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A Study of the Regulatory and Environmental Factors Affecting Trimethylamine Oxide Accumulation in Marine Organisms

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A STUDY OF THE REGULATORY AND
ENVIRONMENTAL FACTORS AFFECTING
TRIMETHYLAMINE OXIDE ACCUMULATION IN
MARINE ORGANISMS

BY

ABIGAIL BRITTANY BOCKUS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
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UNIVERSITY OF RHODE ISLAND

2016

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

Trimethylamine oxide (TMAO) was first described in marine organisms as an osmolyte, involved in the balance of water and solutes. After its discovery, it was found to be part of a subset of osmotic constituents termed counteracting solutes. These solutes exhibit stabilizing properties and can preserve protein functionality against biological and environmental perturbations. TMAO acts as a universal stabilizer, protecting macromolecular structure and function in response to numerous stressors, including urea destabilization, hydrostatic pressure, temperature and salinity. The studies presented in this dissertation address the regulatory and environmental factors affecting TMAO accumulation.

Both exogenous and endogenous sources are involved in the maintenance of TMAO. Exogenous TMAO accumulates through absorption from the diet while endogenous TMAO is synthesized from dietary or cellular precursors with the flavin-containing monooxygenase trimethylamine oxidase (TMAoxi). Species without a physiologically relevant synthetic capacity are hypothesized to rely entirely on dietary contributions for accumulation. Chapter 1 examines the necessity of an exogenous TMAO source on long-term maintenance in elasmobranch species with and without the ability for endogenous synthesis. These data show that presence or absence of TMAoxi cannot be used as a proxy to determine the importance of dietary TMAO on prolonged conservation. It seems that all species, regardless of synthesizing potential, rely to an extent on contributions from the diet.

Chapter 2 further examines the regulatory factors affecting TMAO. This study provides evidence for endogenous production via an understudied synthetic pathway

whereby TMAO is accumulated as a byproduct during lipid storage. The existence of this pathway is supported by a correlation between TMAO content and total lipid in a variety of Hawaiian mid-water fishes. The regulatory role of evolutionary relatedness on accumulation potential is also addressed in this chapter. Phylogenetic independent contrasts (PIC) showed no relationship between phylogeny and TMAO content across 27 species spanning nine orders. This suggests that environmental factors impart a larger influence on TMAO retention than evolutionary history.

Chapter 2 goes on to examine TMAO's role in combatting the environmental stress associated with increasing hydrostatic pressure. TMAO was shown to increase with increasing depth of occurrence across all species of Hawaiian mid-water fishes studied. These data support previous reports of TMAO accumulation as an environmental adaptation to combat the destabilizing effects of elevated hydrostatic pressure.

Chapter 3 explores TMAO's ability to counteract environmental fluctuations in temperature. Previous *in vitro* studies showed intracellular transport and accumulation of TMAO with increasing temperature in elasmobranch red blood cells. Further, this was shown to suppress the traditional heat shock response of heat shock protein 70 (HSP70) upregulation. However, we saw no increase in plasma or tissue TMAO in response to elevated temperature for two shark species *in vivo*. Either mechanisms established *in vitro* are not applicable at the organismal level or additional regulatory factors are limiting TMAO accumulation.

Lastly, a brief study examining regulation of TMAO through ontogeny in an elasmobranch species, *Squalus acanthias*, is presented in the Appendix. Pups of this

species exhibit low levels of urea and TMAO, their two primary osmolytes. However, total osmotic pressure is maintained at adult levels. Therefore, a shift in the osmotic milieu occurs sometime between birth and adulthood. These findings are in contrast to those reported for the little skate, *Leucoraja erinacea*, which expresses adult levels of these osmotic constituents early in development. These data point to divergence in the early osmoregulatory strategies of differing elasmobranch groups.

In the enclosed chapters, key objectives regarding the regulatory and environmental factors influencing TMAO are addressed. Specifically, this research examines how contributing sources, evolutionary restrictions and environmental stress affect TMAO accumulation. These studies elucidate TMAO's multifaceted role in marine organisms and provide insight into the factors regulating its adaptive potential.

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DEDICATION

For T.V. – for wondering what my voice sounded like, then helping me find it

PREFACE

This dissertation is prepared in manuscript format. Chapter 1, entitled “Synthetic capacity does not predict elasmobranchs ability to maintain trimethylamine oxide levels without a dietary contribution,” is being prepared for submission to the journal of *Comparative Biochemistry and Physiology*. Chapter 2, entitled “Trimethylamine oxide accumulation as a function of depth in Hawaiian mid-water fishes,” was published in *Deep-Sea Research I* in 2016. Chapter 3, entitled “Trimethylamine oxide and HSP70 regulation during acute temperature stress in elasmobranchs,” is being prepared for submission to the *Journal of Experimental Biology*. The appendix includes a supplementary study in support of the chapters presented in this dissertation. It is written as a short communication for publication in *The Biological Bulletin* under the title “Ontogenetic osmotic shift in spiny dogfish, *Squalus acanthias*.”

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	v
DEDICATION	vii
PREFACE	viii
TABLE OF CONTENTS	ix
CHAPTER 1: Synthetic capacity does not predict elasmobranchs ability to maintain trimethylamine oxide without a dietary contribution	1
Abstract	2
Introduction	3
Materials and Methods	7
Results	11
Discussion	14
Acknowledgements	32
References	33
CHAPTER 2: Trimethylamine oxide accumulation as a function of depth in Hawaiian mid-water fishes	39
Abstract	40
Introduction	41
Materials and Methods	44
Results	47
Discussion	49
Acknowledgements	70
References	71
CHAPTER 3: Trimethylamine oxide and HSP70 regulation during acute temperature stress in elasmobranchs	82
Abstract	83
Introduction	84
Materials and Methods	87
Results	90
Discussion	93
Acknowledgements	106
References	107
APPENDIX: Ontogenetic osmotic shift in spiny dogfish, <i>Squalus acanthias</i>	113
Abstract	114
Introduction	115
Materials and Methods	117
Results and Discussion	119
Acknowledgements	125

References.....	126
BIBLIOGRAPHY	128

CHAPTER 1

SYNTHETIC CAPACITY DOES NOT PREDICT ELASMOBRANCHS ABILITY TO MAINTAIN TRIMETHYLAMINE OXIDE WITHOUT A DIETARY CONTRIBUTION

Prepared for submission to the journal of *Comparative Biochemistry and Physiology*

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Key Words: trimethylamine oxide (TMAO), urea, elasmobranch, feed, diet, synthesis

Abstract

Trimethylamine oxide (TMAO) is an organic osmolyte that also acts as a universal protein stabilizer. Its role as a cytoprotectant is particularly important in elasmobranchs that accumulate high levels of urea, a macromolecular destabilizer and their primary form of nitrogenous waste. Feeding is a key component in the turnover and maintenance of these nitrogenous compounds; however, previous studies examining the endogenous and exogenous sources involved in TMAO regulation have been completed using starved animals, when nitrogen balance is altered. Here, we test the ability of three elasmobranch species with differing TMAO production capacities to maintain levels independent of a dietary contribution for 56 days. Smoothhounds (*Mustelus canis*), spiny dogfish (*Squalus acanthias*), and little skates (*Leucoraja erinacea*) exhibited species-specific differences in their ability to conserve TMAO when fed a low TMAO diet. Additionally, these differences were not obviously dependent on a species TMAO synthetic capacity. Spiny dogfish, a species with no ability for synthesis, showed a decrease in plasma TMAO when fed a low TMAO diet. However, plasma TMAO was maintained in both the smoothhound and little skate. Further, smoothhounds, the only species examined with the ability to endogenously produce TMAO, showed a decrease in muscle TMAO when fed a low TMAO diet. It is possible that all species rely to an extent on absorption of TMAO from the diet or that alternate endogenous synthetic pathways exist that have not yet been identified.

1. Introduction

Trimethylamine oxide (TMAO) is a small compound accumulated as an intracellular osmolyte in a diversity of marine organisms (Norris and Benoit, 1945; Bickel, 1969; see Seibel and Walsh, 2002; Yancey, 2005 for reviews). It serves an additional role as a universal protein stabilizer (Yancey and Siebenaller, 1999; Yancey et al., 2001; Yancey et al., 2004), protecting structure and function against a multitude of environmental stressors. TMAO can counteract perturbations in protein function due to salinity (Pillans et al., 2005; Hammerschlag, 2006; Deck et al., 2016), temperature (Raymond and DeVries, 1998; Treberg et al., 2002), hydrostatic pressure (Yancey et al., 2002; Bockus and Seibel, 2016) and the nitrogenous waste compound, urea (Somero, 1986; Baskakov et al., 1998; Yancey, 2001; Zou et al., 2002). TMAO is retained at exceptionally high levels by elasmobranchs (sharks, skates and rays) that accumulate urea as their primary osmolyte (Smith, 1929; Forster and Goldstein, 1976; Withers, 1998; Trischitta et al., 2012). These species maintain a 2:1 ratio of urea to TMAO + other stabilizing osmolytes (Yancey and Somero, 1979; Treberg et al., 2006) to promote macromolecular stability (Barton et al., 1999).

There are two general mechanisms used by elasmobranchs to regulate TMAO. Some possess the flavin-containing monooxygenase, trimethylamine oxidase (TMAoxi), and have the ability to synthesize TMAO from endogenous or dietary precursors such as choline (Ágústsson and Strøm, 1981; Raymond, 1998; Schlenk, 1998; Seibel and Walsh, 2002). Species without this enzyme are thought to accumulate TMAO from the diet alone (Benoit and Norris, 1945; Treberg and Driedzie, 2002; Treberg et al., 2006), requiring prey items rich in TMAO to maintain

intracellular levels. However, there is little direct evidence for dietary absorption and it is possible that alternate synthetic pathways exist that have yet to be described.

Several previous studies have examined TMAO maintenance during extended starvation. One study showed that spiny dogfish, *Squalus acanthias*, were unable to synthesize TMAO from radiolabeled precursors and postulated that constant plasma levels were achieved during 20 days of starvation through active reabsorption at the kidneys and release from tissue pools (Goldstein et al., 1967). The winter skate, *Leucoraja ocellata*, was also found to maintain plasma TMAO over 45 days of starvation (Treberg and Driedzic, 2006). Maintenance in this case was attributed to decreased excretion with no increase in TMAO synthesis found. Treberg and Driedzic (2007) also examined muscle TMAO in the winter skate over 28 days of starvation and speculate that constant levels were due to release from tissue catabolism and subsequent recycling. Another study showed stable plasma TMAO and urea over 56 days of starvation in spiny dogfish. These authors also suggest that the large pools of TMAO stored in various tissues, primarily muscle, supply adequate amounts for plasma maintenance (Kajimura et al., 2008). Another possible explanation for prolonged maintenance was provided by Seibel and Walsh (2002), who suggested that TMAO could be synthesized in the absence of dietary contributions from choline, released during hydrolysis of membrane phospholipids, whether endogenous or dietary.

