Correlation of Trimethylamine Oxide and Habitat Depth within and among Species of Teleost Fish: An Analysis of Causation

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ABSTRACT

Most shallow-water teleosts have moderate levels of trimethylamine N-oxide (TMAO; ~50 mmol/kg wet mass), a common osmolyte in many other marine animals. Recently, muscle TMAO contents were found to increase linearly with depth in six families. In one hypothesis, this may be an adaptation to counteract the deleterious effects of pressure on protein function, which TMAO does in vitro. In another hypothesis, TMAO may be accumulated as a by-product of acylglycerol (AG) production, increasing with depth because of elevated lipid metabolisms known to occur in some deep-sea animals. Here we analyze muscle TMAO contents and total body AG (mainly triacyglycerol [TAG]) levels in 15 species of teleosts from a greater variety of depths than sampled previously, including eight individual species caught at two or more depths. Including data of previous studies (total of 17 species, nine families), there is an apparent sigmoidal increase in TMAO contents between 0- and 1.4-km depths, from about 40 to 150 mmol/kg. From 1.4 to 4.8 km, the increase appears to be linear ($r^2 = 0.91$), rising to 261 mmol/kg at 4.8 km. The trend also occurred within species: in most cases in which a species was caught at two or more depths, TMAO was higher in the deeper-caught specimens (e.g., for *Coryphaenoides armatus*, TMAO was 173, 229, and 261 mmol/kg at 1.8, 4.1, and 4.8 km, respectively). TMAO contents not only were consistent within species at a given depth but also did not vary with season or over a wide range of body masses or TAG contents. Thus, no clear link between TMAO and lipid was found. However, TMAO contents might correlate with the rate (rather than content) of TAG production, which could not be quantified. Overall, the data strongly support the hypothesis that TMAO is adaptively regulated with depth in deep-sea teleosts. Whether lipid metabolism is the source of that TMAO is a question that remains to be tested fully.

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Introduction

Deep-sea organisms face a unique combination of environmental stresses relative to inhabitants of overlying surface waters, including low or no sunlight, hypoxia in some regions, low food energy, low temperatures, and high hydrostatic pressure. Thus, deep-sea organisms have unique physiological and biochemical adaptations (Siebenaller and Somero 1989; Childress 1995; Seibel and Drazen 2007). In recent years, a striking linear correlation has been found between contents of trimethylamine N-oxide (TMAO) in some marine animals and depth of capture. In teleost fish, for example, muscle TMAO contents ranged from less than 50 mmol/kg in shallow species to more than 260 mmol/kg in a species from 4,850 m (Gillett et al. 1997; Kelly and Yancey 1999; Yancey et al. 2004). Similar increases in TMAO with depth of capture were found in shrimp, crabs, and elasmobranchs (Kelly and Yancey 1999; Treberg and Driedzic 2002), although not in cephalopods (Seibel and Walsh 2002). Possible reasons for this depth trend are currently being investigated.

TMAO has a variety of possible functions in marine animals, but it is best known as an organic osmolyte. Most marine organisms are osmoconformers; their cells prevent osmotic shrinkage by accumulating organic osmolytes to elevate osmotic pressure to that of the environment (about 1,000 mOsm compared with the roughly 300 mOsm resulting from basic cellular solutes). The main osmolytes in marine invertebrates are certain neutral amino acids and, to a lesser extent, methylamines such as glycine betaine and TMAO; in elasmobranchs, the primary osmolytes are urea and TMAO. Organic solutes are probably selected over inorganic solutes as osmolytes because the latter can perturb macromolecules while the former usually do not; that is, they are "compatible" with cellular functions (Brown and Simpson 1972). More important, some osmolytes such as TMAO actually stabilize macromolecules and can counteract

perturbants. In shallow marine animals, the highest levels of TMAO are found in elasmobranchs, in which TMAO is thought to counteract the effects of urea, the other major osmolyte in these animals and a solute well known to perturb proteins (Yancey et al. 1982).

In contrast to osmoconformers, bony fish and "higher" vertebrates are osmoregulators, relying on osmoregulatory organs such as gills or kidneys to maintain a stable internal osmotic pressure. Most osmoregulators are highly hypoosmotic compared with seawater; for example, marine bony fish are generally characterized as having internal osmotic pressures of about 300–400 mOsm (Lange and Fugelli 1965) and only low levels of organic osmolytes. Deep-sea teleosts, however, appear to be an exception because of TMAO accumulation.

Two sets of hypotheses have been proposed to explain the TMAO depth trend. The first set proposes that high TMAO levels are directly adaptive in the deep sea. First, higher levels could enhance buoyancy, because TMAO solutions are less dense than solutions of most organic solutes (Withers et al. 1994). Perhaps this property is energetically useful in the deep sea. Second, as an osmolyte, TMAO accumulation could reduce the energy costs of osmoregulation in deep-sea teleosts. However, there are no apparent reasons why these properties would not also be useful in shallow-water animals (Kelly and Yancey 1999). Third, TMAO may counteract the inhibitory effects of hydrostatic pressure, which it has been shown to do in vitro for enzyme kinetics, protein structure, and yeast growth (Gillett et al. 1997; Yancey and Siebenaller 1999; Yancey et al. 2001, 2002, 2004). This property might explain the increase of TMAO with depth, since pressure also increases with depth.

The second set of hypotheses suggests that high TMAO levels result from diet or metabolism and thus are not necessarily directly adaptive (or are secondarily adaptive). Seibel and Walsh (2002) proposed that TMAO concentrations are a by-product of the production and storage of acylglycerol lipids, which are important energy stores in a variety of animals. Specifically, phosphatidylcholine (PtdCholine) hydrolysis results in diacylglycerol either for lipid storage as triacylglycerol (TAG) or for energy metabolism. The hydrolysis releases choline, which is then converted into trimethylamine (TMA). This highly toxic compound is accumulated in at least one invertebrate as a buoyancy mechanism (Sanders and Childress 1988). However, it is more typically oxygenated into TMAO, which is either excreted or accumulated (e.g., as an osmolyte). Thus, high levels of nondietary TMAO might occur in an animal if there is a high rate of TAG synthesis. In support of this idea, cephalopods show a strong correlation between muscle TMAO content, digestive gland lipid percentage (of body mass), and total lipid percentage but no clear relationship to depth (Seibel and Walsh 2002). Furthermore, Childress and Nygaard (1974) found that deep-sea crustaceans (at least some of which have elevated TMAO levels; Kelly and Yancey 1999) tend to accumulate greater lipid levels with increasing depth. Therefore, increased levels of TMAO with depth in some taxa may be a by-product of higher lipid production with depth (Seibel and Walsh 2002). Alternatively, more than one hypothesis may be correct; for example, TMAO might be produced as a by-product of lipid metabolism but then might be actively retained rather than excreted because of its adaptive properties (e.g., pressure counteraction).

