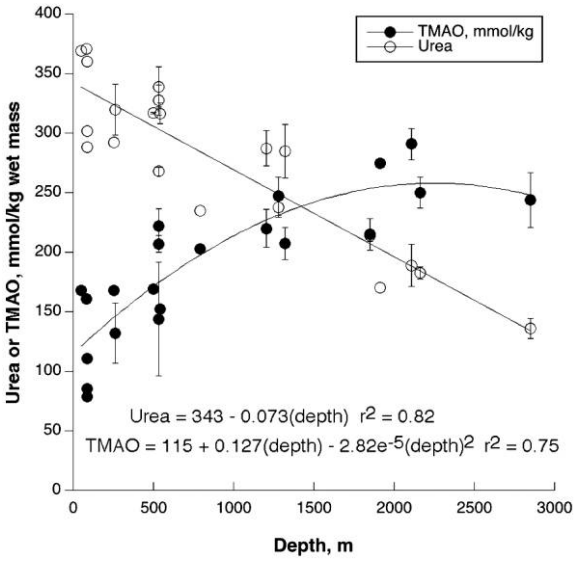

Figure 1. Muscle trimethylamine N-oxide (TMAO) and urea contents per wet mass in Chondrichthyes from various depths. The data for 2,850 m are from Kelly and Yancey (1999). Error bars represent standard deviations. A linear fit for urea and a second-order polynomial fit for TMAO are shown.

in all sharks and the ray to 40 mmol/kg in the skate *B. abyssicola.* Creatine showed a marginally significant decrease with depth (linear fit of $r^2 = 0.21$, $P = 0.05$). Small amounts of other free amino acids were found, primarily taurine, glycine, and alanine. These were all <10 mmol/kg wet mass with no depth trends, except that taurine levels were lower in the deepest two groups (data not shown).

Ratios and Totals

The U : T ratio declined significantly with depth, from 2.96 in the shallowest group to 0.67 in the deepest (Table 1). The ratio plot in Figure 3 exhibits an exponential decline, with a curve fit giving $r^2 = 0.70$, $P < 0.0001$. This is expected because, even if both urea and TMAO change linearly and/or asymptotically with depth, the ratio would decrease exponentially, at least to a point.

Because GB is nearly as strong as TMAO in counteracting urea, it has generally been added to TMAO when ratios are calculated for the urea-methylamine counteraction hypothesis. Here, the ratio of urea to TMAO + GB for the shallowest group was 2.12 ± 0.58, virtually identical to that found by previous research and in line with the approximate 2 : 1 thermodynamic optimum for urea counteraction. The urea : (TMAO + GB) ratio, also plotted in Figure 3, had a fit similar to that for U : T, with $r^2 = 0.70$, $P < 0.0001$.

The changing compositions with depth appear to have kept the total osmotic contribution of organic osmolytes at about the same level (presumably maintaining osmoconformation). The total of the measured organic osmolytes averaged 542 (±52 SD) mOsm/kg at all depths, with a virtually flat linear fit as follows: TMAO (mmol/kg) = −0.018 × depth (m) + 554.7 ($r^2 = 0.01$; $P = 0.69$). Complete data are not available for the 1999 specimen at 2,850 m, and so that species was not included.

Rajidae

Skate (Rajidae) data were analyzed separately, as noted in "Material and Methods." Figure 4*A* shows osmolyte values for this subgroup of Chondrichthyes, revealing again an apparent linear decrease in urea content, with $r^2 = 0.93$, $P = 0.0001$. In contrast, there is a distinct leveling off in TMAO content. A second-

