1 Trimethylamine oxide accumulation as a function of depth in Hawaiian
2 mid-water fishes

3 Abigail B. Bockus^{a,*} and Brad A. Seibel^a

4 ^a*Biological Sciences Department, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881, USA*

5 ^{*}corresponding author, email: abockus@my.uri.edu

Abstract

Trimethylamine oxide (TMAO) is a common osmolyte and counteracting solute. It is believed to combat the denaturation induced by hydrostatic pressure as some deep-sea animals contain higher TMAO levels than their shallow water counterparts. It has also been proposed that TMAO may accumulate passively during lipid storage resulting in a correlation between lipid content and TMAO levels in some groups. Previous research showed that lipid content decreased with depth in species of Hawaiian fishes presenting a novel test of these competing hypotheses. TMAO ranged from 20.4 to 92.8 mmol/kg. Lipid content ranged from 0.50 to 4.7 % WW. After completing a comprehensive search for depths available in the literature, provided here, we analyzed TMAO and lipid as a function of average, minimum and maximum depth of occurrence for 27 species of fishes from nine orders. We found that TMAO is positively correlated with all measures of habitat depth (hydrostatic pressure) but the relationship is strongest with average depth. We further showed using phylogenetic independent contrasts that this relationship was not influenced by the evolutionary relatedness of these species. Interestingly, we found that lipid content increased with depth, in direct contrast to previous studies. TMAO is thus also positively correlated with lipid content. While we are unable to distinguish between these hypotheses, we show that TMAO is strongly correlated with depth in mid-water fishes.

Key Words: trimethylamine oxide (TMAO); lipid; chemical composition; hydrostatic pressure; depth; Hawaiian fish

1.1 Introduction

Trimethylamine oxide (TMAO) is an important cellular component in a wide range of taxa, from bacteria to humans (Chen et al., 2011; Treacy et al., 1995). It was first described in marine organisms (Bickel, 1969 in ref. Suwa, 1909; Norris and Benoit, 1945) as a prominent

osmolyte (Cholette and Gagnon, 1973; Forster and Goldstein, 1976). Later, it was shown to be a strong counteracting solute, (Yancey and Somero, 1979) protecting protein structure (Yancey and Siebenaller, 1999; Qu and Bolen, 2003) and function (Baskakov et al., 1998) from various environmental perturbants including, hydrostatic pressure (Gillett et al., 1997), urea and ammonia toxicity (Yancey and Somero, 1980; Minana et al., 1996), and temperature stress (Treberg et al., 2005; Villalobos and Renfro, 2007).

TMAO increases with habitat depth inter- and intraspecifically in benthic fishes and skates as well as some invertebrate groups (Kelly and Yancey, 1999; Yancey et al., 2001; Yancey et al., 2002; Laxson et al., 2011; Samerotte et al., 2007), suggesting that this molecule is used to combat the increasing stress of hydrostatic pressure. Most recently, Yancey et al. (2014) showed a hadal snailfish at 7,000 m with a TMAO content of 386 mmol/kg, almost eight times higher than the average fish in the euphotic zone. These observed correlations with depth have been further supported by evidence that TMAO prevented hydrostatic pressure denaturation in vitro (Yancey and Siebenaller, 1999).

However, not all taxa show an increase in TMAO with depth (Seibel and Walsh, 2002). Some shallow-living squids have TMAO levels that approach that reported for the hadal snailfish. These authors suggest a novel mechanism of TMAO synthesis leading to accumulation as a byproduct of lipid metabolism and storage and that TMAO is not necessarily retained as a specific adaptation to high hydrostatic pressure. This hypothesis was supported by a strong correlation between total lipid content and TMAO in cephalopods as well as anecdotal evidence in a variety of other groups. For example, lipid content is often higher in deep-living and polar species, which may explain the tendency of species in those habitats to accumulate large quantities of TMAO. However, a subsequent study did not find a relationship between mean

TMAO and triacylglycerol content in fishes (Samerotte et al., 2007), perhaps due to the differing time courses of accumulation and retention that resulted in differing size-scaling relationships of these two compounds.