Although it is possible that each of these processes may contribute to conservation of TMAO, all aforementioned studies examined TMAO flux in elasmobranchs during starvation when nitrogen metabolism is significantly altered

(Wood et al., 2005). Fasted skates have been shown to suppress TMAO excretion during starvation (Treberg and Driedzic, 2006) and dogfish exhibit decreases in plasma urea and total osmolarity during fasting (Leech et al., 1979). In another study, spiny dogfish were able to preserve urea during long-term starvation, although it was reported to come at the cost of significant protein catabolism. It was estimated that 69.5 g of protein would need to be broken down over 56 days for continued urea synthesis under starvation conditions (Kajimura et al., 2008). Further, urea synthesis is an energy-expensive process requiring 5 moles of ATP per 1 mol urea (Anderson, 2001; Lee et al., 2006).

It is thought that spiny dogfish must feed every 5-6 days to sustain nitrogen balance (Kajimura et al., 2006). In fact, the rate of nitrogen loss does not increase after feeding, suggesting these animals are nitrogen limited (Wood et al., 2005, 2007; Treberg and Driedzic, 2006). After a meal, there is a switch from net urea efflux to net intestinal absorption (Liew et al., 2013) and plasma urea spikes 20 hours postprandially in spiny dogfish (Kajimura et al., 2006; Kajimura et al., 2008) indicating absorption from dietary constituents or elevated synthesis. Similarly, plasma TMAO also increases 20 hours after feeding in this species (Kajimura et al., 2006; Wood et al., 2010 for review).

It has been suggested that elasmobranchs excrete between 4-14% of their whole body TMAO per day (Goldstein and Palatt, 1974). Treberg and Driedzic (2006) published a more conservative estimate for the winter skate, *Leucoraja ocellata*, of less than 1% whole body TMAO lost per day. However, the rate of TMAO loss decreased after one week of starvation, which would have reduced Treberg and

Driedzic's estimate. Even if elasmobranchs lose an average of 1% TMAO per day, this would amount to losses greater than 50% of total body stores over 56 days without an endogenous or dietary input. As elasmobranchs are already nitrogen limited (Armour et al., 1993; Wood et al., 2005), questions of TMAO flux and maintenance are better addressed under less stressful physiological conditions when individuals are actively feeding.

Here, we directly test the effect of diet on TMAO content in three elasmobranch species with differing synthetic capacities. The smoothhound, *Mustelus canis*, a shark with TMAoxi activity was compared to the spiny dogfish, *Squalus acanthias*, and little skate, *Leucoraja erinacea*, two species with negligible synthesis (Treberg et al., 2006). Individuals were fed a high or low TMAO diet for 56 days to examine the effects of an exogenous source on long-term TMAO maintenance.

2. Materials and Methods

2.1. Collection and transport

Three elasmobranch species were obtained by otter trawl off the commercial fishing vessel *Virginia Marise*. Smoothhounds, *Mustelus canis* (n=10), spiny dogfish, *Squalus acanthias* (n=13), and little skates, *Leucoraja erinacea* (n=19), were captured in Narragansett Bay, RI during summers 2013 – 2015. Males and females were placed in 150 L insulated coolers provided with chilled, aerated seawater and transported to holding facilities less than an hour away. Individuals were placed in a random fashion into one of two 2.4 m diameter, 2850 L continuous flow circular holding tanks. Sharks were housed up to n=5 and skates up to n=10 per tank.

2.2. Feed trials

Seawater temperature was maintained at $17 \pm 0.22^{\circ}\text{C}$ (mean $\pm$ SEM) and light on a 12h:12h light/dark cycle for the duration of the experiment. Individuals were acclimated for a minimum of 72 hours before initiation of a feeding trial. After acclimation, animals were fed diets high or low in TMAO content for 56 days. The high TMAO diet was comprised of a mixture of herring (*Clupea harengus*) and squid (*Doryteuthis pealei*) fed at 2.5% body weight, twice a week. Rations were chosen in accordance with previous studies (Wood et al., 2005; Wood et al., 2010; Liew et al., 2013). Herring and squid are part of the regular diet consumed in the wild (Stehlik, 2007) and a rich source of TMAO ($\sim 50\text{--}80\text{ mmol kg}^{-1}$, Carr et al., 1996; Treberg and Driedzic, 2007; Supplementary Fig. 1). Individuals placed on a low TMAO diet were fed brook trout (*Salvelinus fontinalis*) at 2.5% body weight, twice a week. Brook trout contain negligible levels of TMAO ($<0.5\text{ mmol kg}^{-1}$, Supplementary Fig. 1) and

constitute a low TMAO diet. Freshwater fish tissue is known to contain lower concentrations of essential vitamins compared to marine tissue (Käkelä et al., 1999); therefore, as brook trout are a freshwater species that would not contribute to the regular diet experienced by these marine elasmobranchs in the wild, a vitamin supplement (SEA TABS for Birds, Turtles, Fish and Sharks by Pacific Research Laboratories) was included in the low TMAO diet to ensure individuals experienced no confounding deficiencies. Three separate feeding trials were conducted and data pooled for analysis. Five spiny dogfish, two smoothhounds and eight little skates were included in trial one which ran for 56 days from 2/20/13 – 4/17/13. 11 little skates were included in trial two which ran for 56 days from 9/08/14 – 11/03/14. Eight smoothhounds and seven spiny dogfish were included in trial three which ran for 56 days from 7/29/15 – 9/23/15. Test species included in each trial were dependent on local availability.

2.3. Sampling

Blood samples were taken by the caudal method at time 0 and once monthly using an 18-gauge hypodermic needle. Prior to blood sampling, specimens were anaesthetized with 0.05 g l⁻¹ MS-222 dissolved in a seawater bath. Red blood cells were separated by centrifugation at 10,000 rpm for three minutes and discarded. Plasma was flash frozen in liquid nitrogen and stored at -80 for later analysis. Measurements of weight, standard and total lengths were taken on each sampling day. Sex and spiracle length were also recorded. These parameters were used to assess growth and as an estimate of age to help assess the influence of size, ontogeny or sex-

related differences on feeding habits and TMAO content (Alonso et al., 2002; Bockus and Seibel, in prep).

On day 56, individuals were euthanized with MS-222 (0.15 g l^{-1}) dissolved in a seawater bath. Length and weight measurements were taken. Plasma samples were obtained by the caudal method as described above. White muscle ($\sim 1 \text{ g}$) was harvested from the left dorsolateral epaxial muscle. The whole liver was removed and weighed. A sample of liver tissue ($\sim 1 \text{ g}$) was harvested from the periphery of the major lobe. All samples were duplicated for each individual and immediately flash frozen for later analysis. The presence of maturing embryos was recorded to take into account any gestational influence. All analyses were conducted in accordance with IACUC #AN12-07-026.

2.4. Analytical techniques

Plasma, muscle and liver samples were analyzed for TMAO and urea. Samples were deproteinized and homogenized 1:5 in 5% trichloroacetic acid solution. TMAO content was determined spectrophotometrically with ferrous sulfate and 2% picric acid as described by Wekell and Barnett (1991). Homogenates were further used to assess urea using the diacetyl monoxime method (Rahmatullah and Boyde, 1980). Total lipid was measured in liver tissue using a 2:1 chloroform to methanol extraction modified for small sample mass (Lee et al., 1996).

2.5. Statistical analysis

Where variables scaled with tissue or body weight, individual values were normalized to a common mass before analysis. Means of individuals fed a high or low TMAO diet were compared between time points by two-way-, or two-way-RM,

ANOVA followed by a Holm-Sidak post-hoc test. Terminal samples were compared between diets within species using one-way unpaired student's t-test. Significance was set at $p < 0.05$. Statistics and graphs were generated using GraphPad Prism 7.0.

3. Results

3.1. Lipid

Liver lipid content was not different between diets within species (Fig. 1). Smoothhounds fed a high TMAO diet had a mean lipid content of 36.93% wet wt. ± 9.29 SEM compared to low TMAO diet individuals at $18.67 \pm 7.62\%$. Spiny dogfish fed a high TMAO diet had a mean lipid content of $62.11 \pm 11.22\%$ compared to low TMAO diet individuals at $51.96 \pm 12.91\%$. Little skates fed a high TMAO diet had a mean lipid content of $22.38 \pm 5.13\%$ compared to low TMAO diet individuals at $23.57 \pm 4.26\%$.

3.2. Plasma

TMAO

Plasma TMAO (mmol kg^{-1}) values at day 0, 28 and 56 listed in Table 1. Plasma TMAO dropped significantly from $78.38 \pm 4.07 \text{ mmol kg}^{-1}$ at day 0 to $45.36 \pm 3.61 \text{ mmol kg}^{-1}$ at day 56 in low TMAO diet spiny dogfish (two-way ANOVA, $p=0.02$). Plasma TMAO was lower at day 56 in low TMAO diet spiny dogfish than high TMAO diet individuals ($73.02 \pm 4.79 \text{ mmol kg}^{-1}$; two-way ANOVA, $p=0.04$). There were no further differences across time points within diets or between diets at an individual time point within species (Fig. 2a-c).

Urea

Plasma urea (mM) values at day 0, 28 and 56 listed in Table 1. Little skates fed a low TMAO diet showed an increase in plasma urea from day 0 at 368.91 ± 11.15 mM to day 28 at 416.97 ± 9.08 mM (two-way RM ANOVA, $p=0.01$) and day 56 at

433.70 $\pm$ 21.17 mM (p=0.002). There were no further differences across time points within diets or between diets at an individual time point within species (Fig. 3a-c).

3.3. Tissue

Scaling

TMAO in liver (y) decreased with increasing liver mass (x) in spiny dogfish (linear regression $y = -0.05588x + 35.80$, $r^2 = 0.37$, $p = 0.04$, Fig. 4). Liver mass ranged from 148.87 - 432.40 g and TMAO was normalized to a common mass of 250 g before further analysis. Urea concentration (x) decreased in the liver with increasing liver mass (y) in smoothhounds (linear regression $y = -2.014x + 319.0$, $r^2 = 0.47$, $p = 0.03$) and little skates (linear regression $y = -5.849x + 491.5$, $r^2 = 0.26$, $p = 0.03$; Supplementary Fig. 2). Liver mass ranged from 14.33 – 79.50 g in smoothhounds and 5.82 – 41.72 g in little skates, and urea values were normalized to a common mass of 30 and 22 g respectively. Little skate muscle urea (x) decreased with increasing total body wet weight (y) (range 0.26 - 0.80 kg; linear regression $y = -245.8x + 557.4$, $r^2 = 0.24$, $p = 0.03$; Supplementary Fig. 3) and was normalized to an average body weight of 0.60 kg before analysis.

TMAO

Tissue TMAO (mmol kg⁻¹) values at day 56 listed in Table 2. Smoothhound muscle TMAO was lower in low TMAO diet individuals at 100.63 $\pm$ 9.91 mmol kg⁻¹ than high TMAO diet individuals at 133.07 $\pm$ 22.76 mmol kg⁻¹ (one-way unpaired student's t-test, $p = 0.03$). There were no further differences between diets within tissue types for individual species (Fig. 5).

Urea

Tissue urea (mM) values at day 56 listed in Table 2. Liver urea was lower in low TMAO diet spiny dogfish at 120.48 ± 13.94 mM than high TMAO diet dogfish at 195.72 ± 23.67 mM (one-way unpaired student's t-test, $p=0.04$). There were no further differences between diets within tissue types for individual species (Fig. 6).