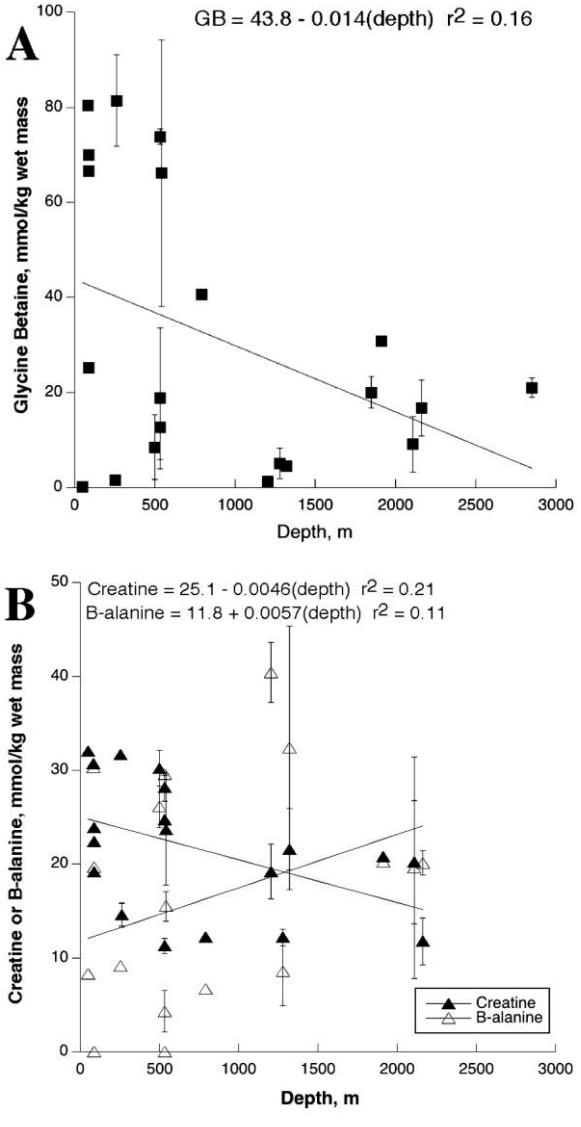

Figure 2. Muscle contents of other major organic osmolytes per wet mass in Chondrichthyes from various depths. The data for 2,850 m are from Kelly and Yancey (1999). Error bars represent standard deviations. *A*, Glycine betaine (GB), with a linear fit. *B*, Creatine and β-alanine, with linear fits.

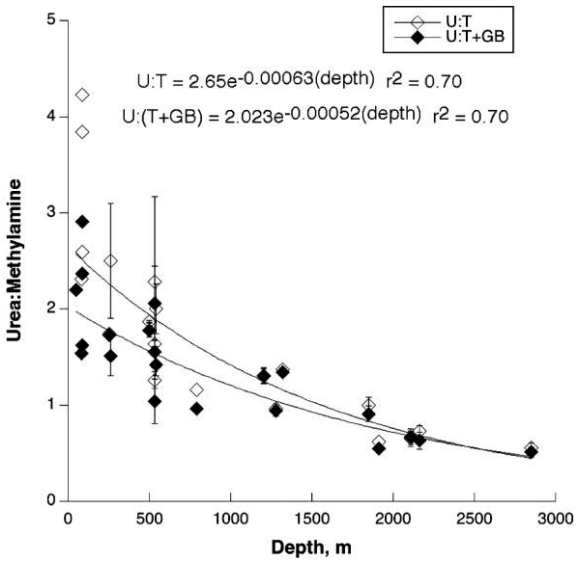

Figure 3. Urea : methylamine ratios (U : T = urea : trimethylamine N-oxide [TMAO]; U : (T + GB) = urea : (TMAO + glycine betaine)) in Chondrichthyes from various depths, from the data in Figures 1 and 2. The data for 2,850 m are from Kelly and Yancey (1999). Error bars represent standard deviations. Exponential fits are shown.

order polynomial fit gave $r^2 = 0.76$, ANOVA $P < 0.0001$. Figure 4*B* shows the U : T and urea : (TMAO + GB) ratios for these rajids. The exponential fits gave $r^2 = 0.71$ ($P < 0.0001$) and 0.93, respectively ($P < 0.0001$).

Water Content and Osmolyte Content by Dry Mass

Water content of white muscle is shown in Table 1. It ranges from 77.8% in *R. stellulata* to 87.3% in *A. brunneus*. However, there were no depth trends, with all species having 78%–82% except for *S. pacificus* at 533 m and *A. brunneus* at 793 m, which were more watery at 83.6% ($n = 2$) and 87.3% ($n = 1$), respectively.