Furthermore, an evolutionary relationship has been suggested for TMAO synthetic capacity between elasmobranchs and chimaeras (Treberg et al., 2006), which may impose inherited limitations on accumulation potential. If phylogeny also plays a role in TMAO accumulation in teleosts, it is possible that depth-related differences are driven by evolutionary history rather than environmental selection or substrate availability. Alternatively, a relationship to phylogeny may coexist and mask environmental trends making analyses between distantly related taxa difficult.

In (1990), Childress et al. examined a population of Hawaiian mid-water fishes that exhibited decreasing lipid content with increasing habitat depth (and hydrostatic pressure). Here, we examine TMAO and lipid content in 27 species of Hawaiian fishes from the same region studied in Childress et al. (1990) to test the competing hypotheses of hydrostatic pressure and lipid content on TMAO accumulation. A stronger relationship to hydrostatic pressure should elicit an increase in TMAO with habitat depth while a decrease with depth may be seen if TMAO is primarily accumulated as a by-product of lipid metabolism. Alternatively, an increase in lipid and TMAO with depth could represent a situation in which fishes accumulate TMAO passively during lipid storage with deeper fishes retaining the molecule for further pressure counteraction.

1.2 Materials and Methods

1.2.1 Collection and Sampling

Fishes were collected aboard the *R/V Kilo Moana* (University of Hawaii) in June 2012 off the west coast of Oahu in the Hawaiian Islands. Specimens were captured using a modified opening-closing Mother Tucker trawl with 3 m² mouth (Childress et al., 1978) between depths of 50-2000 meters. Animals were recovered in a 30-l thermally insulated cod end and immediately processed for later analysis. Individuals from 17 different species were gently blotted dry then flash frozen whole for determination of total lipid content. Additionally, muscle tissue was excised from similar specimens of the same and additional species, for a total of 27 species, and flash frozen for subsequent analysis of TMAO. All samples were collected in accordance with IACUC #AN12-07-026 and stored at -80°C until experimentation was conducted. Representatives of each species were preserved in 5% formalin or photographed for later identification using taxonomic and identification references available in the literature.

1.2.2 Analytical Techniques

Total lipid content for whole body was measured using a similar method to the 2:1 chloroform to methanol extraction described by Bligh and Dyer (1959) paired down for small sample mass (Lee et al. 1996). Muscle tissue samples were deproteinated and homogenized in 5x volume 5% trichloroacetic acid (TCA) followed by spectrophotometric determination of TMAO using the ferrous sulphate-EDTA assay (Wekell and Barnett, 1991). All values represent averages taken from replicate individuals from n = 1 to 12.

1.2.3 Depth analysis

Habitat range was determined according to currently published literature values describing the depth distribution of each species. Average depth is reported as the median of the habitat range, especially in highly migratory species or as average depths specifically reported in the literature. The average depth of a species can be considered as the depth at which the fish

spends most of its time (in non-migratory animals) or as a depth that represents the average level of depth stress (e.g. hydrostatic pressure) encountered by the species (migratory species). Minimum depth of occurrence (MDO) is defined as the depth below which 90% of the population of each species can be found (Childress and Nygaard, 1973). Here, MDOs were taken directly from the literature. Where no MDO was available, the shallowest reported depth for the species was used, substituting 10 m for those reported at the surface. TMAO, lipid and size were further analyzed against capture depth with no correlations found (data not shown).

Due to the limited amount of data available for these fishes, references were taken from studies conducted circumglobally (Supplementary Table 1). For some species, reported depths vary widely between publications; in such cases, the depths chosen for use in this study were based on the most recent and regionally specific data available. Occasionally a species vertical distribution changes with size, where smaller fish are frequently found at shallower depths (Collins et al., 2008). In these instances, reported depths are specific to the size of fish analyzed in this study; therefore, authors should be cautious when reporting these listed depths elsewhere.