To test these hypotheses in more detail with respect to teleosts, we have now examined TMAO contents along with lipid compositions for a greater variety of species and families from a greater variety of depths than examined in previous studies. Furthermore, this study includes several individual species caught at different depths, unlike previous work on TMAO in which most individual species were caught at only one depth (Gillett et al. 1997; Kelly and Yancey 1999). We also test for any association between TMAO and TAG contents, season of capture, and body mass. This analysis had the potential to rule out one set of hypotheses or the other. Our results support the proposal that TMAO accumulation is regulated as a direct adaptation. In contrast to cephalopod data (Seibel and Walsh 2002), no simple correlations between TMAO and lipids in these fish were found. However, the actual rate of lipid metabolism could not be quantified to test the hypothesis fully.

Material and Methods

Specimen Collection

Fifteen species of fish, ranging in capture depths between 191 and 1,406 m, were collected by a Nor'eastern bottom trawl with a 37.4-m footrope. This was a part of the National Marine Fisheries slope survey conducted in November 1996 off the Oregon and California coasts (latitudes and longitudes available on request). *Coryphaenoides armatus*, *Coryphaenoides yaquinae*, and *Coryphaenoides acrolepis* were captured using free vehicle traps and longlines seasonally from 1996 to 1998 in the abyssal north Pacific (4,100 m) and the San Diego Trough (1,170 m; for collection details, see Drazen 2002). Onboard the ship, white muscle samples were removed from each fish under the dorsal fin and frozen at −196°C using liquid nitrogen; on shore, samples were stored at −80°C.

Analytical Procedures

A piece of partially thawed muscle between 0.06 and 0.10 g was homogenized at 4°C with 7% perchloric acid at nine times the tissue volume. Proteins were allowed to precipitate overnight at 4°C. The supernatant was separated from the protein pellet after centrifugation at 15,000 *g* for 20 min at 4°C. TMAO concentrations were measured as described by Wekell and Barnett (1991), using an iron-EDTA reagent to reduce the TMAO to trimethylamine. The TMA was extracted into toluene and reacted with 0.02% picric acid, creating a yellow product measured at 410 nm. TMAO standards of 0, 1, 2, and 3 mM were

Table 1: Teleost species with capture depths and muscle composition per wet mass

Species	Family	*n*	Depth (m)	TMAO (mmol/kg)	Water (%)	Protein (mg/g)
Parophrys vetulus	Pleuronectidae	4	203	41.4 ± 9.5	83.0 ± .83	93.9 ± 5.32
Merluccius productus	Merlucciidae	5	191–290	53.1 ± 7.13	82.0 ± .61	96.5 ± 4.03
Sebastes diploproa	Scorpaenidae	5	347	43.4 ± 8.6	78.7 ± 3.57	110 ± 3.46
Glyptocephalus zachirus	Pleuronectidae	5	507	57.0 ± 11.3	84.1 ± .66	90.2 ± 4.10
Microstomus pacificus	Pleuronectidae	3	511	45.6 ± 4.9	86.6 ± 3.12	71.4 ± 7.99
Sebastolobus alascanus	Scorpaenidae	5	598	97.0 ± 9.2	81.1 ± 1.96	99.8 ± 2.76
S. alascanus	Scorpaenidae	4	801	109 ± 8.7	81.8 ± .19	97.1 ± 3.97
Anoplopoma fimbria	Anoplopomatidae	5	687–691	74.16 ± 6.9	70.0 ± 2.03	86.0 ± 10.2
Sebastolobus altivelis	Scorpaenidae	4	773	110 ± 13.7	83.4 ± .98	92.1 ± 11.0
S. altivelis	Scorpaenidae	5	853	133 ± 9.9*	82.9 ± .79	95.6 ± 8.81
Albatrossia pectoralis	Macrouridae	3	853	*54.7 ± 7.5*	*93.0 ± .43*	*44.3 ± 1.42*
A. pectoralis	Macrouridae	4	924	*53.7 ± 9.7*	*91.2 ± .96*	*44.7 ± 3.31*
A. pectoralis	Macrouridae	2	1,156–1,224	*80.4 ± 1.7**	*90.9 ± .94*	*56.6 ± 1.61*
Alepocephalus tenebrosus	Alepocephalidae	5	860	*90.9 ± 7.2*	*90.2 ± 2.95*	*57.0 ± 3.97*
A. tenebrosus	Alepocephalidae	2	1,046	*91.6 ± 13.4*	*87.2 ± 7.2*	*52.3 ± 11.4*
Coryphaenoides acrolepis	Macrouridae	2	924	100 ± 5.3*	82.7 ± .49	99.8 ± 5.31
February 1998		5	1,170	130 ± 14.1	83.7 ± .95	108 ± 7.05
November 1998		5	1,170	134 ± 7.6	83.4 ± .50	116 ± 3.76
C. acrolepis	Macrouridae	4	1,224	123 ± 11.0	83.1 ± .65	96.7 ± 8.47
Coryphaenoides cinereus	Macrouridae	5	1,169	116 ± 13.0	82.9 ± .39	91.0 ± 6.06
C. cinereus	Macrouridae	3	1,406	152 ± 18.4*	83.8 ± .46	83.0 ± 3.57
Antimora microlepis	Moridae	5	1,173–1,206	*165 ± 21.8*	83.1 ± .90	84.2 ± 6.57
Coryphaenoides armatus	Macrouridae					
April 1998		5	4,100	229 ± 25.1	82.9 ± .94	116 ± 5.64
December 1998		5	4,100	228 ± 17.1	84.0 ± .77	113 ± 4.02
Coryphaenoides yaquinae	Macrouridae	5	4,100	250 ± 19.5	82.5 ± .59	103 ± 9.20

Note. All fish were collected in November 1996 unless otherwise noted. Values in italic are outliers.

* Difference ($P < 0.05$) between depths within species.