TMAO content was divided by dry mass in order to correct for TMAO values in species with high water content (see "Material and Methods"). The results are plotted in Figure 5, with a polynomial fit ($r^2 = 0.68$, ANOVA $P < 0.0001$). The resulting patterns were the same as with wet-mass values, with the exception of the high–water content individuals (*S. pacificus* and *A. brunneus*). Their high TMAO content per dry mass may be due to high extracellular TMAO concentrations (see "Discussion"). For skates only (Fig. 5*B*), the data appear to follow a sigmoidal pattern, with a third-order polynomial fit ($r^2 = 0.90$, ANOVA $P < 0.0001$).

Discussion

The physical and ecological conditions of the abyssal ocean present a unique set of problems that all species there must overcome. Two problems are of particular relevance to this study. First, hydrostatic pressure is a perturbant of cellular structure and function. Second, low energy levels (except in relatively small, energy-rich habitats such as methane seeps and hydrothermal vents) limit the growth of primary producers. Consequently, deep-sea organisms often adapt energy-saving structures and metabolisms. This metabolic efficiency must be balanced against the additional costs associated with overcoming pressure effects. Our current osmolyte analyses were designed to test whether the dearth of Chondrichthyes in the abyssal ocean—they are scarce below 3,000 m and absent below about 4,000 m (Priede et al. 2006)—might be associated with these two variables. Specifically, we asked (1) whether a

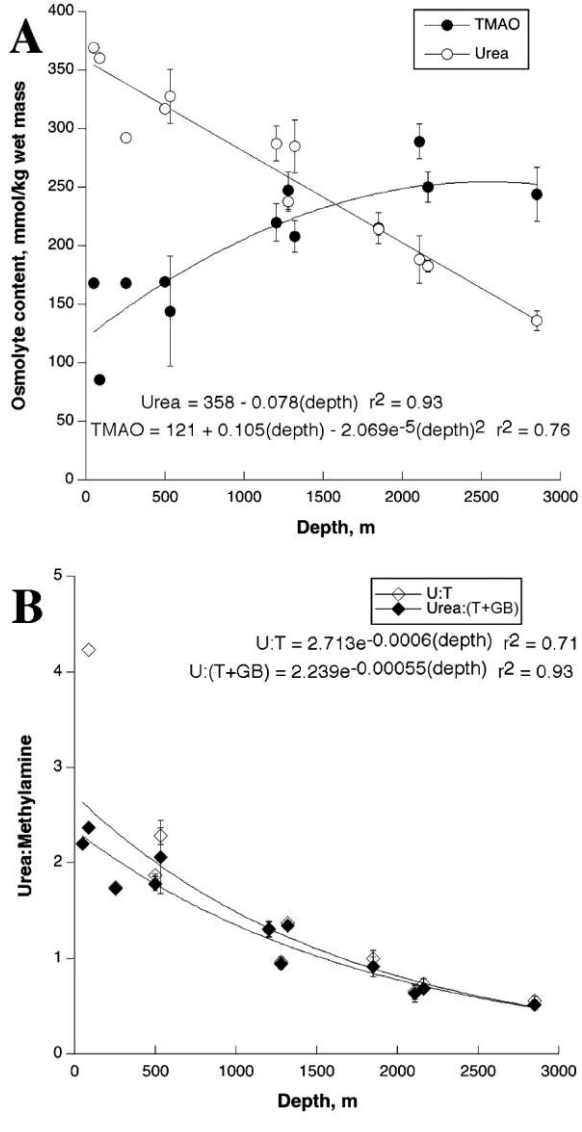

Figure 4. Muscle trimethylamine N-oxide (TMAO) and urea contents in Rajidae from various depths. The data for 2,850 m are from Kelly and Yancey (1999). Error bars represent standard deviations. *A*, Muscle contents, with a linear fit for urea and a second-order polynomial fit for TMAO. *B*, Urea : methylamine ratios (U : T = urea : TMAO; U : (T + GB) = urea : (TMAO + glycine betaine)); exponential fits are shown.