1.2.4 Phylogenetic Comparison and Statistical Analysis

TMAO data were subjected to independent contrasts phylogenetic analysis (PIC) to determine if the phenotypic trends seen in this study could be explained by evolutionary relationships among fish species (Felsenstein, 1985; Seibel and Carlini, 2001). The phylogenetic tree used for this analysis was a compilation of trees previously published in the literature (Stiassny et al., 1996; Harold, 1998; Miya and Nishida, 1998; DeVaney, 2008; Davis, 2010; Kenaley, 2010; Betancur-R, 2013; Denton, 2014). The tree was further rooted in the outgroup Chondrichthyes; however, this group is not included in the analysis as elasmobranch values deviate significantly from all teleost values. All data concerning TMAO, lipid, depth of

occurrence and weight were further analyzed using regression analysis to assess whether any statistically significant relationships occurred. Statistics and graphs were generated using GraphPad Prism 6.0 and the phylogenetic tree used for PIC was made with statistical package R. Estimated TMAO and depth values were calculated for all ancestral nodes assuming equal branch lengths (punctuated model) and included in Supplementary Fig. 1. Further, contrast values were calculated for each node, which indicate both TMAO and depth after points have been made independent by accounting for any phylogenetic signal.

1.3 Results

1.3.1 Fish collection

We collected 27 species of mid-water fishes from 15 trawls ranging in depth from 50 to 2000 m. The species represent 12 families from 9 orders. The habitat depths of each species (average, minimum and maximum) are listed in Table 1.

Family	Species	TMAO (mmol/kg)	Lipid (% wet wt.)	Average depth (m)	MDO (m)	Maximum depth (m)	Vertical migrator
Anoplogastridae	<i>Anoplogaster cornuta</i>	73.9 (1)	3.20*	725	550	900	no
Eurypharyngidae	<i>Eurypharynx pelecانoides</i>	80.7 (1)	--	975	650	1300	no
Giganturidae	<i>Gigantura indica</i>	69.1 (1)	--	875	750 (750)	1000	no
Gonostomatidae	<i>Cyclothone pallida</i>	53.0±11.4 (7)	1.21±0.44 (2)	600	600 (600)	1000	no
	<i>Gonostoma atlanticum</i>	74.5±23.4 (8)	4.70**	520	481 (150)	560	yes
	<i>Gonostoma elongatum</i>	58.6±8.3 (8)	1.18±0.30 (10)	643	560 (200)	725	no
Melamphaidae	<i>Poromitra macrophthalma</i>	92.1±21.3 (6)	3.50*	820	640	1000	no
Myctophidae	<i>Ceratoscopelus warmingi</i>	56.2±14.6 (5)	1.76±0.30 (2)	700	600 (50)	900	yes
	<i>Diaphus perspicillatus</i>	79.9±19.2 (8)	3.52±1.95 (8)	700	500	900	yes ^a
	<i>Hygophum proximum</i>	54.5±26.5 (2)	2.11 (1)	500	10	1000	yes
	<i>Lampanyctus niger "H"</i>	52.1±5.0 (4)	0.98±0.21 (4)	300	100 (sp b, 165)	500	no ^b
	<i>Lampanyctus tenuiformis</i>	92.8±14.5 (4)	1.76±0.60 (4)	800	700 (250)	900	yes ^c
	<i>Taaningichthys bathyphilus</i>	65.2±21.4 (10)	1.57±0.35 (4)	852	582 (600)	1122	no
Oneirodidae	<i>Danaphryne nigrifilis</i>	72.1±4.6 (2)	--	1082	1082	1082	no
Opisthoproctidae	<i>Opisthoproctus soleatus</i>	72.4±12.6 (3)	1.85±0.94 (2)	600	500 (450)	700	no
Paralepididae	<i>Magnisudis atlantica</i>	45.4 (1)	--	468	445	490	--
Serrivomeridae	<i>Serrivomer sector</i>	67.3±40.3 (8)	0.79±0.49 (5)	700	700	1800	no
Sternoptychidae	<i>Argyropelecus affinis</i>	49.2±15.1 (6)	0.79±0.16 (2)	350	200 (225)	500	no