4. Discussion

Animals in this study readily fed on the high and low TMAO diets. Lipid levels were conserved across groups in all species (Fig. 1), as would be expected in actively feeding individuals not relying on protein or lipid stores to support metabolism. Plasma TMAO fell by day 56 in dogfish fed the low TMAO diet but not in the smoothhound or little skate (Fig. 2a-c). This suggests that spiny dogfish (a nonsynthesizing species) do rely, to an extent, on dietary contributions for TMAO maintenance. When faced with an absence of TMAO in the diet, they may transport plasma TMAO to alternate tissues, such as muscle or liver, that depend on TMAO for preserved cellular function. Given these results, one would also expect to see a drop in plasma TMAO in the little skate that similarly exhibit no TMAO synthetic capacity. However, TMAO was maintained in the plasma of the little skate regardless of diet. Skates rely to a lesser extent on TMAO to support osmotic potential. Skates retain plasma TMAO at half the content present in spiny dogfish. Further, they exhibit similar concentrations of urea but higher levels of alternative amino acid osmolytes (King and Goldstein, 1983). It is possible there is a greater discrepancy in the way sharks and skates regulate TMAO than previously thought. TMAO accumulation patterns are different through spiny dogfish and little skate ontogeny, perhaps supporting disparities in the way this molecule is utilized between the two species (Steele et al., 2004; Bockus and Seibel, submitted).

Surprisingly, muscle TMAO fell in smoothhounds fed a low TMAO diet, the only species in this study to exhibit a TMAO synthetic capacity (Treberg et al., 2006), but was maintained in spiny dogfish and the little skate (Fig. 5). Feeding provides

ample precursors, such as choline (Seibel and Walsh, 2002), to support endogenous production supposedly minimizing this species reliance on dietary TMAO for long-term maintenance. It is possible that all species, whether they can synthesize TMAO or not, rely on dietary contributions for absorption and retention. Alternatively, species that can readily synthesize TMAO may not express the regulatory pathways for reabsorption and retention. Spiny dogfish and little skates appear to exhibit a greater capacity for TMAO conservation than smoothhounds via such possible mechanisms as reabsorption at the gill and kidney, transfer between tissue pools, and recycling through catabolism, leading to a greater capacity for TMAO retention over time. In accordance with this view, spiny dogfish and little skates maintained muscle TMAO regardless of whether TMAO was present in the diet or not. These data support previous findings showing TMAO maintenance during prolonged starvation in spiny dogfish and the winter skate *L. ocellata* (Goldstein et al., 1967; Treberg and Driedzic, 2006, 2007; Kajimura et al., 2008) and likely result from a combination of the proposed retention mechanisms. The possibility of unidentified TMAO synthetic pathways cannot be ruled out as a source contributing to extended preservation.

Urea excretion accounts for 90% or more of the total nitrogen excreted by elasmobranchs (Wood et al., 2005; Kajimura et al., 2006). Urea conservation depends largely on selective impermeability at the gills (Wood et al., 1995; Part et al., 1998; Wood et al., 2013); however, there is an unavoidable “leakiness” (Kajimura et al., 2008) and urea stores must be supplemented through exogenous absorption or endogenous synthesis from dietary precursors. Dietary urea may be provided by the low concentrations retained in some marine fishes or by the observed cannibalism of

other marine sharks rich in urea (Stehlik, 2007). In this study, there was no difference in plasma urea between diets in any species. Although there were subtle, but significant, differences in plasma urea concentration over time in the little skate (Fig. 3c), all values fell within the range reported previously for wild caught specimens of this species.

There was a decrease in liver urea in spiny dogfish fed a low TMAO diet but not the other two species studied (Fig. 6). Again, although the difference in spiny dogfish was significant, it fell within the previously reported range and is not likely physiologically relevant. There is no reason to expect the low TMAO diet would restrict the urea synthetic pathway (ornithine-urea cycle) or affect retention mechanisms. However, freshwater fishes exhibit lower concentrations of urea, ~1.5mM in brook trout (Rehulka and Minarik, 2007), compared to marine fish with reported concentrations up to ~25mM (Raymond, 1994). Therefore, less urea was available for direct dietary absorption in our low TMAO diet, perhaps explaining the difference.

A number of the given explanations for TMAO and urea regulation are likely involved in the conservation of these molecules. However, the presence or absence of TMAoxi cannot be used as a metric to assess a species reliance on dietary TMAO. Treberg et al. (2005) found TMAoxi activity did not correlate to TMAO content in smelt (*Osmerus mordax*). Likewise, although FMO activity was present in the winter skate (*L. ocellata*), there was no evidence of TMA oxidation (Treberg and Driedzic, 2006). This suggests that although TMAoxi has been shown to oxidize TMA to TMAO it may not be the key enzyme regulating TMAO synthesis and retention. In

fact, there is some discrepancy in the literature regarding the ability of dogfish and skates to synthesize TMAO (Schlenk and Li-schlenk, 1994; Schlenk, 1998; Treberg et al., 2006) although these concerns were addressed by Treberg and Driedzic (2006) that support the conclusion that these species are incapable of physiologically relevant production. However, this disagreement demonstrates the lack of understanding with regard to relevant sources involved in TMAO production. There may be alternate enzymes capable of oxidizing TMAO such as cytochrome P450 monooxygenases (Ágústsson and Strøm, 1981; Raymond, 1998) or others that have not been identified. Additionally, TMAO oxidation by gut microflora (Koeth et al., 2013) may diminish a species reliance on dietary contributions from prey tissue.

When accumulation of an organic osmolyte is limited, animals have been shown to replace the osmotic deficit with alternate compounds. Treberg et al. (2006) showed an increase in betaine, another methylamine, to offset TMAO losses and maintain osmotic balance in the little skate. Similarly, NaCl replaced urea in the plasma of nitrogen-limited European dogfish (Armour et al., 1993). Although these alternatives pose possible short term solutions, increasing NaCl intracellularly has been shown to destabilize protein function (Yancey et al., 1982) and betaine, although a protein stabilizer, is not as effective at preserving protein structure as TMAO (Yancey et al., 2004). Therefore, the concentration of betaine needed to affect the same degree of stability would require an increase in total osmolarity or decrease in other osmolytes. The vitamin supplement we added to our low TMAO diet did include taurine, an alternative counteracting solute, which may have been accumulated in

place of TMAO. However, this would not explain the differences we saw between species and tissue types of individuals fed the low TMAO diet.

This study shows species specific differences in elasmobranchs ability to regulate TMAO without an exogenous source. Further, there was no clear delineation between synthesizing potential and ability to regulate TMAO without a dietary contribution. Therefore, presence or absence of TMAoxi activity cannot be used as a proxy to determine whether a dietary contribution is needed for long-term TMAO maintenance. Diet was also important for urea conservation, although effects seem to differ between tissue type and species. Although alternative compounds may be substituted to maintain osmotic balance when TMAO or urea accumulation are limited, these molecules serve a variety of functions and fluctuations caused by shifts in diet may affect a number of physiological processes in these animals. More evidence is needed to determine the mechanistic regulatory pathways involved in preservation of these nitrogenous compounds, specifically those controlling TMAO

Figure 1. Total liver lipid (% wet weight) in elasmobranchs fed a high or low TMAO diet for 56 days. Smoothhound (*Mustelus canis*, n=10), spiny dogfish (*Squalus acanthias*, n=7) and little skate (*Leucoraja erinacea*, n=19) means $\pm$ SEM. No significant differences (one-way unpaired student's t-test, $p < 0.05$) between diets within species.

(days)	TMAO (mmol kg ⁻¹)			Urea (mM)		
	0	28	56	0	28	56
<i>Smoothhound</i>						
high TMAO (4)	60.18 ± 10.88	56.65 ± 5.76	54.94 ± 5.17	358.87 ± 13.23	345.11 ± 9.31	302.85 ± 32.48
low TMAO (6)	58.26 ± 5.09	42.46 ± 3.69	46.38 ± 4.00	353.32 ± 19.73	357.20 ± 20.34	361.28 ± 23.23
<i>Spiny dogfish</i>						
high TMAO	75.33 ± 0.81 (2)	60.59 ± 15.87 (2)	73.02 ± 4.79 (7)	385.35 ± 34.22 (2)	368.00 ± 18.84 (2)	388.42 ± 35.24 (7)
low TMAO (5)	78.38 ± 4.07	54.01 ± 9.1	45.36 ± 3.61 ^{#*}	391.68 ± 17.69	363.51 ± 21.43	337.28 ± 32.61
<i>Little skate</i>						
high TMAO (9)	33.14 ± 7.25	33.48 ± 5.71	36.32 ± 3.28	407.22 ± 15.03	375.15 ± 17.98	420.96 ± 23.17
low TMAO (10)	35.08 ± 8.11	32.66 ± 4.14	29.92 ± 2.76	368.91 ± 11.15	416.97 ± 9.08 [#]	433.70 ± 21.17 [#]

Table 1. Plasma contents over time in elasmobranchs fed a high or low TMAO diet. Smoothhound (*Mustelus canis*), spiny dogfish (*Squalus acanthias*) and little skate (*Leucoraja erinacea*) plasma TMAO (mmol kg⁻¹) and urea (mM) ± SEM. n values given in parentheses. # indicates a significant difference within a diet from time 0. * indicates a significant difference between high and low TMAO diet means at an individual time point within a species (two-way or two-way RM ANOVA, p<0.05).

Figure 2a-c. Plasma TMAO (mmol kg⁻¹) over time in elasmobranchs fed a high or low TMAO diet. Smoothhound (*Mustelus canis*, n=10), spiny dogfish (*Squalus acanthias*, n=12) and little skate (*Leucoraja erinacea*, n=19) plasma TMAO $\pm$ SEM at time 0, 28 and 56 days. # indicates a significant difference from time 0 within a diet. *

indicates a significant difference between high and low TMAO diet means at an individual time point within a species (two-way or two-way RM ANOVA, $p < 0.05$).

Figure 3a-c. Plasma urea (mM) over time in elasmobranchs fed a high or low TMAO diet. Smoothhound (*Mustelus canis*, n=10), spiny dogfish (*Squalus acanthias*, n=12) and little skate (*Leucoraja erinacea*, n=19) plasma urea $\pm$ SEM at time 0, 28

and 56 days. # indicates a significant difference from time 0 within a diet (two-way or two-way RM ANOVA, $p < 0.05$).

Figure 4. Spiny dogfish liver TMAO (mmol kg⁻¹) decreases with increasing liver mass (g). TMAO in spiny dogfish (*Squalus acanthias*, n=12) liver decreased as liver mass increased from 148.87 – 432.40 g. Linear regression $y = -0.05588x + 35.80$, $r^2 = 0.34$, $p = 0.04$. Livers ranged from 148.87-432.40 g and TMAO was normalized to a common liver mass of 250 g before further analysis. Reference results section 3.3 and Supp. Fig. 2 - 3 for complete list of variables shown to scale with mass.