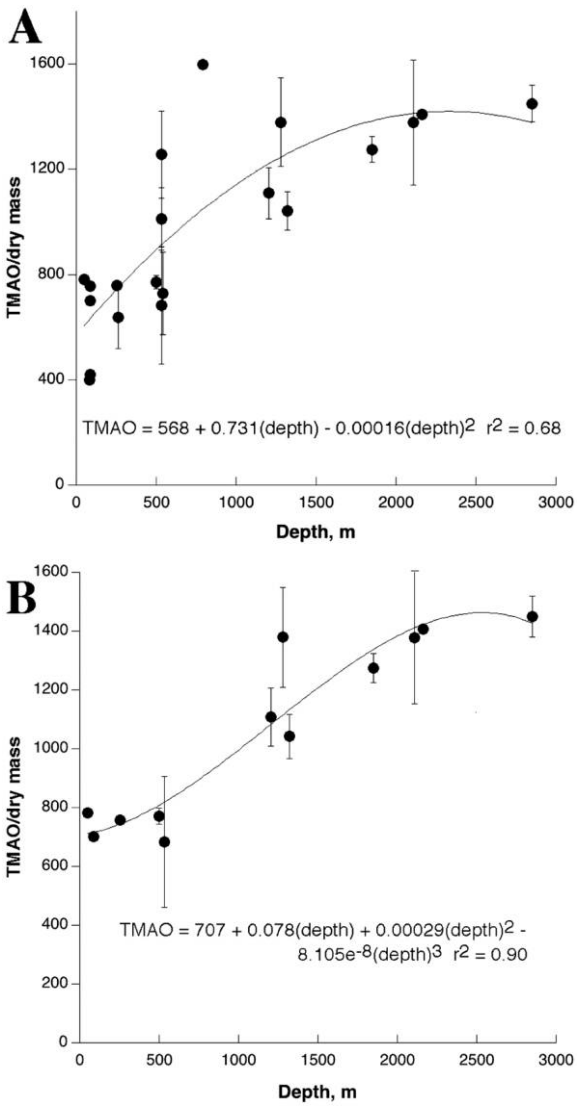

Figure 5. Muscle trimethylamine N-oxide (TMAO) content per dry mass in Chondrichthyes from various depths. The data for 2,850 m are from Kelly and Yancey (1999). Error bars represent standard deviations. *A*, All species, with a second-order polynomial fit; *B*, Rajidae only, with a third-order polynomial fit.

previously reported depth trend of increasing TMAO and decreasing urea in three species of elasmobranchs—a trend hypothesized to be a pressure adaptation—is a widespread phenomenon in Chondrichthyes and (2) whether that trend, if real, shows any depth limits that might relate to the absence of Chondrichthyes at greater depths.

Decreasing U : T Ratio with Depth: "TMAO-Osmotic" Deep-Sea Chondrichthyes

For the first question, our data confirm that Chondrichthyes below 1,500 m are likely to be universally "TMAO-osmotic," utilizing TMAO as their major organic osmolyte while keeping the total content of major organic osmolytes about the same as that in shallow species. The trend of increasing TMAO and decreasing urea with depth has now been shown to be consistent in a variety of sharks and skates from the Pacific and Atlantic Oceans (this study; Kelly and Yancey 1999; Treberg and Driedzic 2002). Moreover, deep-sea Greenland sharks have been reported to have considerably higher TMAO levels than shallow sharks, although urea content was not reported (Anthoni et al. 1991).

The high TMAO per unit dry mass in the sharks *Apristurus brunneus* and *Somniosus pacificus* (Fig. 5*A*) could be due to high TMAO content both extra- and intracellularly, since TMAO in their extracts would be higher than that in extracts from species in which TMAO is found primarily inside cells. This was found for one deep-sea teleost species that had very high plasma TMAO concentrations and also much higher TMAO concentrations in extracts than other species with low plasma TMAO levels from the same depth (Gillett et al. 1997; Samerotte et al. 2007). Moreover, shallow sharks are routinely reported as having higher plasma TMAO concentrations than shallow skates and holocephalans (e.g., Robertson 1975, 1976; King and Goldstein 1983). However, we do not know whether deep-sea elasmobranchs have relatively higher plasma TMAO concentrations.