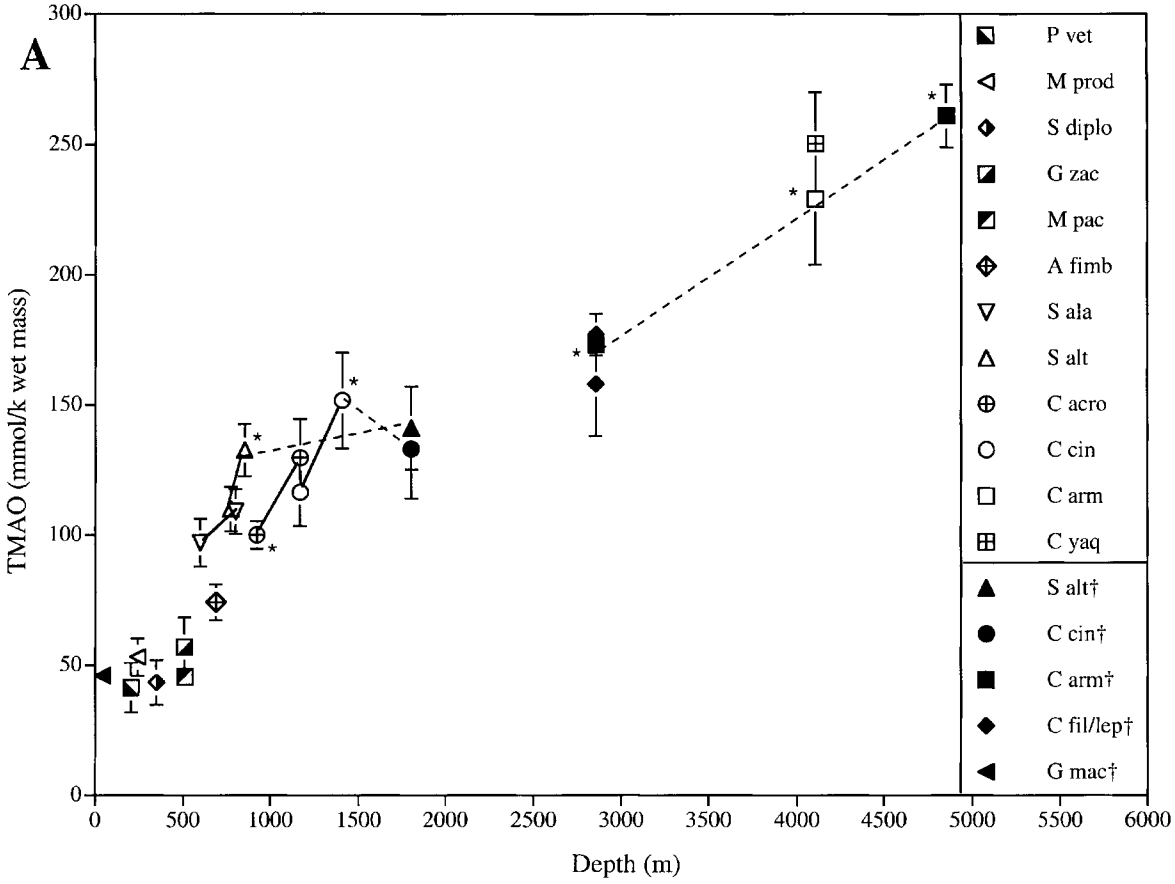
run with each trial. To account for tissue levels of TMA that are, by default, also measured by the above procedure, TMA levels were separately measured using the TMAO procedure without the iron-EDTA reagent. TMA levels also indicate whether the muscle samples have been decomposing over the years of storage. Other potential osmolytes (neutral amino acids, betaine, polyols) and creatine (a major constituent of vertebrate muscle) were measured by high performance liquid chromatography using a Waters Sugarpak column, as described by Wolff et al. (1989).

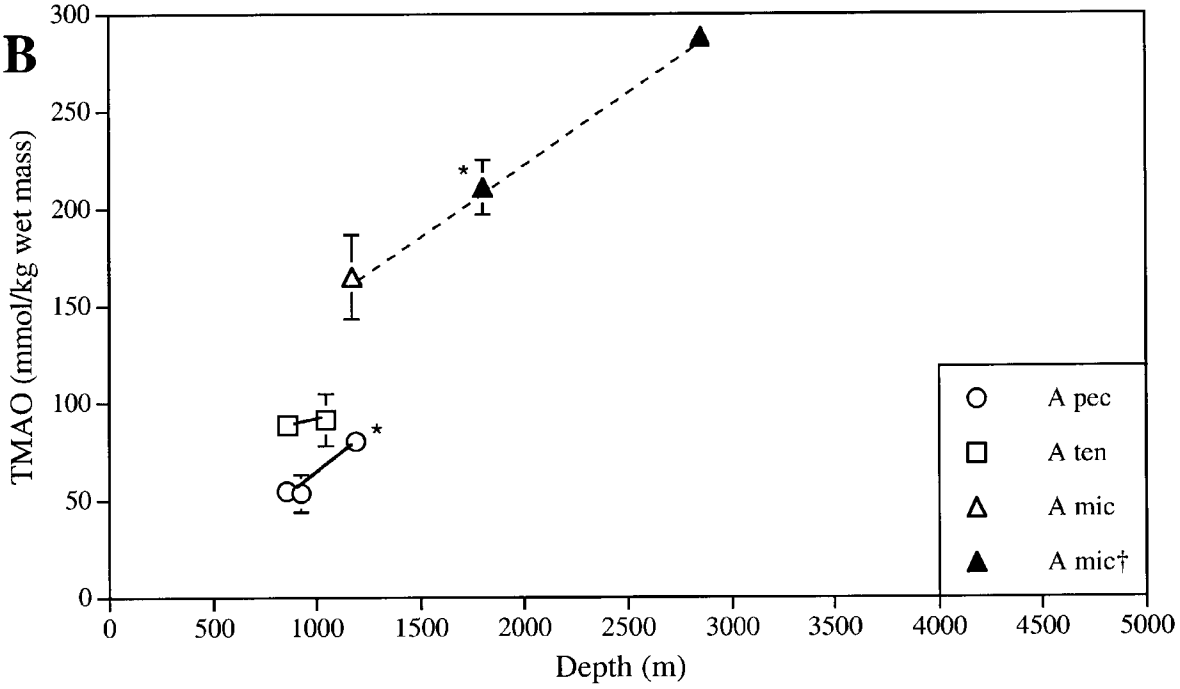
Lipid, water, and protein contents for the same fish specimens were determined as by Drazen (2002). Triplicate samples of muscle were freeze dried, and water content was determined by difference in weight. Protein and lipid assays were performed on tissue homogenized in distilled water in triplicate. The bicinchoninic acid protein assay (Smith et al. 1985) was used with bovine serum albumin as a standard. Lipids were extracted according to Bligh and Dyer (1959), and lipid composition was determined using the Iatroscan technique (Fraser et al. 1985; Volkman and Nichols 1991). Cholesteryl oleate, triolein, oleic acid, cholesterol, diolein, and phosphotidycholine were used as standards for steryl esters, triglycerides, free fatty acids, sterols, diglycerides, and phospholipids, respectively. Standards were run for each set of 10 SIII chromorods, and standard curves were best fit as either linear or power functions. Lipids were concentrated, resuspended in chloroform, and spotted (1 μL) in duplicate. Rods were developed in 85 : 15 : 0.01 (muscle lipids) or 90 : 10 : 0.01 (liver lipids) hexanes, diethyl ether, and formic acid for 20–25 min. Rods were dried for 8–10 min in an oven at 110°C before scanning on a Mark V Iatroscan. Each frame was scanned once to quantify the lipid classes and a second time to remove residual material. In this article, only acylglycerol levels are reported in detail since they are proposed to be related to TMAO production (other lipid classes will be reported in a separate article).