	TMAO (mmol kg ⁻¹)		Urea (mM)	
	muscle	liver	muscle	liver
<i>Smoothhound</i>				
high TMAO (4)	133.07 ± 22.76	47.76 ± 7.71	296.07 ± 25.07	226.88 ± 19.59
low TMAO (6)	100.63 ± 9.91*	41.01 ± 3.13	329.87 ± 21.43	272.53 ± 44.08
<i>Spiny dogfish</i>				
high TMAO (7)	162.93 ± 8.43	21.89 ± 4.52	378.40 ± 18.06	195.72 ± 23.67
low TMAO (5)	144.72 ± 20.79	19.94 ± 2.90	313.45 ± 36.68	120.48 ± 13.94*
<i>Little skate</i>				
high TMAO (9)	102.10 ± 15.06	50.13 ± 5.91	417.41 ± 40.74	367.46 ± 47.24
low TMAO (10)	90.18 ± 12.73	33.71 ± 3.23	429.81 ± 23.09	374.58 ± 47.06

Table 2. Tissue contents in elasmobranchs fed a high or low TMAO diet for 56 days. Smoothhound (*Mustelus canis*), spiny dogfish (*Squalus acanthias*) and little skate (*Leucoraja erinacea*) muscle and liver TMAO (mmol kg⁻¹) and urea (mM) ± SEM. n values given in parentheses. * indicates a significant difference between diets within a tissue type of an individual species (one-way unpaired student's t-test; p<0.05).

Figure 5. TMAO (mmol kg⁻¹) in muscle and liver of elasmobranchs fed a high or low TMAO diet for 56 days. Muscle and liver means $\pm$ SEM for smoothhound (*Mustelus canis*, n=10), spiny dogfish (*Squalus acanthias*, n=12), and little skate (*Leucoraja erinacea*, n=19). Spiny dogfish liver TMAO scaled with liver mass (Supp. Fig. 3) and was normalized using regression $y = -0.05588x + 35.80$ before analysis. Significant differences (*) determined between diets within tissue types for individual species (one-way unpaired student's t-test, $p < 0.05$).

Figure 6. Urea (mM) in muscle and liver of elasmobranchs fed a high or low TMAO diet for 56 days. Muscle and liver means $\pm$ SEM for smoothhound (*Mustelus canis*, n=10), spiny dogfish (*Squalus acanthias*, n=12), and little skate (*Leucoraja erinacea*, n=19). Skate urea concentration scaled with body weight and was normalized to an average of 0.6 kg before analysis (regression $y = -245.8x + 557.4$, Supp. Fig. 3). Smoothhound and skate liver urea scaled with liver mass and were normalized (regression $y = -2.014x + 319.0$ and $y = -5.849x + 491.5$ respectively, Fig. 4). Significant differences (*) determined between diets within tissue types for individual species (one-way unpaired student's t-test, $p < 0.05$).

Supplementary Figure 1. Muscle TMAO (mmol kg⁻¹) in high and low TMAO diet feed components. The high TMAO diet was made up of squid (*Doryteuthis pealei*) and herring (*Clupea harengus*). The low TMAO diet consisted of brook trout (*Salvelinus fontinalis*). Squid (n=4) exhibited muscle TMAO at 101.60 ± 0.17 mmol kg⁻¹, herring (n=4) at 61.88 ± 0.41 mmol kg⁻¹ and brook trout (n=4) at 0.05 ± 0.03 mmol kg⁻¹.

Supplementary Figure 2. Urea concentration decreases with increasing liver mass. Little skate (*Leucoraja erinacea*, n=19) and smoothhound (*Mustelus canis*, n=10) liver urea (mM) scaled with liver mass (g). Regression lines for skate ($y = -5.849x + 491.5$, $r^2 = 0.26$, $p = 0.03$) and smoothhound ($y = -2.014x + 319.0$, $r^2 = 0.47$, $p = 0.03$) used to normalize urea to common masses of 22g and 30g respectively before analysis.

Supplementary Figure 3. Little skate muscle urea (mM) decreases with increasing body wet weight (kg). Urea decreased in little skate (*Leucoraja erinacea*, n=19) muscle from a body weight of 0.26 to 0.80 kg and was normalized to an average weight 0.60 kg before analysis. Linear regression $y = -245.8x + 557$, $r^2 = 0.24$, $p = 0.03$.

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CHAPTER 2

TRIMETHYLAMINE OXIDE ACCUMULATION AS A FUNCTION OF DEPTH IN HAWAIIAN MID-WATER FISHES

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

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.

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.

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.

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

(Seibel, 2011; Seibel et al., 2014) so further evidence is needed to support this supposition.

1.4.2 Phylogenetic Comparison

The only study to examine the evolutionary history of TMAO synthetic capacity, as described by the activity of trimethylamine oxidase (TMAoxi), found it to be a derived characteristic in elasmobranchs and chimaeras (Treberg et al., 2006). Species lacking measurable TMAoxi activity must rely on dietary contributions to accumulate TMAO (Treberg and Driedzic, 2002), potentially placing ecological restrictions on their ability to use TMAO as a counteracting solute. If teleosts were to exhibit a similar phylogenetic pattern, it would suggest differing capacities for TMAO regulation between clades and could influence inherent TMAO 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.

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 Pearcy, 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.

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.

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.

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</i> "H"	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							

Sternoptychidae	<i>Serrivomer sector</i>	67.3±40.3 (8)	0.79±0.49 (5)	700	700	1800	no
	<i>Argyropelecus affinis</i>	49.2±15.1 (6)	0.79±0.16 (2)	350	200 (225)	500	no
	<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
Stomiidae	<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

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

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

* data taken from Neighbors, 1988; ** data taken from Childress et al., 1990

a Rao, 2010; b Clarke, 1978; c Hulley, 1990; d Inferred for genera by Kenaley, 2008

Figure 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 $y = 0.02539x + 39.69$ ($r^2 = 0.2520$, $p < 0.0076$).

Figure 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 $y = 0.06965x + 5.225$ ($r^2 = 0.4036$, $p = 0.0009$).

Figure 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 $y = 0.002599x + 0.3926$ ($r^2 = 0.2496$, $p = 0.0152$).

Figure 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 $y = -0.7241x + 3.559$ ($r^2 = 0.8834$, $p = 0.0175$).

Figure 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 $y = 10.27x + 40.80$ ($r^2 = 0.4328$, $p = 0.0006$).

Supplementary Figure 1. Phylogenetic tree used to determine contrast values.

For simplicity, only species with TMAO and depth values from this study are shown.

TMAO (mmol kg⁻¹) and average depth (m) are shown to the right of the taxon name.

Ancestral TMAO and depth values calculated by PIC are plotted at the nodes. Due to a

lack of phylogenetic data all branch lengths were considered equal and a punctuated

model of change was assumed. Contrast values calculated from this tree are shown in

Fig. 2.

Family	Species	Depth (m); Reference	Type	Region
Anoplogastridae	<i>Anoplogaster cornuta</i>	600; Meek and Childress, 1973** 550-900; Childress, 1975* 550; Neighbors, 1988 550; Janssens et al., 2000	MDO MDO, max MDO MDO	Southern California Southern California Southern California NE North Pacific
Eurypharyngidae	<i>Eurypharynx pelecanoides</i>	2300; Vaillant, 1883 650-1300; Clarke and Wagner, 1976*,** 971,1532; Owre and Bayer, 1970 1020; Campbell and Gartner, 1982 500-2750; Nielsen et al., 1989	C C average C C	SE North Atlantic Hawaii SW North Atlantic NW North Atlantic Atlantic Basin
Giganturidae	<i>Gigantura indica</i> ^a	750-1000; Clarke and Wagner, 1976*,** 750; Childress et al., 1990* 947; Tomiyama et al., 2008	C MDO C	Hawaii Hawaii Japan
Gonostomatidae	<i>Cyclothone pallida</i>	500-1000; Badcock, 1982 400-1000; Miya and Nemoto, 1987 600; Childress et al., 1990* 600-1000; Craddock et al., 1992*,** 500-700; McClain et al., 2001** 400-1377; Ross et al., 2010	C C MDO C C C	Tropical Atlantic Japan Hawaii NW North Atlantic Tropical Atlantic Gulf of Mexico
	<i>Gonostoma atlanticum</i>	200-560; Clarke, 1974** 150; Childress et al., 1990* 481-560; De Forest and Drazen, 2009*	C MDO C	Hawaii Hawaii Hawaii
	<i>Gonostoma elongatum</i>	560-725; Clarke, 1974*,** 80-350; Hopkins et al., 1981	C night/day	Hawaii SW North Atlantic

Melamphaidae		25-325; 425-725; Lancraft et al., 1988 200; Childress et al., 1990* 50-500; Ross et al., 2010 500-1200; Sutton et al., 2010	night/day MDO C C	Gulf of Mexico Hawaii Gulf of Mexico Sargasso Sea
	<i>Poromitra macrophthalma</i> ^b	450; Ebeling and Cailliet, 1974 400; Ebeling, 1975 640-1000; Clarke and Wagner, 1976*,** 655; Kotlyar, 2010	MDO C C C	Southern California Indo-Pacific Hawaii Indian/Pacific Oceans
Myctophidae	<i>Ceratoscopelus warmingi</i>	80-700; Hopkins et al., 1981 50; Childress et al., 1990* >600; Craddock et al., 1992*,** 500-900; Hulley, 1992* 50-100; 400-500; Saito and Murata, 1996 1000; Boxshall, 2000	night/day MDO C C night/day C	SW North Atlantic Hawaii NW North Atlantic SE North Atlantic Japan tropical South Pacific
	<i>Diaphus perspicillatus</i>	40-400; Balachandran and Abdul Nizar, 1990 surface; Gartner et al., 1989 500-900; Hulley, 1992* 132-353; Ross et al., 2010	C C C C	India Sargasso Sea SE North Atlantic Gulf of Mexico
	<i>Hygophum proximum</i>	20-75; Hartmann and Clarke, 1975 larvae below 50; Ropke, 1993 SSL; Tsarin, 1997** shallow; De Forest and Drazen, 2009 0-150; 500-1000; Drazen et al., 2011*	C C C C night/day	Tropical Pacific Arabian Sea Arabian Sea Hawaii Hawaii
	<i>Lampanyctus niger "H"</i>	100-500; Hartmann and Clarke, 1975*	C	Tropical Pacific

		165 (sp b); Childress et al., 1990*	MDO	Hawaii
	<i>Lampanyctus tenuiformis</i>	0-200; Kinzer and Schulz, 1985 250; Childress et al., 1990* 700-900; Hulley, 1992* 81-262; Ross et al., 2010	C MDO C C	Tropical Atlantic Hawaii SE North Atlantic Gulf of Mexico
	<i>Taaningichthys bathyphilus</i>	650; Paxton, 1967** 800; Ebeling and Cailliet, 1974 600; Childress et al., 1990* 582-1122; Garcia and Morgan, 2002* 1000-1550; Gartner et al., 1987	C MDO MDO C C	Southern California Southern California Hawaii SW South Atlantic Gulf of Mexico
Oneirodidae	<i>Danaphryne nigrifilis</i>	1082; Moller et al., 2010* ,** in ref to Stearn and Pietsch, 1995 0-1011; Moore et al., 2003	C C	Labrador Sea NW North Atlantic
Opisthoproctidae	<i>Opisthoproctus soleatus</i>	500-700; Krefft, 1976 500-600; Clarke and Wagner, 1976** 450; Childress et al., 1990* 500-700; Gagnon et al., 2013*	C C MDO C	Tropical Atlantic Hawaii Hawaii N/A
Paralepididae	<i>Magnisudis atlantica</i>	445-490; Maslenikov et al., 2013*	C	Bering Sea
Serrivomeridae	<i>Serrivomer sector</i>	600; Williams and Weiss, 1973 300; Janssens et al., 2000 700-1800; Robison et al., 2010* ,**	C MDO C	Southern California Eastern North Pacific Eastern North Pacific
Sternoptychidae	<i>Argyropelecus affinis</i>	100-350,350-600; Somiya, 1976 400-550; Hopkins et al., 1981	night/day night/day	Indian/Pacific Oceans SW North Atlantic