Stomiidae	<i>Danaphos oculatus</i>	61.1±22.3 (12)	2.60 [*]	540	430 (430)	650	no
	<i>Sternoptyx diaphana</i>	45.3±4.2 (7)	2.26±1.22 (3)	660	422 (450)	899	no
	<i>Aristostomias grimaldi</i>	58.3±15.7 (2)	1.02 (1)	425	100	750	yes
	<i>Chauliodus sloani</i>	45.1±10.1 (2)	1.40 ^{**}	300	100 (175)	500	yes
	<i>Flagellostomias boureei</i>	39.5±11.6 (3)	0.89±0.31(4)	450	10	900	--
	<i>Idiacanthus antrostomus</i>	44.4±6.0 (4)	0.66±0.19 (3)	225	150	300	yes
	<i>Photostomias liemi</i>	59.1±5.6 (3)	1.63±0.74 (3)	386	10	762	yes ^d
	<i>Photostomias lucingens</i>	40.4 (1)	0.71±0.21 (2)	63	10	115	yes ^d
	<i>Thysanactis dentex</i>	20.4 (1)	0.50 ^{**}	280	10 (75)	550	yes

132 **Table 1. Composition and habitat parameters of Hawaiian mid-water fishes.** Depth and migration data derived from the literature

133 (Supplementary Table 1). Minimum depth of occurrence (MDO) listed as updated values used in this study with Childress et al. (1990) values
134 listed in parentheses where available. TMAO and lipid values reported as averages ± standard deviation with number of individuals analyzed in
135 parentheses.

136 * data taken from Neighbors, 1988

137 ** data taken from Childress et al., 1990

138 a Rao, 2010

139 b Clarke, 1978

140 c Hulley, 1990

141 d Inferred for genera by Kenaley, 2008

1.3.2 TMAO vs. depth

Average TMAO content ranged between 20-93 mmol/kg wet mass (Table 1), which is consistent with values reported for fishes elsewhere (Carr et al., 1996). TMAO content increased linearly with all measures of habitat depth. The relationship was strongest with a species' average depth ($r^2 = 0.5309$, $p < 0.0001$; Fig. 1a) but was also significant as a function of MDO ($r^2 = 0.5074$, $p < 0.0001$; Fig. 1b) and maximum depth ($r^2 = 0.2520$, $p = 0.0076$; Fig. 1c). Separating fishes into non-migrating and vertically migrating species did not strengthen the trend with depth and variance between these groups was not significantly different (data not shown). Additionally, a phylogenetically independent analysis of the data (Phylogenetic Independent Contrasts) also resulted in a significant positive relationship between TMAO and habitat depth ($r^2 = 0.4036$, $p = 0.0009$; Fig. 2), which suggests the trend is independent of any phylogenetic relationships across these 27 species.

1.3.3 Lipid vs. depth and TMAO

Lipid content ranged between 0.5 – 4.7% wet weight in these fishes. Lipid values showed a significant increase with increasing average depth ($r^2 = 0.2888$, $p = 0.0261$) in the 17 species analyzed for lipid in this study. Additional lipid values taken from the literature ($n = 6$) strengthened this relationship ($r^2 = 0.2496$, $p = 0.0152$; Table 1, Fig. 3). Lipid values from the literature were only included for species in this study where lipid was not measured directly. Lipid also significantly increased with MDO but not maximum depth (data not shown). When divided into non-migrating and vertically migrating species, groups did not exhibit significant differences in variance (data not shown). Further, lipid was positively correlated with size in the family Myctophidae ($r^2 = 0.4145$, $p = 0.0009$) and negatively correlated with size in the species, *Sternoptyx diaphana* ($r^2 = 0.8834$, $p = 0.0175$; Fig. 4). However, size was not related to any

measure of habitat depth for these species (data not shown). TMAO increased linearly with increasing lipid content ($r^2 = 0.2744$, $p = 0.0309$) across the 17 fish species analyzed. Adding lipid values from the literature ($n = 6$) strengthened this relationship ($r^2 = 0.4328$, $p = 0.0006$; Fig. 5).