Results

TMAO versus Depth

TMAO contents ranged from 41 mmol/kg wet mass in the shallowest species (*Parophrys vetulus*) to 250 mmol/kg in the


A
TMAO (mmol/k wet mass)
Depth (m)
P vet
M prod
S diplo
G zac
M pac
A fimb
S ala
S alt
C acro
C cin
C arm
C yaq
S alt†
C cin†
C arm†
C fil/lep†
G mac†


B
TMAO (mmol/kg wet mass)
Depth (m)
A pec
A ten
A mic
A mic†

deepest (*Coryphaenoides yaquinae*; Table 1). The results for 11 species are plotted in Figure 1*A* (open or half-filled symbols). Also plotted on Figure 1*A* are the results from previous studies (solid symbols). There was a general pattern of increased TMAO contents with depth across all species.

Three species (*Albatrossia pectoralis*, *Alepocephalus tenobrosus*, *Antimora microlepis*), plotted separately on Figure 1*B*, were considered outliers for the following reasons. Most species had robust muscles with protein contents in the range of about 80–110 mg/g and water contents less than 87% (Table 1). However, *A. pectoralis* and *A. tenobrosus* both had much lower muscle protein contents, 44–57 mg/g, and water contents usually more than 90%. TMAO is thought to be mostly intracellular in deep-sea fish because plasma TMAO values are low in most species that have been analyzed (including *A. pectoralis*; Gillett et al. 1997). Moreover, studies on watery muscles of deep-sea fish such as *A. pectoralis* show that they have greater extracellular volumes than other fish muscles (Crapo et al. 1999). Thus, on homogenization of muscle tissue, TMAO concentrations are diluted by the extracellular fluid (ECF), such that the contents reported (Table 1) are significantly less than actual intracellular concentrations. For very watery muscles with higher ECF volume, this dilution would be considerably greater. Consistent with this prediction, TMAO values from these species are lower than for the others, especially in the case of *A. pectoralis* (Fig. 1*B*). For *A. microlepis*, the situation is reversed: this species was found to have very high TMAO levels in plasma (159 mM) as well in whole tissue extracts (Gillett et al. 1997); thus, TMAO would be diluted very little during homogenization. Consistent with this, TMAO contents in extracts from this species were considerably higher than from the other species (Fig. 1*B*). However, intracellular concentrations should be much more similar to those of other species.

Sebastolobus alascanus, *Sebastolobus altivelis*, *A. pectoralis*, *A. tenobrosus*, *Coryphaenoides acrolepis*, and *Coryphaenoides cinereus* were caught at two or more different depths (symbols connected by solid lines in Figs. 1, 2). In most cases, these displayed an increase of TMAO contents with depth, significant in the case of *S. altivelis*, *C. acrolepis*, *C. cinereus* (Table 1; Fig. 1*A*), and *A. pectoralis* (Fig. 1*B*). In addition, *S. altivelis*, *C. cinereus*, *Coryphaenoides armatus*, and *A. microlepis* specimens had been analyzed from other depths in previous studies; those values are plotted as solid symbols in Figures 1 and 2. For three of these four species, there was again an increase in TMAO contents with depth, significant in the case of *C. armatus* (from three depths and two oceans, connected by dashed line in Fig. 1*A*) and *A. microlepis* (three depths, connected by dashed line in Fig. 1*B*). For *C. cinereus*, the older value from 1,800 m (*solid circle*, Fig. 1*A*) was somewhat lower than the new value from 1,406 m (*open circle*, Fig. 1*A*), but the difference was not significant.

The new data combined with the old data are replotted in Figure 2 for curve fits. A simple linear fit has a correlation r^2 of 0.91 (*dashed line and equation*, Fig. 2*A*), with the regression line fitting well with species at depths greater than 1,400 m. However, the line passes below points in the 600–1,400-m range and above points in the shallowest range. A sigmoidal curve (third-order polynomial fit) for the 0–1,400-m range fits the data better (solid curve for 0–1,400 m; $r^2 = 0.85$). At greater depths, there appears to be a linear increase (Fig. 2*A*; 1,400–4,850-m range; $r^2 = 0.90$).

Values for TMAO per dry mass were also calculated. This allows the outliers *A. pectoralis* and *A. tenebrosus* to be included with other species, since this derivation should correct for the problem of watery tissues (again, assuming that TMAO is primarily located intracellularly in all but *A. microlepis*). The results are shown in Figure 2*B* for the current specimens (dry masses were not available from previous studies). Again, a two-part relationship is apparent, with a possible sigmoidal (third-order polynomial) fit for 0–1,400 m. At greater depths, there are insufficient points to determine whether the fit is linear, but a possible linear relationship is shown.

TMAO levels were not related to season of capture. *Coryphaenoides acrolepis* (1,170 m) and *C. armatus* (4,100 m) were caught at the same depth during different seasons and had stable levels of TMAO (about 130 and 228 mmol/kg, respectively; $P > 0.2$, Mann-Whitney U test for each species; Table 1; 1,998 specimens). TMAO contents did not correlate with body mass across species ($r^2 = 0.011$, slope $= -0.007$; $P > 0.05$; see Table 2 for body mass ranges). Intraspecific comparisons of TMAO and body mass were also made and are discussed with lipids below.

Figure 1. *A*, Muscle TMAO contents as a function of depth in teleost fish. Open and half-filled symbols are from this study; solid lines connect same species at different depths. Solid symbols for species marked with a dagger are from previous work (Gillett et al. 1997; Kelly and Yancey 1999; Yancey et al. 2004); dashed lines connect same species from previous and current work. Asterisk indicates significant (ANOVA, $P < 0.05$) difference between depths (within species). Species abbreviations: *P vet* = *Parophrys vetulus*; *M prod* = *Merluccius productus*; *S dip* = *Sebastes diploproa*; *G zach* = *Glyptocephalus zachirus*; *M pac* = *Microstomus pacificus*; *A fim* = *Anoplopoma fimbria*; *S ala*, *S alt* = *Sebastolobus alascanus*, *altivelis*; *C acro*, *C cin*, *C arm*, *C yaq*, *C fil/lep* = *Coryphaenoides acrolepis*, *cinereus*, *armatus*, *yaquinae*, *fillifer/leptolepis*; *G mac* = *Gadus macrocephalus*. *B*, Muscle TMAO contents of outliers. See *A* for other details. Species abbreviations: *A pec* = *Albatrossia pectoralis*; *A ten* = *Alepocephalus tenebrosus*; *A mic* = *Antimora microlepis*.