Stomiidae

	200-400; Bailey and Robison, 1986**	C	Eastern North Pacific
	200; Neighbors, 1988	MDO	Southern California
	400-500; Kinzer and Schulz, 1988*	C	Tropical Atlantic
	225; Childress et al., 1990*	MDO	Hawaii
	200; Janssens et al., 2000*	MDO	Eastern North Pacific
<i>Danaphos oculatus</i>	430-650; Clarke, 1974*,**	C	Hawaii
	430; Childress et al., 1990*	MDO	Hawaii
	183-914; Shinohara et al., 1994	C	Indian/Pacific Oceans
	in ref to Eschmeyer et al., 1983		
<i>Sternoptyx diaphana</i>	600-900; Baird, 1971	C	circumglobal
	600-800; Badcock and Baird, 1980	average	Tropical Atlantic
	150-500; Hopkins et al., 1981	night/day	SW North Atlantic
	500-1200; Bailey and Robison, 1986**	C	Eastern North Pacific
	500-800; Kinzer and Schulz, 1988	C	Tropical Atlantic
	450; Childress et al., 1990*	MDO	Hawaii
	600-1000; Craddock et al., 1992**	C	NW North Atlantic
	625-725; Baird and Jumper, 1995	average	Hawaii
	422-899; Ross et al., 2010*	C	Gulf of Mexico
	700-1200; Sutton et al., 2010	C	Sargasso Sea
<i>Aristostomias grimaldi</i>	100-750; Clarke, 1974*	C	Hawaii
<i>Chauliodus sloani</i>	175-600; Clarke, 1974	C	Hawaii
	70-450; Hopkins et al., 1981	night/day	SW North Atlantic
	175; Childress et al., 1990*	MDO	Hawaii
	450-950; Sutton and Hopkins, 1996	average	Gulf of Mexico
	100-500; Butler et al., 2001*	C	Arabian Sea
	984-2169; Cartes and Carrasson, 2004	C	Mediterranean Sea

	400; Dalyan and Eryilmaz, 2008	C	Mediterranean Sea
<i>Flagellostomias boureei</i>	0-900; Sutton and Hopkins, 1996* 1460; Vazquez et al., 2013	average C	Gulf of Mexico NW North Atlantic
<i>Idiacanthus antrostomus</i>	150-600; Bailey and Robison, 1986** 150; Neighbors, 1988* 300; Smith-Beasley, 1992* 250; Janssens et al., 2000 500-2000; Gagnon et al., 2013	C MDO C MDO C	Eastern North Pacific Southern California Southern California Eastern North Pacific N/A
<i>Photostomias liemi</i>	0-762; Kenaley, 2009*,**	C	Hawaii
<i>Photostomias lucingens</i>	0-115; Kenaley, 2009*,**	C	Hawaii
<i>Thysanactis dentex</i>	150-700; Clarke, 1974 0-550; Jorgensen and Munk, 1979* 75; Childress et al., 1990*	C C MDO	Hawaii Tropical Atlantic Hawaii

Supplementary Table 1. Depth (m) data for individual teleost species. Data represent available reported depths for each species circumglobally. Type of depth is reported as minimum (MDO), maximum (max), capture (C), average or day/night depths for vertical migrators. Region of each study is also included. Some reported depth values are specific to the size of fish observed and analyzed in this study.

* reference used for depth analysis in this study

** reference used for presence or absence of vertical migration

^a *Bathyleptus lisae* is a junior synonym used in older publications

^b previously reported as *megalops*

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CHAPTER 3

TRIMETHYLAMINE OXIDE AND HSP70 REGULATION DURING ACUTE TEMPERATURE STRESS IN ELASMOBRANCHS

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Abstract

Trimethylamine oxide (TMAO) and heat shock protein 70 (HSP70) are intracellular components directly involved in the thermal stress response, both protecting protein function at elevated temperatures. Due to their functionally similar roles, we address the simultaneous response of these constituents to increasing temperature *in vivo*. We use two elasmobranch species, which possess innately high levels of TMAO, to address if regulation of TMAO and HSP70 is coordinated during a 6°C increase in temperature for 72 hours. The spiny dogfish, *Squalus acanthias*, a species with no endogenous synthetic capacity for TMAO, was compared to the smoothhound, *Mustelus canis*, a synthesizing species. There was no increase in plasma or tissue TMAO with elevated temperature in either species. HSP70 accumulation was observed with increasing temperature in white muscle of *S. acanthias* but not *M. canis*. The HSP70 response in *S. acanthias* demonstrates that the high TMAO content in this species does not confer sufficient protection to offset the denaturing effects of elevated temperature. The lack of heat-shock response in *M. canis* HSP70 was surprising and may be explained by species-specific differences in thermal tolerance, maintenance of high constitutive levels of HSP70 or preferential accumulation of alternate counteracting solutes. Our findings are in contrast to previous studies conducted with elasmobranch cells in vitro that show accumulation of TMAO with thermal stress and subsequent suppression of a HSP70 response.

1. Introduction

Trimethylamine oxide (TMAO) is a small intracellular osmolyte and cytoprotectant. It acts as a chemical chaperone to conserve protein structure and function (Yancey and Somero, 1979; Yancey and Siebenaller, 1999; Yancey et al., 2001) against a number of destabilizing biotic and abiotic factors including urea (Treberg et al., 2006), salinity (Pillans et al., 2005; Deck et al., 2016), hydrostatic pressure (Yancey et al., 2014; Bockus and Seibel, 2016), and temperature (see Seibel and Walsh, 2002; Yancey, 2005 for reviews). The majority of studies examining TMAO's ability to combat thermal fluctuations *in vivo* have focused on teleost fishes and their response to cold acclimation. Rainbow smelt (*Osmerus mordax*) increased serum TMAO in response to winter-acclimatization (Raymond, 1994; Treberg et al., 2002). Similarly, cold-water Pacific herring (*Clupea harengus*) express elevated serum TMAO compared to their temperate water counterparts (Raymond, 1998). In fact, some of the highest values recorded for teleosts were found in Antarctic fishes, with TMAO increasing total osmolarity and depressing the freezing point (Raymond and DeVries, 1998). TMAO accumulation has also been linked to thermal acclimation in response to warming temperatures. The presence of TMAO increased RNA melting temperatures (Pincus et al., 2008) and addition of TMAO to growth medium enhanced heat resistance in *Escherichia coli* (Velliou et al., 2010) and spiny dogfish, *Squalus acanthias*, cells in vitro (Villalobos and Renfro, 2007; Kolhatkar et al., 2014).

An alternative response to elevated temperature is accumulation of heat shock proteins (HSPs). Upregulation of the inducible isoforms of HSPs has traditionally been referred to as the “heat shock response” (Ritossa, 1962), with preferential

accumulation of HSP70 (Luft et al., 1996) a highly conserved and widely studied biomarker. Under a variety of stress conditions, HSP70 expression is induced to counteract protein misfolding (Freeman et al., 1999). HSP70 is an ATP-dependent protein chaperone that functions as part of the proteostasis network to prevent aggregation and promote refolding of misfolded and denatured proteins in collaboration with several co-chaperone partners and other chaperone systems (Feder and Hofmann, 1999; Powers and Balch, 2013; Finka et al., 2015 for reviews). A number of fish species increase hsp70 mRNA transcript levels and HSP70 protein in response to thermal stress (Basu et al., 2002; Iwama et al., 2004 for review), including tilapia (Molina et al., 2000), rainbow trout (Currie et al., 2000), brook trout (Lund et al., 2003), gilthead seabream (Feidantsis et al., 2009) and common carp (Sung et al., 2014). Additionally, fish preconditioned to sublethal elevated temperatures accumulate HSP70 resulting in enhanced thermotolerance (Sung et al., 2014).

As TMAO and HSP70 serve similar functions, albeit by different mechanisms, in the thermal stress response, recent attention has focused on potential interactions between these pathways. *S. acanthias* subjected to hyposmotic stress showed decreasing levels of TMAO in gill tissue and subsequent accumulation of HSP70, while muscle TMAO and HSP70 did not change (MacLellan et al., 2015). The authors suggest that protein stabilization provided by TMAO, when present, may inhibit the HSP70 response. A similar relationship was found in *S. acanthias* choroid plexus tissue, with addition of TMAO to incubation medium suppressing HSP70 accumulation in response to increased temperature (Villalobos and Renfro, 2007). Presence of extracellular TMAO also inhibited HSP70 accumulation following heat

shock in *S. acanthias* red blood cells (Kolhatkar et al., 2014). Although these studies provide evidence of cooperation between the two pathways, to our knowledge no work has been done to examine these interactions in response to elevated temperatures *in vivo*.

Here, TMAO and HSP70 were measured in two elasmobranch species during 72 hours at elevated temperature. Marine elasmobranchs were used as a model system as they retain exceptionally high levels of TMAO ($\sim 180 \text{ mmol kg}^{-1}$) due to their unique osmoregulatory strategy of accumulating urea, a protein denaturant (Withers, 1998; Trischitta et al., 2012). The smoothhound (*Mustelus canis*), a species capable of TMAO synthesis, was compared to *S. acanthias*, which requires dietary TMAO input (Treberg et al., 2006). This study examines whether TMAO can be accumulated as an alternative to HSP70 during the thermal stress response in these species and whether endogenous TMAO production leads to preferential use of TMAO over HSP70.

2. Materials and methods

2.1. Experimental animals

S. acanthias (n=14) and *M. canis* (n=16) of mixed sex were captured by otter trawl from Narragansett Bay, RI off the F/V *Virginia Marise* in summers 2013 – 2015. Individuals ranging from 0.54 to 4.68 kg were obtained from a field site near the mouth of the bay with an average bottom temperature of 16.19°C. Animals were placed in 150 l insulated coolers with flow-through seawater and aeration up to n=3 per cooler. Coolers were transported from Galilee, RI marina to the Graduate School of Oceanography, University of Rhode Island within one hour of capture. Individuals were tagged using a tag gun (Avery Monarch SG) with markers inserted near the base of the first dorsal fin. Animals were housed in 2.4 m diameter, 2850 l flow-through circular holding tanks up to n=5. Tanks were provided with temperature regulated (15°C) filtered seawater at a flow rate of 12 l minute⁻¹. Individuals were fed a mixed diet of herring and squid twice a week at 2.5% body weight in accordance with previous studies (Wood et al., 2005; Wood et al., 2010; Liew et al., 2013). All animals were acclimated for a minimum of 72 hours before initiation of a temperature trial.