In the two counteracting-osmolytes hypotheses, TMAO counteracts the protein-perturbing effects of both urea and pressure. However, this begs the question of why Chondrichthyes do not simply use TMAO as their main osmolyte at all depths. The mechanisms by which such counteracting organic osmolytes function are only partially understood. It is known that TMAO and other stabilizing osmolytes are largely excluded from interaction with the protein's peptide backbone because of interactions with water molecules. This characteristic, termed the osmophobic effect, favors the compact, folded conformation of a protein over its denatured state (Bolen and Baskakov 1995). In comparison, urea favorably binds to the peptide backbone, promoting its exposure and thus the destabilization of the folded protein. Since the solutes do not interact directly with one another, their effects are additive. The Gibbs free energy, Δg, for transfer of the universal peptide backbone into a 1M urea solution is negative (favorable) and about half that of the positive (unfavorable) value for 1M TMAO (Street et al. 2006). The equilibrium of the biological system results from a favorable mixture of these two cosolutes. High concentrations of stabilizers at shallow depths, where hydrostatic pressure is not a significant perturbant, may be just as disadvantageous as urea alone, favoring protein aggregation and inhibiting conformational changes in enzymatic reactions (Yancey and Somero 1980; Lin and Timasheff 1994; Yancey 2005).

TMAO with Depth: A Sigmoidal Increase?

Previous work on 17 species of teleosts showed that there was a sigmoidal increase in TMAO content, with little change between 0 and 500 m, a steep rise between 500 and 1,500 m, and a linear increase at greater depths (Samerotte et al. 2007). It

was hypothesized that this increase reflects the effects of pressure on proteins, which also generally follow a sigmoidal pattern, with little effect in the 0–50-atm range (equivalent to 0–500-m depth). Our current data for Chondrichthyes can neither confirm nor eliminate a similar sigmoidal pattern, primarily because of the wide variation in the data of the species sampled within the 50–543-m range, as seen in Figures 1 and 5*A*. Similarly, in the skate plots (Figs. 4, 5*B*), there appears to be a sigmoidal pattern with little difference in TMAO content between 50 and 533 m, but again, the data are too varied to confirm this trend. Moreover, urea content might not change in a simple linear way with depth but could be relatively flat over the 50–543-m range (Fig. 1), decreasing only at greater depths.

At least four reasons could account for such scatter. First, previous work on Chondrichthyes has shown a wide variation in compositions of stabilizing osmolytes thought to counteract urea. Sharks, for example, typically use TMAO and lesser amounts of GB; some skates have been found to have a mixture of TMAO, GB, sarcosine (N-methylglycine), and β-alanine (King and Goldstein 1983); and TMAO is the main methylamine in one species of holocephalan (Robertson 1976), but GB is in another (Bedford et al. 1998). Within the skate family, a mixture of stabilizing osmolytes counteracts urea about as well as pure TMAO (Yancey and Somero 1980). Thus, TMAO values vary considerably in shallower species, where stabilizing osmolytes serve primarily to counteract urea. The lower variation in TMAO content at greater depths (Fig. 1) may be due to an increasing preference for TMAO over other solutes because of its superiority as a pressure stabilizer (Yancey et al. 2004).

Second, some moderate-depth species may migrate up and down the continental slope and may not regulate their urea and TMAO contents precisely or immediately for optimal conformation to any particular depth. Typically, benthic fish such as skates are regarded as sedentary, maintaining a relatively high degree of site fidelity. However, a few studies have actively tracked the diel vertical movements of individual skates. In one study on the movement patterns of the northeast Atlantic common skate (*Dipturus batis*), electronic tagging data of six females revealed that individuals often migrated up to 100 m vertically in a day (Wearmouth and Sims 2009). Such daily migrations may explain some of the variation in osmolyte concentrations in the 50–543-m depth range: their osmolyte levels might reflect their recent average depth rather than the exact depth of capture.

Third, the lower variation in TMAO content at greater depths may be due to the decreasing number of species in our data set. Finally, the pressure hypothesis could be wrong, with the depth relationships due to other factors, such as dietary or metabolic limits unrelated to pressure.