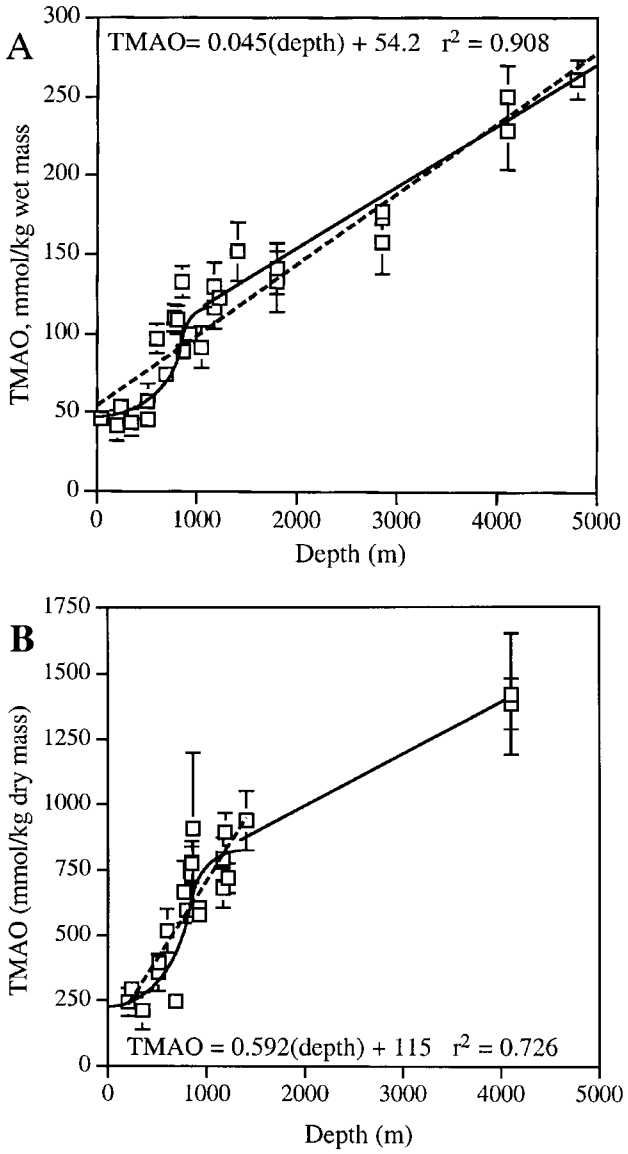


Figure 2. Curve fits for the TMAO data of Figure 1. *A*, Dashed line shows a linear fit for all the data of Figure 1*A* (equation at top). Solid curve shows a sigmoidal (third-order polynomial) fit for 0–1,400 m: TMAO = 42.8 − 0.03 × depth + 0.000199 × depth^2 − (9.16 × 10^{-8}) × depth^3 (r^2 = 0.85). Solid line for 1,400–4,850 m shows a linear fit: TMAO = 0.039 × depth + 71.2 (r^2 = 0.90). *B*, TMAO per dry mass derived from current specimens of Figure 1*A*, plotted with *Albatrossia pectoralis* and *Alepocephalus tenobrosus* included from Figure 1*B*. Values are not available from previous studies. Two separate linear fits are shown, as is a sigmoidal fit (third-order polynomial) for the 0–1,400-m range.

Other Solutes

TMA contents were found to be less than 2 mmol/kg wet mass in all cases, indicating that the samples had not deteriorated significantly during storage. TMA, though volatile, is well known to build up within tissues to high levels during spoilage; a fish fillet is typically considered by the food industry to be unspoiled if TMA is <2.3 mM (Huss 1988). Nevertheless, some slight deterioration could account for some of the variability in Figure 1.

Creatine and neutral amino acids were also analyzed, using high performance liquid chromatography. Due to limited sample size, only one or two individuals from each species were analyzed. Most amino acids were at very low levels, with no significant differences with depth. Taurine was the most concentrated amino acid, ranging from 0.1 to almost 16 mmol/kg, but had no correlation with depth. Most species were found to have substantial levels of creatine, as expected for white skeletal muscle, ranging from 12 to 29 mmol/kg for all but two species. Again, *A. pectoralis* was a clear outlier, having only 6–9 mmol/kg, and *A. tenebrosus* was the second lowest, with 11 mmol/kg. These values are consistent with these fish having more extracellular space than the other species.

Lipid Contents

Lipid contents of the fish are shown in Table 2. Diacylglycerol contents were negligible in all specimens, so only TAG was considered further. First, we tested for an interspecific depth trend in lipids. While mean TAG content as a percent of body mass (TAG%) did have an upward trend across species as a function of depth, the relationship was not significant ($P > 0.05$; $r^2 = 0.21$). We then tested TMAO as a function of TAG%, following the analysis of Seibel and Walsh (2002), who found a highly significant relationship between TMAO content and body lipid percentage across cephalopod species. For these teleosts, mean TMAO content as a function of TAG% showed a slight upward trend across species, but again, it was not significant ($P > 0.05$; $r^2 = 0.06$).

We also examined intraspecific data for TMAO in relation to TAG%. TMAO contents were consistent across a wide range of TAG% within each species (Fig. 3). We also examined TAG% and TMAO as a function of body mass, similar to the analysis of Seibel and Walsh (2002), who reported that both TMAO and lipid contents (as a percent of body mass) were highly correlated with body mass in gonatid squids. In teleosts, TAG% varied with body mass in different ways in different species, while TMAO generally did not. For *C. armatus* (Fig. 4*A*), *S. alascanus*, and *A. microlepis*, TAG% increased significantly with body mass while TMAO did not vary (see legend of Fig. 4*A*). In *Microstomus pacificus*, *Anoplopoma fimbria*, *A. tenebrous*, and *C. acrolepis*, neither TMAO nor TAG% varied significantly with body mass. Finally, for *A. pectoralis*, TAG% did not vary significantly with body mass, while mean TMAO contents increased with size but only because the larger specimens were deeper living (Fig. 4*B*).

Table 2: Teleost species with capture depths, body mass, and lipid compositions per wet mass and as a percent of body mass