2.2. Treatment

Control individuals were maintained at 15°C ± 0.86 for 72 hours. Temperature was increased from 15°C to 21°C at a rate of 2°C every two hours in treatment tanks and held constant at 21°C ± 0.78 for 72 hours. At time 0 control specimens were anaesthetized with 0.075 g l⁻¹ MS-222 dissolved in a seawater bath one at a time. Blood samples were obtained from the caudal vein using an 18-gauge hypodermic needle pretreated with 30 units heparin. Blood was centrifuged at 10,000 rpm for three

minutes and the plasma isolated. The sex, weight and length were recorded for each animal. Treated individual were similarly sampled at 2 and 20 hours. Muscle biopsies were also taken from the dorsal epaxial muscle of treated individuals using a 5 mm biopsy punch (Alimed). Tissue samples (20-50 mg) were removed and the procedure completed in less than two minutes. All animals were fed mixed herring and squid at 2.5% body weight 30-48 hours after initiation of a temperature trial to ensure TMAO availability for *S. acanthias* individuals lacking a synthetic capacity.

At the end of 72-hours, control and treatment animals were euthanized with 0.25 g l⁻¹ MS-222 dissolved in a seawater bath one at a time and subsequently cervically transected. The liver was weighed and blood plasma separated by centrifugation at 10,000 rpm for three minutes. Blood plasma, white and red muscle, and liver were flash frozen in liquid nitrogen and stored in a -80 °C freezer for later analysis. Terminal samples were collected in triplicate.

2.3. Analyses

Samples were homogenized 1:5 in 5% trichloroacetic acid (TCA) with a glass homogenizer or mortar and pestle on ice. Supernatant was obtained by centrifuging at 10,000 rpm for five minutes. TMAO was measured in duplicate using the ferrous sulfate/EDTA method described by Wekell and Barnett (1991). Homogenates were run in triplicate for urea using diacetylmonoxime (Rahmatullah and Boyde, 1980).

Soluble protein was extracted from white muscle tissue by homogenization in a buffer containing 50mM Tris-HCl (pH 7.5), 2% sodium-dodecyl sulfate (SDS), and protease inhibitor cocktail (88666 ThermoFisher Scientific). Following homogenization, sample protein concentrations were determined by the bicinchoninic

acid (BCA) assay (BCA-1KT Sigma Aldrich). Samples (15µg) were separated by SDS-PAGE using 4-12% Bis-Tris 10-well acrylamide gradient gels and transferred to a nitrocellulose membrane. Samples were immunoblotted with rabbit polyclonal anti-Hsp70/Hsc70 primary antibodies (Agrisera, AS05 083A) and goat anti-rabbit IgG secondary antibodies (Invitrogen, G-21234) at 1:10000 dilution. Hsp70 bands were detected with SuperSignal West Femto Chemiluminescent substrate (Thermo Scientific 34095). Specificity was not tested as only minor non-specific bands distinguishable from 70 kDa were detected. Images were captured using autoradiography film (Life Technologies) and band intensities quantified through densitometry analysis using ImageJ. Bands were normalized to a *S. acanthias* control sample run adjacent to each set of samples included in the analysis.

2.4. Statistics

Time points within control groups were compared using one-way paired student's t-test. Where sample sizes differed between time points, data were compared using one-way unpaired student's t-test. Time points within treatment groups were compared using one-way RM ANOVA with Holm-Sidak post-hoc test. Tissue comparisons between treatments within species were determined using one-way unpaired student's t-test. Linear regression was performed to assess variable scaling with mass. Significance was set at $p < 0.05$. All analyses and graphs were generated with GraphPad Prism 7.0.

3. Results

3.1. TMAO

After comparing two temperature treatments, 15°C and 21°C, we observed no significant difference in plasma TMAO content. Plasma TMAO was 74.43 ± 4.42 mmol kg⁻¹ and 76.41 ± 10.10 mmol kg⁻¹ between 0 and 72 hours in control *S. acanthias*. Plasma TMAO was 77.08 ± 1.72 mmol kg⁻¹, 68.54 ± 4.58 mmol kg⁻¹ and 74.42 ± 3.15 mmol kg⁻¹ between 2, 20 and 72 hours in treated *S. acanthias* (Fig. 1). Plasma TMAO was 64.52 ± 2.05 mmol kg⁻¹ and 68.21 ± 4.33 mmol kg⁻¹ between 0 and 72 hours in control *M. canis*. Plasma TMAO was 60.61 ± 1.8 mmol kg⁻¹, 58.76 ± 1.64 mmol kg⁻¹ and 67.54 ± 5.46 mmol kg⁻¹ at 2, 20 and 72 hours in treated *M. canis* (Fig. 1).

Similarly, there was no significant difference between treatments in white muscle TMAO. White muscle TMAO was 162.30 ± 5.98 mmol kg⁻¹ at 72 hours in control *S. acanthias*. White muscle TMAO was 139.61 ± 5.42 mmol kg⁻¹, 129.57 ± 11.71 mmol kg⁻¹ and 166.98 ± 11.87 mmol kg⁻¹ at times 2, 20 and 72 hours in treated *S. acanthias* (Fig. 2). White muscle TMAO was 179.51 ± 9.60 mmol kg⁻¹ at 72 hours in control *M. canis*. White muscle TMAO was 153.48 ± 4.96 mmol kg⁻¹, 147.58 ± 4.72 mmol kg⁻¹ and 188.30 ± 5.69 mmol kg⁻¹ at 2, 20 and 72 hours in treated *M. canis*. White muscle TMAO increased significantly in treated *M. canis* from 2 to 72 hours ($p < 0.0001$, Fig. 2).

TMAO was not significantly different between 15°C and 21°C for any tissue in *S. acanthias* or *M. canis* at 72 hours. *S. acanthias* TMAO decreased from 37.27 mmol

kg⁻¹ to 10.01 mmol kg⁻¹ as liver weight increased from 87.37 to 432.40 g. The scaling relationship was defined by $y = -0.06856x + 39.81$ with an r^2 of 0.59 and $p = 0.0012$ (Fig. 3). Liver TMAO was corrected to a common weight of 250 g in *S. acanthias* before further analysis. Plasma TMAO was 74.67 ± 5.07 mmol kg⁻¹ in control and 72.41 ± 3.36 mmol kg⁻¹ in treated *S. acanthias*. White muscle TMAO was 165.88 ± 4.74 mmol kg⁻¹ in control and 157.84 ± 7.86 mmol kg⁻¹ in treated *S. acanthias*. Red muscle TMAO was 101.72 ± 6.45 mmol kg⁻¹ in control and 97.10 ± 9.77 mmol kg⁻¹ in treated *S. acanthias*. Liver TMAO was 21.37 ± 4.38 mmol kg⁻¹ in control and 24.04 ± 3.02 in treated *S. acanthias* (Fig. 4A). Plasma and white muscle TMAO for control and treated *M. canis* at 72 hours are the same as reported above. Red muscle TMAO was 123.21 ± 5.87 mmol kg⁻¹ in control and 118.15 ± 3.10 mmol kg⁻¹ in treated *M. canis*. Liver TMAO was 41.28 ± 1.61 in control and 46.83 ± 3.02 in treated *M. canis* (Fig. 4B).

3.2. Urea

There was no significant difference in urea concentration between treatments within tissue types for either *S. acanthias* or *M. canis* at 72 hours. Urea was around 400 mM in all tissues (plasma, white and red muscle) except liver where it was closer to 200-300 mM. Plasma urea was 387.37 ± 27.23 mM in control and 407.60 ± 20.25 mM in treated *S. acanthias*. White muscle urea was 378.84 ± 22.93 mM in control and 388.54 ± 10.37 mM in treated *S. acanthias*. Red muscle urea was 429.75 ± 22.08 mM in control and 391.25 ± 24.62 mM in treated *S. acanthias*. Liver urea was 185.61 ± 13.41 mM in control and 185.61 ± 13.41 mM in treated *S. acanthias* (Fig. 5A). Plasma urea was 407.77 ± 12.71 mM in control and 363.51 ± 20.14 mM in treated *M. canis*.

White muscle urea was 404.47 ± 24.30 mM in control and 379.88 ± 21.01 mM in treated *M. canis*. Red muscle was 405.34 ± 27.19 mM in control and 384.23 ± 11.37 mM in treated *M. canis*. Liver urea was 265.04 ± 22.88 mM in control and 297.39 ± 25.31 mM in treated *M. canis* (Fig. 5B).

3.3. HSP70

There was no significant difference in HSP70 between 15°C and 21°C white muscle in *M. canis* (Fig. 6A and C) or between either *M. canis* group and *S. acanthias* 15°C control. In contrast, 21°C *S. acanthias* exhibited an almost 3 fold higher relative HSP70 concentration than 15°C individuals at 72 hours (Fig. 6B and D, $p=0.0008$).

Discussion

Both *S. acanthias* and *M. canis* are acutely temperature sensitive with seasonal thermal fluctuations regulating migration patterns both in - offshore and latitudinally (Bigelow and Schroeder, 1948; Compagno, 1984; Rountree and Able, 1996; Stehlik, 2007; Ulrich et al, 2007). Adult *S. acanthias* in the northwest Atlantic express a thermal preference of ~10-11°C (McMillan and Morse, 1999; Stehlik, 2007) with catches occurring at temperatures as low as 6°C (Stehlik, 2007). This population of *S. acanthias* was found off the coast of South Carolina within a range of 10.5 – 29.1°C and exhibited a mean catch temperature of 15.45°C. *S. acanthias* appeared in this area when temperatures dropped to 13°C and departed when temperatures reached 19°C (Ulrich et al., 2007).

Catch rates for the northwest Atlantic population of *M. canis* decline significantly at temperatures above 21°C (Skomal, 2007). *M. canis* was shown to occur within a narrower thermal range of 12.2 - 24.5°C with a mean of 17.72°C. Temperature dictated migration in this region with *M. canis* arriving when temperatures dropped to 18°C and disappearing when temperatures rose above 19°C (Ulrich et al., 2007). The inner waters of estuaries serve as nurseries for this species (Skomal, 2007), and likely represent the upper end of their recognized thermal range. These upper temperatures do not accurately describe the preferred habitat of adults that are found deeper and further offshore - up to depths greater than 300m (Zagaglia et al., 2011).

Both species co-occurred in this study. The capture site (41°26.3' N, 71°25.4' W) in Narragansett Bay, RI had May – September bottom temperatures

ranging from 7.54°C to 21.09°C and averaging 16.19°C. Although adult *M. canis* is found further inside the bay than *S. acanthias* (personal observation), surface temperatures in this area average 16-17°C in spring-summer (Collie et al., 2008), with temperatures experienced by these demersal species notably lower. The northwest Atlantic population of *M. canis* seems to prefer temperatures slightly above those of *S. acanthias* but both species favor temperatures well below 21°C. Based on previous reports of these populations' recognized thermal range and tolerance as well as our own observations, both species are likely stressed at the treatment temperature of 21°C.