1.4 Discussion

1.4.1 TMAO vs. depth

TMAO increases with a species' habitat depth in a number of different clades including, anemones (Yancey et al., 2004), crustaceans (Zerbst-Boroffka et al., 2005), Chondrichthyes (Laxson et al., 2011) and teleosts (Kelly and Yancey, 1999; Yancey et al., 2002). Yancey and colleagues hypothesize that these groups have converged on a similar mechanism of using TMAO to counteract the perturbing effects of hydrostatic pressure on protein function. TMAO is able to protect protein function against pressure better than other osmolytes such as betaine, glycine, taurine and myo-inositol. These compounds do show some stabilizing potential against hydrostatic pressure (Yancey et al., 2004) but higher concentrations are required to counteract comparable pressures. Additionally, TMAO acts as a universal cytoprotectant and is able to stabilize different types of proteins (Yancey and Somero, 1979) as well as protein homologs from distantly related species (Yancey and Siebenaller, 1999) against denaturation.

Samerotte et al. (2007) found a sigmoidal pattern in the relationship between TMAO and habitat depth in benthic teleost fishes between 0-1400 m and a linear relationship at greater depths to at least 7,000 m (Yancey et al., 2014). The TMAO values we report fall near those found in the fishes previously examined but increase linearly with depth to 1,200 m. This supports the hypothesis that TMAO is being used to counteract hydrostatic pressure but that the relative accumulation needed for stabilization may be different between groups, ecotypes or

locations. Alternatively, extracellular to intracellular volume ratios may be different between the mid-water fishes studied here and the demersal fishes examined previously, which would imply similar intracellular TMAO contents between these groups.

Fig. 1a-c. TMAO as a function of depth. TMAO increased significantly with increasing habitat depth. Each data point ($n = 27$) represents the average calculated for an individual species. Depth values were calculated from the literature (Table 1 and Supplementary Table 1). **A** Average depth is defined as the depth at which the species can most commonly be found or the median depth for highly migratory species. Linear regression $y = 0.05022x + 31.20$ ($r^2 = 0.5309$, $p < 0.0001$). Values are plotted against the analysis performed by Samerotte et al. (2007), which found a sigmoidal relationship between TMAO and capture depth in the upper 1,400 m for fishes in the eastern Pacific. **B** The MDOs were taken from previously reported literature values. Where a MDO has not been reported, the shallowest reliable observation was used. Linear regression $y = 0.04275x + 42.35$ ($r^2 = 0.5074$, $p < 0.0001$). **C** Linear regression $y = 0.02539x + 39.69$ ($r^2 = 0.2520$, $p < 0.0076$).

TMAO showed increases when examined against average, minimum and maximum habitat depth of these fishes (Fig. 1a-1c), with the strongest relationship to average depth. MDO is commonly used to relate metabolic rate to depth as metabolic rates in strongly visually-orienting taxa seem to be largely dependent on light and visual predator-prey interactions that are most important at the upper depth limit of the organism (Childress, 1995; Drazen and Seibel,

207 2007; Seibel and Drazen, 2007). Conversely, one might expect TMAO to correlate most
208 strongly with the maximum pressure experienced by a species if accumulation is being driven by
209 pressure counteraction. However, it is interesting to note that TMAO accumulation is most
210 tightly coupled to average depth, where fishes may spend the majority of their time. TMAO
211 fluctuations may be inhibited by time-course restrictions, especially in diel vertical migrators,
212 which could impose limitations on their ability to match TMAO to minimum and maximum
213 depths and explain the strong relationship to average depth. Therefore, it is possible that these
214 fishes are experiencing modest conformational changes to protein structure during their time
215 spent at maximum depth. This has been shown to occur during dormancy (Muir et al., 2008) and
216 other circumstances of urea destabilization (Yancey and Somero, 1979). These changes could be
217 used to facilitate metabolic suppression and energy conservation during the time spent at daytime
218 depths among vertically migrating species. However, metabolic suppression has only been
219 demonstrated for vertical migrators living in pronounced oxygen minimum zones (Seibel, 2011;
220 Seibel et al., 2014) so further evidence is needed to support this supposition.