Species	Depth (m)	Body Mass (g)	Liver Mass (% body)	Muscle TAG (mg/g)	Liver TAG (mg/g)	TAG (% body)
Parophrys vetulus	203	238–390	1.09 ± .17	.61 ± .72	1.31 ± 1.32	.03 ± .04
Merluccius productus	191–290	522–970	4.94 ± .88	.63 ± .87	350 ± 26.3	1.77 ± .40
Sebastes diploproa	347	96–278	2.58 ± .53	.32 ± .33	116 ± 11.9	.31 ± .10
Glyptocephalus zachirus	507	202–416	.504 ± .220	.12 ± .11	47.3 ± 31.2	.03 ± .01
Microstomus pacificus	511	338–1,210	.903 ± .278	.19 ± .26	19.8 ± 17.2	.04 ± .04
Sebastolobus alascanus	598	434–2,182	2.53 ± .99	2.39 ± 3.11	163 ± 55.0	.56 ± .44
S. alascanus	801	266–370	1.19 ± .49	.64 ± 1.0	153 ± 21.8	.21 ± .11
Anoplopoma fimbria[a]	687–691	1,162–1894	2.20 ± .52	86.6 ± 17.1	226 ± 57.9	4.82 ± .86
Sebastolobus altivelis	773	190–266	1.37 ± .79	.09 ± .04	89.2 ± 42.1	.15 ± .14
S. altivelis	853	60–222	1.47 ± 1.00	3.07 ± 3.41	134. ± 74.3	.35 ± .22
Albatrossia pectoralis	853	714–1,250	2.51 ± .25	.01 ± .003	454 ± 12.8	1.14 ± .08
A. pectoralis	924	894–1,236	4.18 ± 1.09	.02 ± .004	459 ± 34.6	1.93 ± .58
A. pectoralis	1,156–1,224	3,886, 4,456	2.47 ± .88	.01 ± .001	376 ± 36.2	1.00 ± .61
Alepocephalus tenobrosus	860	252–666	.490 ± .430	8.78 ± 8.61	32.5 ± 18.9	.38 ± .48
A. tenobrosus	1,046	304, 320	.627 ± .260	15.3 ± 21.1	77.3 ± 67.9	.82 ± .89
Coryphaenoides acrolepis	924	506, 1,096	4.89 ± .92	.03 ± .000	318 ± 122	1.48 ± .84
February 1998	1,170	612–2,321	4.56 ± 2.21	.02 ± .01	314 ± 71.6	1.54 ± 1.14
November 1998	1,170	832–1,260	3.92 ± 1.09	.01 ± .002	266 ± 60.2	.78 ± .51
C. acrolepis	1,224	718–1,220	3.92 ± 1.09	.03	384 ± 72.8	1.55 ± .51
Coryphaenoides cinereus	1,169	147–249	5.29 ± 2.83			
C. cinereus	1,406	227–484	2.61 ± 2.08	.08 ± .10	319 ± 59.6	.91 ± .89
Antimora microlepis	1,173–1,206	214–1,010	7.15 ± 2.13	.11 ± .11	358 ± 42.5	2.60 ± .86
Coryphaenoides armatus:						
April 1998	4,100	434–2,439	8.77 ± 5.35	.03 ± .02	400 ± 194	3.87 ± 3.13
December 1998	4,100	497–1,608	8.94 ± .71	.02 ± .006	421 ± 43.1	3.75 ± .37
Coryphaenoides yaquinae	4,100	231–481	2.66 ± 1.73	.02 ± .01	139 ± 139	.58 ± .70

Note. Whole-body triacylglycerol (TAG) as a percent of body mass was calculated assuming muscle comprises 50% of body mass (Bone 1978). TAG was the dominant acylglycerol found in these fish.

[a] *Anoplopoma fimbria* contained additional lipids in its bones (Lee et al. 1975); these were not quantified.

Discussion

TMAO versus Depth

The first objective of this study was to determine whether the previously reported increase in TMAO with depth in marine teleosts would be consistent across a greater variety of depths and species and also within species. Six families of teleosts were analyzed for TMAO in previous studies: Gadidae, Merlucciidae, Scorpaenidae, Macrouridae, Moridae, and Zoarcidae. Five of the same families were included in this study, plus three additional ones: Pleuronectidae, Alepocephalidae, and Anoplopomatidae (Table 1). The general depth trend is clearly confirmed by the new data, especially since TMAO contents are generally higher within the same species caught at different depths. This suggests that individuals are actively regulating TMAO contents in direct correlation with their depth.

Previous studies yielded a highly linear correlation between TMAO and depth (TMAO = 0.044 × depth + 44.0; r^2 = 0.99; Yancey et al. 2004). However, only four depths were sampled. This study fills in a gap between 40 and 1,800 m and adds a depth (4,100 m) between 2,850 and 4,850 m (previous work). A linear fit gives a nearly identical equation (shown on Fig. 2*A*). However, a sigmoidal curve appears to be a better fit from 0 to about 1,400 m (Fig. 2*A*). That is, there appears to be little change in TMAO with depth down to about 500 m and then a steep climb from 500 to 1,400 m, with a more gradual linear increase at greater depths (Fig. 2*A*).

Whether the steep increase in TMAO contents between about 500 and 1,400 m is also true of intracellular concentrations depends on how concentrated TMAO is in the ECF. As explained in "Results" for the outlier *Antimora microlepis* (Fig. 1*B*), species with high plasma TMAO will yield higher TMAO concentrations in the analyzed extracts than species with low plasma levels (even if all have identical intracellular concentrations). Plasma samples were not available for this study, and it is possible that some of the species in the steep part of the

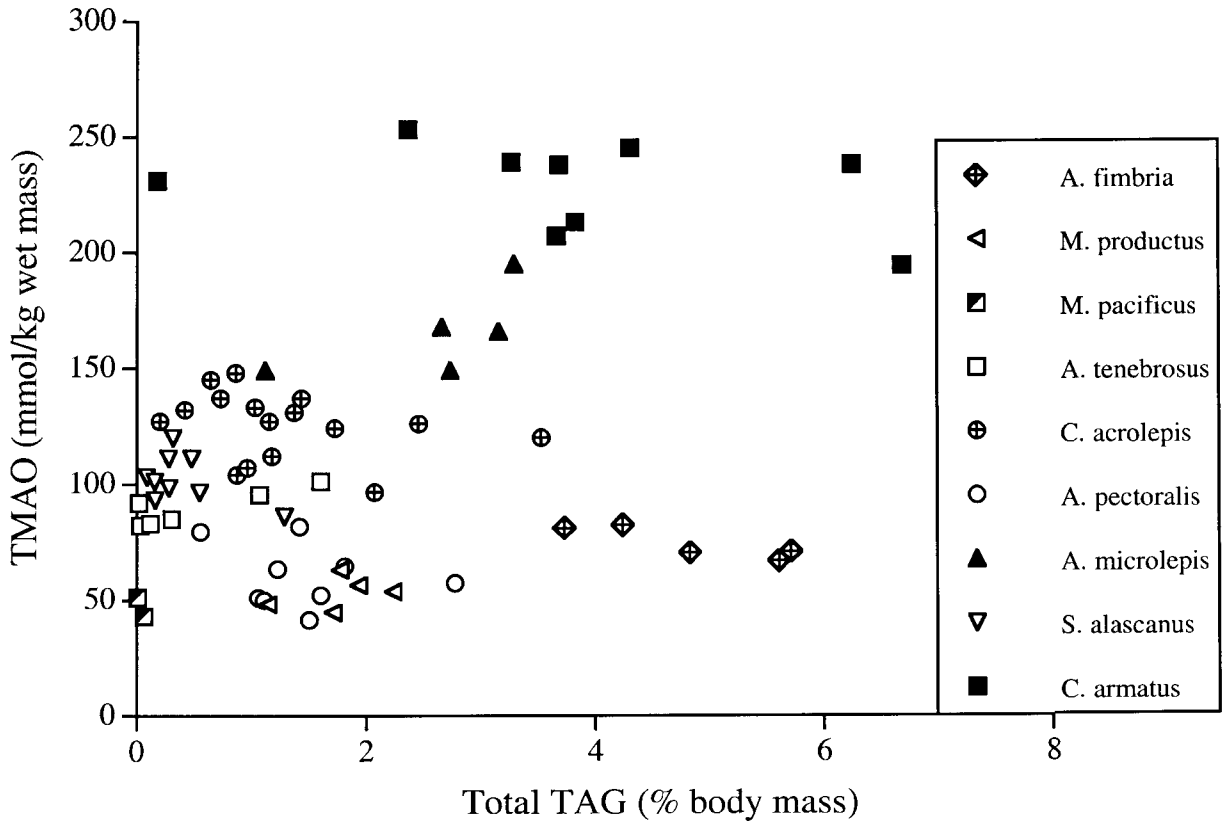


Figure 3. Intraspecies plot of muscle TMAO as a function of body triacylglycerol content, expressed as a percent of body mass. TAG was the dominant acylglycerol found in these fish. Data for each species are from one or two depths.