Previous authors have shown accumulation of both TMAO and HSP70 with elevated temperature stress. A recent in vitro study showed intracellular transport and accumulation of TMAO with further suppression of the HSP70 heat shock response in *S. acanthias* red blood cells (Kolhatkar et al., 2014). At the organismal level, we find no evidence of plasma or tissue TMAO accumulation in 21°C treated individuals relative to 15°C controls in these two shark species (Fig. 1, 2, 4). Although TMAO increased over time in *M. canis* 21°C white muscle (Fig. 2), treated individuals did not exhibit higher TMAO than 15°C controls and thus this increase cannot be directly attributed to a temperature effect. If these sharks do induce TMAO in response to elevated temperature, 72 hours may not have been long enough for the increase to become apparent; although elasmobranch red blood cells in culture were able to accumulate TMAO from the external medium within two hours of encountering thermal stress (Kolhatkar et al., 2014). The dynamics regulating these interactions may be more complex at the organismal level or the +11°C employed in the Kolhatkar

study may have elicited a stronger cellular response than the +6°C administered here. Further, Kolhatkar targeted an elevated temperature of 24°C compared to our 21°C, a condition deviating more significantly from this species thermal optimum and possibly resulting in a higher level of treatment stress.

Elasmobranchs with no endogenous synthetic capacity for TMAO are dependent on dietary contributions for maintenance and accumulation (Treberg and Driedzic, 2002; Treberg et al., 2006). Although food was offered during the experiment, consumption was suppressed in some heat stressed individuals (personal observation), which would have limited availability of dietary TMAO for accumulation. This may have contributed to the lack of increase at 21°C in nonsynthesizing *S. acanthias* (Fig. 4A). In fact, the repressed feed response at elevated temperature is problematic as recent evidence suggests all elasmobranchs depend on dietary contributions for TMAO maintenance regardless of synthetic capacity (Bockus and Seibel, in prep). This could further explain the lack of accumulation with elevated temperature in either species (Fig. 4A-B). However, plasma TMAO spikes 20 hours postprandially (Wood et al., 2010) providing ample time for assimilation in individuals that did feed.

Another possibility is that TMAO accumulation is not initiated as a thermal protective mechanism at the whole organism level, with preferential regulation of other cytoprotective pathways. Accumulation may be restricted by the high levels of urea found in elasmobranch tissue (Fig. 5A-B). Urea is retained in elasmobranchs as a major osmolyte and their primary form of nitrogenous waste (Forster and Goldstein, 1976; Withers, 1998; Trischitta et al., 2012). Here, urea averaged ~400 mM in plasma

and muscle of these species, levels well established in the literature for marine elasmobranchs (Kempton, 1953; Walsh et al., 1994; Wood et al., 2015). Regulation of urea and TMAO is tightly coupled as the two act in an additive capacity for optimal protein stabilization (Mello and Barrick, 2003) and are generally found in a 2:1 ratio of urea to TMAO + other stabilizing osmolytes (Yancey and Somero, 1979). The high concentration of urea in elasmobranch cells may diminish their capacity to adjust TMAO to combat alternate stressors due to co-regulation of these compounds. In comparison, ammonotelic teleost fishes have the ability to synthesize urea, particularly during early development (LeMoine and Walsh, 2013), but retain negligible levels (Wood et al., 1995; Raymond, 1998; Wood et al., 2015). These species may serve as better models for future studies examining TMAO regulatory processes in response to environmental perturbations.

Like TMAO, HSP70 is a known component of the stress response that acts on diverse protein substrates to protect them from inactivation. Elevated HSP70 levels elicited by increasing temperatures have also been shown to provide additional protection against subsequent stresses such as exposure to environmental pollutants (Padmini and Rani, 2008) and ammonia (Sung et al., 2014). Surprisingly, no increase in HSP70 was observed in *M. canis* at 21°C compared to 15°C. However, we observed significantly higher levels of HSP70 in white muscle of *S. acanthias* at 21°C compared to 15°C (Fig. 6). Although *S. acanthias* may transiently experience temperatures in the mid to upper 20s in the wild, the present study shows they are temperature stressed at 21°C, further supported by previous catch data and adults preference for cooler, deeper waters (Stehlik, 2007). As TMAO was not shown to

increase in either species (hypothetically with the ability to combat the HSP70 response), the question remains as to why HSP70 increased in *S. acanthias* but not *M. canis*.

M. canis is not likely more temperature tolerant than *S. acanthias*, based on this species' narrower thermal range. Therefore, the lack of HSP70 accumulation in *M. canis* at 21°C is surprising. As has been shown to occur in Antarctic ice fishes (Place et al., 2004; Place and Hofmann, 2005), constitutively high expression of inducible HSP70 in *M. canis* may impart partial protection against a rise in temperature. Although 15°C *M. canis* HSP70 levels were not significantly higher than 15°C *S. acanthias*, relative expression was around 1.0 compared to 0.5 respectively (Fig. 6). Further, the methods used in this study did not differentiate between constitutive and inducible isoforms, which would provide a clearer understanding of innate differences between these species.

Aside from the regulation of HSPs, *M. canis* may combat thermal stress using different mechanisms altogether. A number of other osmolytes are categorized as “counteracting” solutes (Yancey, 2005) and have the ability to protect cellular function against a variety of stressors, including thermal fluctuations. These include certain methylamines, carbohydrates and amino acids; although TMAO is one of the most effective stabilizers, lowering the K_m of NADH under elevated pressure more than betaine, myo-inositol or glycine (Yancey et al., 2004). Organisms express a wide array of these compounds (Yancey, 2005) with preferential accumulation changing by clade. Some cephalopods and molluscs retain betaine as their primary osmolyte, while crustaceans and decapods accumulate glycine, and euphausiids and fishes express high

levels of TMAO (Carr et al., 1996). However, significant levels of TMAO and other osmolytes, such as taurine, are also retained in many of these organisms.

Accumulation of other protein stabilizers in response to thermal stress and their cooperation with HSP70 is an understudied alternative in need of further investigation. Here, we show no increase in plasma or tissue TMAO in response to thermal stress. These data are in contrast to previous in vitro studies showing elevated TMAO and suppressed accumulation of HSP70. Additionally, our observation of elevated HSP70 in *S. acanthias* at 21°C demonstrates that innate muscle TMAO (around 180 mmol kg⁻¹ in both species) is not sufficient to combat the destabilizing effects of elevated temperature in these elasmobranchs. Although it is possible that the TMAO and HSP70 responses are effected differently by elevated temperature and the pathways are more independent than previously thought. Further, we show differences in these species HSP70 response at 21°C. A more detailed study of the factors contributing to these interactions is warranted. HSP70 genes do not have introns in fishes (Molina et al., 2000) and upregulation can occur in a matter of minutes. However, evidence suggests changes in TMAO may take 2 to 20 hours depending on availability of substrates for synthesis vs. absorption from the diet (Wood et al., 2010; Kolhatkar et al., 2014). TMAO regulation may be further limited by ecological availability of prey items and coupling to urea in ureosmotic organisms. Much remains to be done to elucidate the role of alternate counteracting solutes in the thermal stress response and how these cytoprotectants work in concert in elasmobranchs.

Figure 1. Plasma TMAO (mmol kg⁻¹) during 72 hours at control and elevated temperature. 15°C control (n=7 at time 0, n=3 at time 72) and 21°C treated (n=4) *Squalus acanthias* and 15°C control (n=8) and 21°C treated (n=8) *Mustelus canis*.

Data presented as means $\pm$ SEM. No significant differences found between treatments within species (one-way unpaired student's t-test) or between time points within treatments (one-way RM ANOVA or one-way paired student's t-test, $p < 0.05$).

Figure 2. White muscle TMAO (mmol kg⁻¹) during 72 hours at control and elevated temperature. 15°C control (n=3) and 21°C treated (n=4) *Squalus acanthias* and 15°C control (n=8) and 21°C treated (n=8) *Mustelus canis*. Data presented as means $\pm$ SEM. * indicates a significant difference from 2 hours within a treatment group (one-way RM ANOVA, $p < 0.05$). No significant differences between treatments within species (one-way unpaired student's t-test, $p < 0.05$).

Figure 3. TMAO content (mmol kg⁻¹) decreases with increasing liver weight in *Squalus acanthias*. As liver mass increased TMAO significantly decreased in *S. acanthias* (n=14). Linear regression $y = -0.06856x + 39.81$, $r^2 = 0.59$, $p = 0.0012$.

Figure 4A-B. Tissue TMAO (mmol kg⁻¹) at control and elevated temperature in *Squalus acanthias* and *Mustelus canis* at 72 hours. A) Plasma, white muscle and liver in 15°C control (n=6) and 21°C treated (n=8) *S. acanthias*. Red muscle control (n=3) and treated (n=4) values also included. B) 15°C control (n=7 plasma and liver, n=8 white and red muscle) and 21°C treated (n=8) *M. canis*. Data presented as means

± SEM. No significant differences between treatments within tissue types (unpaired student's t-test, $p < 0.05$).

Figure 5A-B. Tissue urea (mM) at control and elevated temperature in *Squalus acanthias* and *Mustelus canis* at 72 hours. A) Plasma, white muscle and liver in 15°C control (n=6) and 21°C treated (n=8) *S. acanthias*. Red muscle control (n=3) and treated (n=4) values also shown. B) Plasma, white and red muscle, and liver for 15°C control (n=8) and 21°C treated (n=8) *M. canis*. Data presented as means $\pm$ SEM. No significant differences between treatments within tissue types (unpaired student's t-test, $p < 0.05$).

Figure 6. Hsp70 accumulation in 15°C control and 21°C treated *Mustelus canis* and *Squalus acanthias* at 72 hours. Quantitation of Hsp70 in white muscle tissue by densitometry (A and B) and corresponding Hsp70 immunoblots (C and D) for *M. canis* (A and C, n=4) and *S. acanthias* (B and D, n=4) held at 15°C and 21°C. Relative Hsp70 expression (15 µg total protein per lane) was not significantly different between 15°C control and 21°C *M. canis* or between either *M. canis* group and *S. acanthias* control. However, 21°C treated *S. acanthias* was higher than *S. acanthias* 15°C control (two-tailed student's t-test, p=0.0008).

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APPENDIX

ONTOGENETIC OSMOTIC SHIFT IN SPINY DOGFISH, *SQUALUS ACANTHIAS*

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Squalus acanthias

Abstract

As osmoconformers, elasmobranchs possess a suite of osmolytes to maintain water and solute balance. Previous studies have found interspecific differences in the ability of developing elasmobranch embryos to iono- and osmoregulate, which have been largely attributed to alternate reproductive strategies. However, most work has focused on plasma urea and ions while TMAO, the second most common intracellular osmolyte, and tissue osmotic composition have largely been ignored. Here, we provide tissue values for urea and TMAO in late term embryos and neonates of the ovoviviparous spiny dogfish, *Squalus acanthias*. We also present the first recorded tissue osmotic pressure for pups of this species. Our data show that although osmolarity is consistent with adult values, the two primary osmolytes are significantly lower, suggesting a developmental shift in the major osmotic constituents. These findings are in direct contrast with previously published data in *Raja erinacea*, pointing to further divergence in the early osmotic strategies of different elasmobranch groups.

Introduction

Elasmobranchs (sharks, skates and rays) retain a suite of molecules, including sugars, polyols, free amino acids, methylamine compounds and urea, to roughly match the osmotic strength of their tissues to that of seawater (~1,000 mOsm), an osmoregulatory strategy referred to as osmoconformation. These constituents differ in their interactive properties within the cell, and have been categorized as perturbing, compatible (having a minimal macromolecular effect) and counteracting solutes, which stabilize proteins that may be otherwise denatured by cellular or external stressors (Yancey, 2005).