Urea and/or TMAO as Possible Depth Limiters

Our second question was whether the trend of increasing TMAO and decreasing urea shows a depth limit related to the absence of Chondrichthyes from the abyssal ocean. We wondered whether urea cannot be decreased and/or TMAO cannot be increased sufficiently as depth increases, perhaps because of excretory or metabolic limits. If so, pressure perturbation may keep these fishes out of the deep sea. Unfortunately, we did not capture any Chondrichthyes below 2,165 m in this study, so our data do not provide a definitive answer. However, we found some intriguing trends when we included previous skate data from 2,850 m (Kelly and Yancey 1999).

As noted above, the depth limit of Chondrichthyes may be due to an inability to sufficiently reduce the concentration of urea as depth increases. Our plots do not show an indication of this, because urea decreases in a linear pattern down to 2,850 m (Figs. 1, 4*A*). It is possible that urea content plateaus below 3,000 m, but without capturing the rare elasmobranchs that live in the 3,000–4,000-m range, we cannot test this idea.

It is also possible that a limit to TMAO accumulation may prevent chondrichthyans from colonizing the abyssal ocean. Our data show an apparent plateau of TMAO concentrations between 250 and 300 mmol/kg (Figs. 4*A*, 5*B*). Although this is consistent with the hypothesis, the plateau is largely due to the data for one species from 2,850 m. The data for *Amblyraja badia*, a decrease in urea but no change in TMAO from 1,280 m to 2,165 m, also support the possibility of a limit to TMAO accumulation but not a limit to urea reduction. Again, this was the only species caught at two different depths where pressure is a clear perturbant.

A plateau in TMAO content at great depths might be explained in another way. TMAO at 250–300 mmol/kg may be sufficient to counteract pressure effects at depths over the range of 2,000–4,000 m or so if urea drops steadily toward 0 (perhaps at 4,600 m, based on extrapolation of the curve fits in Figs. 1, 4). This possibility is suggested by one teleost fish from 4,800 m having virtually the same TMAO content (261 mmol/kg; Samerotte et al. 2007) as chondrichthyans at 1,911–2,850 m. Deeper chondrichthyans would need other osmolytes to maintain osmoconformation while keeping TMAO constant over a wide depth range. If, however, TMAO accumulation becomes very difficult below 3,000 m, it could still explain why chondrichthyan species and abundance numbers drop precipitously below 3,000 m.

Even if TMAO accumulation becomes limited with depth, this begs the question of why chondrichthyan proteins would not evolve intrinsic pressure resistance at greater depths, obviating the need for more TMAO. As we noted above, deep-sea teleosts—which have been studied biochemically only down to about 5,000 m—have evolved pressure-resistant proteins, but that resistance is often incomplete (e.g., Na^+/K^+-ATPase; Gibbs and Somero 1989). TMAO may be important for counteraction of remaining pressure effects. Perhaps the physicochemical effects of pressure on proteins and water may make complete intrinsic pressure resistance difficult to evolve. Significantly, we do not yet know whether teleosts living at 5,000–8,000 m rely on TMAO accumulation above 300 mmol/kg, intrinsic protein adaptations, or both (or some other adaptation). If TMAO is the key, then the hypothesis that chondrich-

thyans are restricted by inability to accumulate more TMAO is still tenable. However, if intrinsic pressure resistance is the key in the deepest teleosts, then chondrichthyans should also be able to evolve similar resistance. In that case, evolution of resistance could be impaired if urea cannot be decreased at greater depths. Conversely, the evolution of intrinsic pressure resistance might not be limiting with depth, and other factors (rather than TMAO/urea) might be involved in exclusion of Chondrichthyes from the abyss.

Exogenous or Endogenous Origins of TMAO

If TMAO accumulation is a depth limiter, what might make it difficult for Chondrichthyes at great depths to accumulate TMAO to necessary concentrations? At least two scenarios are possible, as noted in "Introduction." If produced endogenously, TMAO may be too metabolically costly to produce. On the other hand, exogenous sources of TMAO may be insufficient because of limited prey in the deep sea.