plot have high plasma TMAO levels. Thus, the sigmoidal pattern could be an artifact. However, all deep-sea species except *A. microlepis* had low plasma TMAO in a previous study, including *Coryphaenoides cinereus* from 1,800 m (solid circle in Fig. 1*A*; Gillett et al. 1997). (This finding also suggests there was no TMAO leakage from cells during capture.) If this pattern holds for most specimens used in this study, the sigmoidal pattern may be true of intracellular values as well as of tissue contents.

At depths greater than about 1,400 m, TMAO contents appear to increase linearly. No teleost has been reported below about 8,400 m (Nielsen 1977). If we extrapolate the linear fits (Fig. 2*A*) to 8,500 m, TMAO would be predicted to be about 400–430 mmol/kg. Recall that TMAO contents are considerably less than intracellular concentrations, such that 400 mmol/kg should be more than 600 mM inside cells. Indeed, extrapolation of a linear fit for *A. microlepis* (Fig. 1*B*), a species for which extracted TMAO contents are much closer to actual concentrations in vivo, gives a TMAO value of 699 mmol/kg at 8,500 m. Since universal cellular solutes (K^+ and other ions, proteins, etc.) typically add up to 300–400 mmol, TMAO levels at depths greater than 8,500 m might make these fish cells hyperosmotic to seawater. Thus, it is tempting to speculate that TMAO contents set the depth limit for teleosts.

Analysis of Causation: Adaptive Explanations for the TMAO/Depth Trend

The second objective was to test hypotheses for increase in TMAO with depth. What might explain this trend, with a possible sigmoidal pattern for the 0–1,400-m range? As noted in the introduction, TMAO accumulation might be a direct adaptation for one or more of the following functions.

Buoyancy. TMAO solutions have a density approximately equal to that of pure water (1.000 g/mL for a 1 M solution), whereas solutions of other organic osmolytes are significantly denser (Withers et al. 1994). Thus, TMAO accumulation might be beneficial in the energy-poor, high-pressure deep sea, where inflating a swim bladder is difficult. However, we discount this hypothesis for three reasons. First, TMAO should not help buoyancy of hypoosmotic osmoregulators since the solute would essentially be replacing water at the same density. However, perhaps the density of a TMAO solution becomes rela-

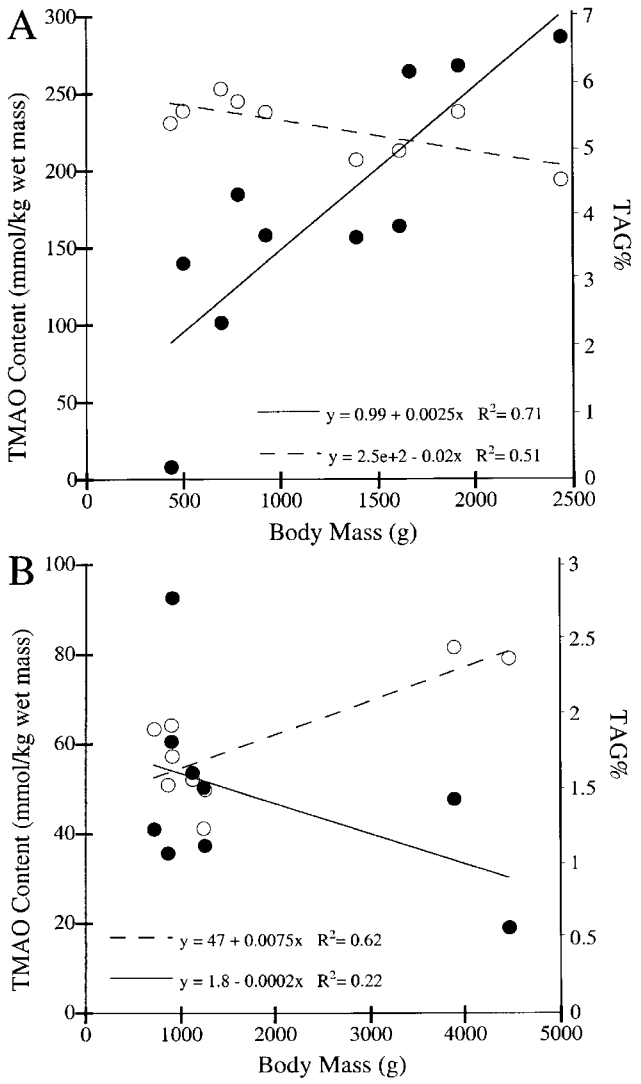


Figure 4. Muscle TMAO content (*open circles*) and TAG% (*solid circles*) as a function of body mass for selected species. *A*, *Coryphaenoides armatus*. TMAO per wet mass (plotted) did have a slight but significant decrease with body size ($P = 0.03$), but this was not significant for TMAO per dry mass ($P = 0.10$). *B*, *Albatrossia pectoralis*. The two upper right TMAO data points are from deeper specimens (1,156–1,224 m) than the other TMAO points (853–924 m).

tively less compared with pure water as pressure increases, which is an unstudied option (Kelly and Yancey 1999). Second, many of the benthopelagic species in this study have swim bladders, including the abyssal species. Finally, benthic fish (which rest on the seafloor with no need for buoyancy) such as scorpaenids (e.g., *Sebastolobus altivelis*) fit the depth trend.

Osmoregulatory Energy Constraints. Metabolism in many animal groups, including pelagic fish, is reduced in deep relative to shallow fish (Childress 1995; Seibel and Drazen 2007). Perhaps as a result, deep-sea teleosts may have become more like osmoconformers by accumulating TMAO in cells and NaCl in plasma (Gillett et al. 1997), thereby reducing the osmotic gradient between the external and internal fluids. Activities of gill sodium-potassium ATPases are reduced in deep-sea fish compared with shallow fish (Gibbs and Somero 1990), suggesting that deep species have lower osmoregulatory costs. This might simply be due to lower metabolic rates in these species but could reflect a lower plasma-seawater ion gradient.