Unlike other marine animals, most elasmobranchs studied to date retain urea, a strong destabilizing agent, as their primary osmolyte. Urea concentrations may reach 400 mM, and account for almost half an individual's osmotic pressure; values far above the threshold needed to impart protein denaturation. To offset urea's perturbing effects, elasmobranchs also accumulate large concentrations of the counteracting solute trimethylamine oxide (TMAO). TMAO content is tightly correlated to accumulation of urea and organisms generally exhibit a ratio of 2:1 Urea:TMAO + other methylamines (Yancey and Somero, 1979), making TMAO the second most common intracellular osmolyte in these organisms.

Three distinct modes of reproduction - oviparity, ovoviparity and viviparity - are present in elasmobranchs and development of the osmoregulatory system in the embryo is hypothesized to depend on the composition of the medium surrounding the egg and, consequently, on the reproductive strategy employed by the species (Kormanik, 1993). The enzymes necessary for urea synthesis are functional early in

both shark and skate ontogeny (Read, 1968) and plasma values reported for late-term embryos suggest a fully developed osmoregulatory capacity (Price and Daiber, 1967; Read, 1968; Evans et al., 1982). However, plasma osmolytes may not represent the osmotic condition of the entire individual. Osmolyte values in embryonic tissue have not, with few exceptions (Read, 1968b; Kormanik et al., 1992; Steele et al., 2004), been previously studied and only one of these conducted in an ovoviviparous species (Kormanik et al., 1992).

In this study, we present osmolyte values, including urea and TMAO, for the muscle tissue and yolk of late term embryos and neonates (less than 24 hours old, hereafter combined and referred to as pups) as well as adults of the ovoviviparous spiny dogfish, *Squalus acanthias*. We sought to determine how the intracellular osmotic state changes through ontogeny and how it compares to literature values of elasmobranch species differing in their reproductive strategies.

Materials and Methods

Sample collection

Adult male and female spiny dogfish (*Squalus acanthias*) were caught in summers 2013 and 2014 in Narragansett Bay, Rhode Island by otter trawl off the F/V *Virginia Marise*. Animals were provided with ventilated, chilled seawater and transported to the seawater facility at the Graduate School of Oceanography, University of Rhode Island. Individuals were kept in 2850 l continuous flow circular holding tanks up to $n = 5$. Holding facilities were provided with course filtered seawater and temperature maintained at 15°C for the duration of the experiment. Adults were held up to two months and fed a mixed diet of herring and squid twice weekly at 2.5% body weight. Neonate dogfish (younger than 24 hours) were berthed in captivity and late term embryos dissected live from *in utero*. As no significant variability was found between pups collected by these various methods all data have been combined. Only late term (>30 cm, yolk sac still present) or fully developed (no yolk sac) individuals were used in this study. Due to collection methodology, it was not possible to discriminate between the two early ontogenetic life stages during analysis.

All animals were euthanized with MS-222 (0.15 g l⁻¹ seawater) in accordance with IACUC #AN13-05-020. White muscle was excised from the dorsal epaxial of adult ($n = 11$) and pup ($n = 37$) spiny dogfish and immediately flash frozen in liquid nitrogen for later analysis. When yolk sacs were present, they were also removed and similarly frozen.

Laboratory analyses

Tissue was homogenized 1:5 (wet weight:volume) in 5% saturated trichloroacetic acid. Homogenates were then assayed for TMAO by the ferrous-sulfate method whereby concentration is determined spectrophotometrically by a colorimetric reaction between reduced TMAO and 2% picric acid (Wekell and Barnett, 1991). TMA is generally very low in marine elasmobranchs ($< 2 \text{ mmol kg}^{-1}$) and no corrections for endogenous TMA have been made. Homogenates were further analyzed for urea, again a colorimetric reaction that spectrophotometrically determines concentration against known standards (Rahmatullah and Boyde, 1980). Fresh muscle tissue was homogenized 1:1 in deionized water and total osmolarity (mOsm) calculated using the freezing point of the solution as determined by the automatic osmometer micro-osmometer model 5004 (Precision Systems Inc.). Group means $\pm$ s.e.m. were compared with two-way Student's t-tests and statistics and graphs generated using GraphPad Prism 7.0. Student's t-test for osmolarity was run with Welch's correction for unequal variance.

Results and Discussion

Adult spiny dogfish, *S. acanthias*, exhibited an average tissue TMAO content of $154.84 \pm 7.43 \text{ mmol kg}^{-1}$ wet wt. In contrast, pups had an average TMAO content of only $42.13 \pm 2.98 \text{ mmol kg}^{-1}$, more than 3.5 times lower than the adult average ($p < 0.0001$) and slightly lower than the whole embryo content of $67.8 \text{ mmol kg}^{-1}$ reported by Kormanik et al. (1992). Urea followed a similar trend with adult values averaging $463.70 \pm 28.00 \text{ mmol kg}^{-1}$ wet wt. and pup concentrations less than half that ($220.95 \pm 15.21 \text{ mmol kg}^{-1}$; $p < 0.0001$, Fig. 1). Yolk TMAO and urea were $58.41 \pm 2.99 \text{ mmol kg}^{-1}$ and $178.13 \pm 10.34 \text{ mmol kg}^{-1}$ respectively, similar to pup tissue values.

The discrepancy between adult and pup values of the two primary osmolytes reported here in *Squalus acanthias* is in contrast to the situation described for skates. Steele et al. (2004) showed little skate, *Raja erinacea*, embryos and adults contain similar concentrations of both osmolytes as early as four months after oviposition and well before the embryo hatches from the egg case at nine months. Big skate, *Raja binoculata*, embryos were also shown to exhibit urea at similar concentrations to adults with no change in urea or TMAO through development (Read, 1968b). The species specific differences between groups may be attributed to alternate modes of reproduction in the ovoviviparous spiny dogfish compared to the oviparous skates. The majority of literature in this area has concluded that early osmoregulatory ability in elasmobranchs can best be ascribed to reproductive strategy (Price and Daiber, 1967; Evans et al., 1982; Kormanik, 1992).

The embryos of oviparous species develop inside a highly permeable egg case deposited on the sea floor, subjecting the developing embryo to full strength seawater

and requiring a working osmotic system soon after fertilization (Price and Daiber, 1967; Kormanik, 1989). Ovoviviparous species on the other hand begin in egg cases, which hatch inside the mother where the embryos finish developing *in utero*. The uterine environment during the first part of development bathes the embryo in a solution similar to the composition of maternal plasma establishing low salt and urea gradients and relaxing pressure on the osmoregulatory needs of the early individual. During the second part of development, the uterine fluid undergoes a compositional change and the embryo is surrounded by a medium with characteristic similarities to seawater (Kormanik and Evans, 1986). At this time the individual must be able to fully osmoregulate in order to maintain water and solute balance (Evans et al., 1982). Therefore, ovoviviparous species have a delayed requirement for osmoregulatory development based on ionic and osmotic gradients established by the surrounding medium. Lastly, viviparous species complete development inside a placental environment with fluid resembling the composition of maternal plasma, greatly reducing the need for embryonic ionic and osmotic regulation (see Price and Daiber, 1967; Kormanik, 1992; Kormanik, 1993 for review).

These differences in embryonic environment readily explain why the oviparous little skate would exhibit early osmoregulatory development. It may also explain why near-term spiny dogfish display a fully operational osmoregulatory system (Kormanik, 1992). In accordance with this view, we found the tissue osmotic strength of the spiny dogfish pup to be 943.3 mOsm and closely match that of adults at 1,009 mOsm ($p = 0.3112$, Fig. 2) and the expected value of seawater around 1,000 mOsm. Therefore, although the two most important osmolytes, urea and TMAO, were found at

concentrations significantly lower than expected, total osmolarity is enough to maintain osmotic balance against seawater. These data suggest a large shift in tissue osmolyte composition between late term / early life pups and adult spiny dogfish, during which urea and TMAO values progressively increase, while some other osmolytes or salts decrease, to adult levels.

Low pup TMAO contents may be explained by limited availability of the compound itself. Spiny dogfish do not exhibit the enzyme responsible for endogenous TMAO synthesis (Treberg et al., 2006) and rely to an extent on absorption from the diet (Treberg and Driedzic, 2002; Bockus and Seibel, in prep); possibly limiting accumulation until the pup has eaten its first meal. This limitation may also facilitate the concomitant observation of low urea in these pups due to a limited counteracting capacity. As mentioned above, optimal protein stabilization is achieved by a 2:1 ratio of urea to TMAO + other methylamines and previous studies have shown *R. erinacea* embryos to maintain this ratio early in embryogenesis (Steele et al., 2004). If TMAO is limiting, perhaps accumulation of urea is restricted until the cell is able to protect against its destabilizing properties via TMAO (or other counteracting solute) accumulation. Steele et al. (2004) also showed *R. erinacea* embryos to rely more heavily on TMAO than other counteracting solutes during embryonic development, further supporting the possibility that dietary regulation of TMAO may be limiting urea accumulation.

We show that pups contain significantly lower tissue urea and TMAO than adults but exhibit a similar total osmotic pressure. These findings imply a large shift in the cellular osmolyte constituents present between birth and adulthood, during which

urea and TMAO increase significantly and take over as primary osmolytes; a shift that may impose additional restrictions on this group. For example, low levels of TMAO, which is the most effective counteracting solute present in the tissue, due to possible accumulation restrictions in early ontogenetic stages may render pups particularly susceptible to environmental stress. Our findings also raise an important question regarding which solute (or solutes), if not urea and TMAO, act as the primary osmolyte in pup spiny dogfish. Additionally, this data is in contrast to the oviparous species previously studied, which retain a similar osmolyte milieu through development and into adulthood. This suggests divergence in the osmotic mechanisms employed by the early life individuals of different elasmobranch groups and confirms the need to differentiate developmental osmoregulatory data based on reproductive strategy.

Figure 1. Primary osmolytes of adult and pup *Squalus acanthias*. TMAO (left Y-axis) and urea concentrations (right Y-axis) were compared in the white muscle of adult and pup spiny dogfish (*S. acanthias*). All values reported as mean $\pm$ s.e.m. and analyzed with two-way Student's t-tests. Adult dogfish (n = 9) exhibited 3.5 fold higher TMAO (mmol kg⁻¹ wet wt.) than dogfish pups (n = 28) and urea concentrations (mmol kg⁻¹) in adults (n = 11) were twice those seen in similar pups (n = 37). TMAO was significantly lower in the white muscle of *S. acanthias* pups (p < 0.0001) than the white muscle of adults, with urea also accumulating at significantly lower concentrations (p < 0.0001). Yolk TMAO (n = 10) and urea (n = 12) values also shown.

Figure 2. Total osmolarity (mOsm) of adult and pup *Squalus acanthias*. Total tissue osmolarity of white muscle in mature spiny dogfish ($n = 8$) and pups ($n = 7$). Values reported as means $\pm$ s.e.m. and analyzed with a two-way Student's t-test with Welch's correction for unequal variance. Osmolarity was not significantly different ($p = 0.3112$) between the two and roughly matched the expected osmotic strength of seawater.

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