222 1.4.2 Phylogenetic Comparison

223 The only study to examine the evolutionary history of TMAO synthetic capacity, as
224 described by the activity of trimethylamine oxidase (TMAoxi), found it to be a derived
225 characteristic in elasmobranchs and chimaeras (Treberg et al., 2006). Species lacking measurable
226 TMAoxi activity must rely on dietary contributions to accumulate TMAO (Treberg and Driedzic,
227 2002), potentially placing ecological restrictions on their ability to use TMAO as a counteracting
228 solute. If teleosts were to exhibit a similar phylogenetic pattern, it would suggest differing
229 capacities for TMAO regulation between clades and could influence inherent TMAO
230 concentrations as well as certain species ability to accumulate TMAO. We found no relationship

between total TMAO content and evolutionary relatedness (Supplementary Fig. 1). Instead, when the interrelatedness between data points imposed by evolutionary history was accounted for (contrast values), there was still a significant increase in TMAO with depth (Fig. 2). Therefore, in these Hawaiian fishes, trends seem to be driven primarily by environmental and ecological variability and not by an innate phylogenetic signal.

Fig. 2. Standardized contrasts of TMAO against standardized contrasts of average depth. Contrast: TMAO increases significantly with increasing Contrast: depth. Contrast values establish phylogenetic independence; calculated using Phylogenetic Independent Contrasts from 26-taxon tree (Supplementary Fig. 1; punctuated model assuming equal branch length). Linear regression $y = 0.06965x + 5.225$ ($r^2 = 0.4036$, $p = 0.0009$).

1.4.3 Lipid vs. depth

High energy materials, such as protein and lipid, decrease with depth in Southern California fishes and are replaced by less expensive materials such as water which lowers organisms' metabolic demands and allows deep-sea species to reach larger sizes with minimal

cost (Childress and Nygaard, 1973). A similar trend was shown for Hawaiian fishes (Childress et al., 1990) where decreasing lipid levels with depth were attributed to lower metabolic rates. However, many species increase lipid levels with depth as has been shown in copepods (Lawrence, 1976), crustaceans (Childress and Nygaard, 1974), zooplankton, fish (Reinhardt and Van Vleet, 1986) and cephalopods (Seibel and Walsh, 2002). We showed increasing lipid with average depth for Hawaiian fishes (Fig. 3). These results are opposite those reported by Childress et al. (1990).

The methods employed for lipid analysis by Childress and colleagues are best for samples of large mass with the size of these fishes averaging less than five grams wet weight. We found the modified protocol for small sample mass to yield more reliable results, perhaps explaining the discrepancy. The three values included in this study do not reflect the overall trend found by Childress of decreasing lipid with depth, most likely due to the large variability found in that study (0.2 to 10% wet weight) and the small number of data points included here. We also found evidence of changing lipid content with size in some species, which may complicate interpretations based on habitat alone (Fig. 4). It is not likely that seasonal variability plays a large role in lipid storage for the warm water fishes studied here (Childress et al., 1990). Alternatively, it is possible that the deeper living species accumulate lipid to sustain them between the intermittent meals experienced in the deep-sea environment or to fuel extensive egg-brooding periods as in the squid, *Gonatus onyx* (Seibel et al., 2000), and the lophogastrid crustacean, *Gnathophausia ingens* (Childress and Price, 1983). The increase in lipid with depth may also be due to replacement of the gas-filled swim bladder with fatty tissue for buoyancy shown to occur in other myctophid species (Butler and Percy, 1972). In such cases, swim bladders are typically filled with wax esters which are derived from metabolic pathways

independent of the diacylglycerol ethers and triacylglycerols whose formation leads to accumulation of TMAO precursors (e.g. choline; Seibel and Walsh, 2002). All these factors can impart selective pressure on lipid content and it is possible the additional taxa included in the Childress study were experiencing different combinations or levels of selection resulting in the opposite trend with depth.

Fig. 3. Total body lipid as it relates to average habitat depth. Lipid increased significantly with increasing depth. Each data point ($n = 23$) is the measured average for an individual species. Closed circles are measured lipid values, triangles are lipid values from Neighbors (1988), x's are values from Childress et al. (1990). Linear regression without literature values $y = 0.001839x + 0.4836$ ($r^2 = 0.2888$, $p = 0.0261$). Linear regression with literature values $y = 0.002599x + 0.3926$ ($r^2 = 0.2496$, $p = 0.0152$).