Our data may be consistent with the hypothesis that TMAO accumulation is favored by energy constraints, but we discount this for several reasons. (1) It is not clear why osmoregulatory energy savings from TMAO accumulation, if real, would not be favored in shallow animals. (2) Calculations by Kirschner (1993) suggest that osmoregulating may not be more energetically costly than osmoconforming. Finally, (3) it has been recently reported that TMAO contents increase with depth in amphipods in Lake Baikal, a freshwater body in which animals would have no need of organic osmolytes (the authors propose the pattern to be a pressure adaptation; Zerbst-Boroffka et al. 2005).

Pressure Counteraction. As discussed earlier, there is good evidence that TMAO can counteract the inhibitory effects of high pressure on biological systems (demonstrated in vitro for actin polymerization, enzyme kinetics, protein stability, and growth of yeast; Gillett et al. 1997; Yancey and Siebenaller 1999; Yancey et al. 2001, 2002, 2004). Thus, it was hypothesized that TMAO accumulation is an adaptation to high pressure. More direct evidence that some osmolytes may be adaptive for pressure tolerance comes from work on deep-sea microbes, some of which have been found to accumulate β-hydroxybutyrate in correlation with laboratory exposure to hydrostatic pressure as well as to osmotic pressure. The investigators proposed the term "piezolyte" for this type of solute (Martin et al. 2002).

Our new data suggest that TMAO contents increase with depth with a sigmoidal pattern, at least at the low-to-intermediate depths. This might be consistent with the pressure counteraction hypothesis, because pressure, though it increases linearly with depth, often has sigmoidal effects on protein functions. For example, assembly of actin from *Coryphaenoides acrolepis* is inhibited by pressure in a sigmoidal pattern (Morita 2003). Studies by physical chemists using pressure as a probe of protein structure (i.e., research unrelated to the deep sea) frequently find sigmoidal effects of pressure on model proteins (e.g., Oliveira et al. 1994; Athès et al. 1998). Thus, if indeed TMAO helps to counter such effects, there might be no need for elevated levels in the shallowest range.

Deep-sea proteins have often been reported to be more resistant to pressure than are homologues from shallow-water species. Actin polymerization from *Coryphaenoides armatus*, for example, is considerably less sensitive to pressure than polymerization of actin from shallow fish (Swezey and Somero 1982;

Morita 2003). Nevertheless, *C. armatus* actin is still somewhat sensitive to pressure, and TMAO at the level found in these fish can completely abolish this pressure effect (Yancey et al. 2001). Thus, adaptations for proper protein function in the deep sea may involve two levels: an intrinsic one based on changes in amino acid structure and an extrinsic one involving the solute environment.

Analysis of Causation: Lipid Metabolism as an Explanation for the TMAO/Depth Trend

As discussed in the introduction, Seibel and Walsh (2002) hypothesize that high TMAO levels in the deep sea might be a result of enhanced lipid metabolism and thus be either nonadaptive or a secondary adaptation (e.g., TMAO is useful but only to the extent that it can be supplied by lipid metabolism as a by-product). As noted previously, evidence for a strong lipid-TMAO correlation has been found in cephalopods, in particular between lipid content (as a percent of body mass) and TMAO content. In addition, both lipid mass (percent) and TMAO increase with body mass in gonatid squids (Seibel and Walsh 2002). If lipid mass correlates with the rate of PtdCholine hydrolysis in these animals (as discussed below), then the cephalopod data support the hypothesis.

This study tested for similar correlations in teleost fish. Unlike in cephalopods, these teleosts revealed no simple, direct correlations between TMAO and acylglycerol contents (here dominated by TAG). The lack of any TAG-TMAO relationships within species (Fig. 3) and the consistency of TMAO levels despite different TAG% scaling patterns among species (Fig. 4) would seem to disprove the lipid hypothesis, at least for teleosts. However, that hypothesis predicts that TMAO will correlate with the rate of PtdCholine hydrolysis (yielding acylglycerols and choline), a variable for which we have no data in these fish. Thus, the hypothesis is not fully tested by our data.

Perhaps the differences between cephalopods and teleosts should not be surprising, given the different life histories and patterns of lipid accumulation in fish and cephalopods. In most cephalopods, lipid accumulation occurs over a 1–2-yr period during their growth and maturation before they enter their reproductive period and die (Rocha et al. 2001). Lipid accumulation (and thus body content of lipid) may be steady and may be a reliable marker of PtdCholine hydrolysis rates (at least before the reproductive period). However, in fish, lipid contents probably do not reflect such rates. In fish, growth and development are accompanied by increasing total lipid content, but after maturity is reached, most fish are iteroparous. Many fish, and certainly the species in this study, have much longer life spans than the cephalopods (Cailliet et al. 2001). This leads to well-recognized cycles in lipid stores related to reproductive events and seasonal variation in food supplies (Jangaard et al. 1967; Dygert 1990; Smith et al. 1990; MacFarlane et al. 1993). PtdCholine metabolism may very well be the mechanism by which TMAO is synthesized in these animals, and there may be some correlation between these two variables. It is possible that future work will reveal a correlation between actual PtdCholine metabolism and TMAO contents.

Yet, even if PtdCholine hydrolysis rates prove to relate to TMAO production in teleosts, other mechanisms (e.g., active retention vs. excretion) would appear to be necessary to regulate TMAO concentration in these animals, as illustrated by the depth changes within species (Fig. 1) and the consistency within species across wide ranges of body mass and TAG% (Figs. 3, 4). TMAO balance in these fish may be similar to osmolyte balance in elasmobranchs. In the latter, urea results from protein degradation, while TMAO arises from either the diet or lipid metabolism. However, the animals have high and stable levels of both urea and TMAO (Yancey et al. 1982) due in part to retention mechanisms (e.g., both solutes are reabsorbed by elasmobranch kidneys; Goldstein et al. 1967). TMAO and urea levels are maintained even during starvation (Wood et al. 2005), and TMAO levels are high in deep-sea sharks lacking the enzyme for TMAO synthesis (Treberg and Driedzic 2002). Thus, these osmolytes are actively regulated, and their levels do not appear to be driven by protein or lipid metabolism. For deep-sea teleosts, the relative rate of PtdCholine hydrolysis should correlate with the total pool of choline available for TMAO synthesis, and without constant replenishment, TMAO levels would decline. However, regulatory mechanisms such as those for TMAO retention and excretion may adjust TMAO levels independently of that choline pool.

Conclusion

Clearly, these types of simple correlations will not fully test the different hypotheses for TMAO-depth trends. However, the data here strongly support the hypothesis that TMAO is actively regulated with depth in deep-sea teleosts; in contrast, no clear support for a link between TMAO and lipid was found. Moreover, the hypotheses are not mutually exclusive, and more than one may be true; for example, phosphatidylcholine hydrolysis may indeed be the mechanism by which methylamines are produced for adaptive use (e.g., pressure counteraction) in deep-sea organisms. Experimental approaches using animal models in pressure chambers, along with direct measurements of PtdCholine metabolism, may be required to fully test the hypotheses.

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