Early metabolic studies of vertebrates indicate that choline, when present in prey, can be converted to TMAO (Baker and Chaykin 1962). Additional studies have elucidated this pathway, demonstrating that diacylglycerol can be converted to phosphatidylcholine, which is then hydrolyzed by enzymes in the liver to choline. Choline is then converted to GB and on to TMA, a highly toxic solute that may then be oxidized into TMAO (Seibel and Walsh 2002). However, this pathway is dependent on an animal's ability to oxidize TMA, a reaction catalyzed by the enzyme trimethylamine oxidase (TMAoxi). TMAoxi has been found to be largely lacking in elasmobranchs. In various studies of levels of TMAoxi in 12 different marine and euryhaline chondrichthyans, only three shallow-living sharks had significant levels of the enzyme, which was undetectable in one holocephalan (*Hydrolagus colliei*), two rays (including *Torpedo californica*), four skates, and two sharks (including *Squalus acanthias* and one deep-sea species with high TMAO; Baker et al. 1963; Goldstein and Dewitt-Harley 1973; Treberg et al. 2006). Therefore, a constraint on TMAO accumulation may be related to a lack of endogenous TMAO synthesis.

It is therefore likely that TMAO must be accumulated exogenously from dietary sources and then actively regulated and retained. In a study on the winter skate (*Leucoraja ocellata*), TMAO levels were maintained for up to 45 days with whole-body losses of less than 1% per day in the absence of feeding and with no detectable TMAoxi (Treberg and Driedzic 2006). Given the low food supply in most of the deep sea, perhaps Chondrichthyes cannot obtain enough exogenous TMAO at these depths to exhibit this degree of maintenance over the long term. However, as we have shown previously (Kelly and Yancey 1999), many deep-sea prey animals that Chondrichthyes eat (shrimp, crabs, cephalopods, teleosts) have elevated TMAO content, which could compensate for increasing scarcity of prey with depth. For example, *Bathyraja abyssicola* feeds on benthic annelid worms, cephalopods, tanner crabs, shrimp, and teleosts; *A. badia* feeds on cephalopods, crustaceans, and small teleosts such as rattails (Zorzi and Anderson 1988; Ebert 2003), although the quantity of prey eaten over time has not been reported. Thus, we cannot determine whether dietary intake sets a limit to TMAO accumulation at greater depths.

If TMAO intake is not limiting but levels higher than 300 mmol/kg are needed to counteract pressure at depths below 3,000 m, a third possibility is that TMAO becomes toxic above the 300-mmol/kg level regardless of whether a protein perturbant is present. This could be due to excessive TMA production or to direct perturbations not related to protein stabilization. Data are lacking to test this possibility (although a recent study found that elevated TMAO concentrations favor the development of atherosclerosis in mammals; Wang et al. 2011).

Glycine Betaine, Creatine, and Depth

While TMAO synthesis is not widespread in Chondrichthyes, GB synthesis is quite common. Choline can be converted to GB via betaine aldehyde dehydrogenase (BADH). Treberg et al. (2006) found that measurable levels of BADH activity were present in six species of Chondrichthyes and that all specimens had significant capacity for GB synthesis. Nevertheless, our results show that GB is a minor contributor to osmolality, when compared to TMAO, and that GB content does not increase with depth (Fig. 2*A*).

Creatine's marginally significant decrease with depth (Fig. 2*B*) may reflect a decline in robust swimming capacity with depth, since creatine in muscle is tightly linked to the energy-storage compound creatine phosphate. A decline with depth in swimming capacity (as indicated by contents of key enzymes such as lactate dehydrogenase) has been documented in both teleosts (Sullivan and Somero 1980) and elasmobranchs (Condon 2011).

Conclusion

Priede et al. (2006) proposed that the scarcity of abyssal Chondrichthyes is due to their high metabolic needs. Our hypothesis, that TMAO accumulation may set a depth limit, does not contradict their hypothesis but rather provides a different metabolic explanation. Clearly, Chondrichthyes from 3,000–4,000 m (as well as teleosts from 5,000–8,000 m) are needed to further test this hypothesis.

Acknowledgments

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