Fig. 4. Total lipid as it relates to body size. Lipid showed a significant increase with increasing size in all myctophid species (triangles) and a significant decrease with increasing body size in the species *Sternoptyx diaphana* (open circles). Each data point represents a measurement for a single individual (n = 23 for myctophids, n = 5 for *S. diaphana*). Myctophid linear regression $y = 0.9395x - 0.03917$ ($r^2 = 0.4145$, $p = 0.0009$). *S. diaphana* linear regression $y = -0.7241x + 3.559$ ($r^2 = 0.8834$, $p = 0.0175$).

1.4.4 TMAO vs. lipid

Although TMAO may be used to combat hydrostatic pressure in many organisms, there are species that do not accumulate TMAO with depth: such as some echinoderms, mollusks, polychaetes, and vestimentiferans (Yancey, 2005). These animals seem to accumulate a plethora of alternative osmolytes with potential stabilizing properties including a serine-phosphate compound, other methylamines and polyols (Yancey et al., 2002). Therefore, a number of mechanisms exist whereby fishes may be combatting hydrostatic pressure aside from TMAO accumulation. In fact, TMAO performs a number of roles including osmotic balance, buoyancy regulation, as well as urea and temperature counteraction, all of which may impart competing selection on TMAO content. However, the ability of TMAO to aid in buoyancy is limited in hypoosmoregulating fishes (Gillett et al., 1997) and plays a larger role in invertebrates and

elasmobranchs. Further, TMAO regulation may be influenced by diet or passive accumulation during lipid storage (Seibel and Walsh, 2002).

The latter hypothesis, passive TMAO accumulation during lipid storage, has received little attention. In 2002, a new synthetic pathway for TMAO was proposed whereby phosphatidylcholine, a compound readily available from the diet or the breakdown of cellular membranes, is converted to diacylglycerol or triacylglycerol (TAG) for lipid storage. During this process a choline moiety is cleaved from phosphatidylcholine, which can then be transformed to TMAO (Seibel and Walsh, 2002). These authors demonstrate a correlation between lipid and TMAO content in cephalopods and discuss the tendency of many organisms from deep and polar environments to accumulate high concentrations of both TMAO and storage lipid. Although no correlation between total TAG and TMAO was found for 15 species of fish caught in the eastern Pacific (Samerotte et al., 2007), the relationship between the two may be confounded by retention or excretion of TMAO and the active use of storage lipid for metabolic purposes. Cell membrane restructuring during growth or osmotic challenges, for example, may also lead to TMAO precursor availability without the accumulation of storage lipid (Seibel and Walsh, 2002). Additionally, dietary TMAO may negate the need for endogenous production. However, little information is available regarding turnover or TMAO content in the diet of these fishes making conclusions speculative. The fishes in this study show increasing levels of TMAO with total lipid content (Fig. 5), supporting evidence for the possible existence of a synthetic pathway whereby TMAO is accumulated during lipid storage.

Fig. 5. TMAO content as a function of total body lipid. TMAO increased significantly with increasing % lipid. Each data point ($n = 23$) represents the average for an individual species. Black circles are measured lipid values, triangles are lipid values from Neighbors (1988), x's are lipid values from Childress et al. (1990). Linear regression with no additional literature values $y = 10.06x + 43.53$ ($r^2 = 0.2744$, $p = 0.0309$). Linear regression with all values $y = 10.27x + 40.80$ ($r^2 = 0.4328$, $p = 0.0006$).

1.4.5 Conclusions

TMAO was positively correlated with depth in the 27 species of Hawaiian teleost fishes studied here. Additionally, this trend was independent of phylogenetic relatedness suggesting that environment, not evolution, is playing a larger role in driving the relationship. As depth and lipid were positively correlated, it was not possible to definitively rule out either the hydrostatic pressure or the lipid accumulation hypothesis although we provide supportive evidence for both. However, the two hypotheses are not mutually exclusive and it is possible the choline substrate produced during lipid accumulation may be converted to TMAO and actively retained to counteract hydrostatic pressure.

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342 **Competing Interests**

343 The authors declare no competing financial interests.

344 **Author contributions**

345 This project was conceived by B.A.S. and A.B.B. Data was collected and analyzed by A.B.B.

346 The final manuscript was prepared by A.B.B. and revised by B.A.S.

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