THE ACCUMULATION, SYNTHETIC CAPACITY AND INTERTISSUE DISTRIBUTION OF TRIMETHYLAMINE OXIDE IN DEEP-SEA FISH AND THE COLD ADAPTED SMELT (Osmerus mordax)

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by

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Abstract

Trimethylamine oxide (TMAO) is common to most marine fishes; however, the role TMAO plays in the physiology of marine fish is not well understood. I have used two distinct TMAO accumulating fish 'types', deep-sea fish and smelt (*Osmerus mordax*), to compare differences in the levels, intertissue distribution and capacity for synthesis of TMAO in fish with high and low levels of TMAO. Several consistencies were found. The intertissue distribution of TMAO showed a trend of locomotory muscle > heart > liver ≅ kidney ≅ brain. Levels of trimethylamine oxidase, the enzyme required for TMAO synthesis, did not correlate with higher tissue TMAO content indicating that enhanced endogenous synthetic capacity is not responsible for elevated TMAO content . Finally, evidence for the active uptake of TMAO into striated muscle and the regulation of TMAO concentration in white muscle is presented, possibly due to some role TMAO plays in muscle function.

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EDTA: Ethylenediaminetetraacetic acid

FAD: Flavin adenine dinucleotide (oxidized form)

$FADH_2$: Flavin adenine dinucleotide (reduced form)

FMO: Flavin containing monooxygenase

K_m: Michelis constant (substrate or cofactor concentration that gives ½ maximal reaction velocity)

NADH: Nicotinamide adenine dinucleotide (reduced form)

$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidized form)

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)

TCA: Trichloroacetic acid

TMA: Trimethylamine

TMAO: Trimethylamine oxide

TMAoxi: Trimethylamine oxidase

U/hr: μmoles of substrate converted (gram wet tissue weight)$^{-1}$ hour^{-1}

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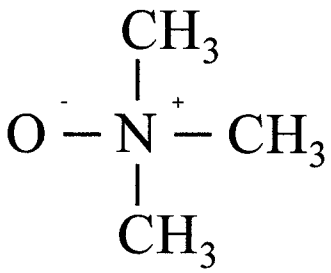
Chapter 1: Overview of the occurrence, metabolism and significance of TMAO in fish.

1.1 Introduction

The muscle of marine fishes has substantial trimethylamine oxide (TMAO) content, varying with species and osmoregulatory strategy. TMAO is a small organic nitrogenous solute (Fig. 1.1); however, little is known about how and why marine fishes accumulate TMAO. This chapter will provide a basic background on the occurrence, metabolism, and potential physiological roles of TMAO in fish. A comprehensive review of the occurrence of TMAO in fish is well beyond the scope of this work (see Hebard et al. 1982 for review); however, the general trends in TMAO levels in teleost and elasmobranch fishes as well as what may affect levels of TMAO is presented as this background is required for the interpretation of the following chapters.

Figure 1.1 Structure of trimethylamine oxide



1.2 Occurrence of TMAO

1.2.1 Teleosts

Marine teleosts typically have between 20-90 mmol/kg TMAO in white muscle. The gadiforms (cods and cod-like fishes) have higher muscle TMAO (40-90 mmol/kg) than most other groups (20-60 mmol/kg). Flatfishes have low to moderate levels, while the highly active tuna and mackerels have low levels. Salmoniformes (salmon and trout) have very low levels (< 15 mmol/kg). TMAO content appears to increase with the size of the fish and thus, presumably, with increasing age (Dyer 1952, Shewan 1951). The red muscle of many species has less TMAO than white muscle; however, in highly active species, such as mackerel and tunas, red muscle has higher TMAO content (Hebard et al. 1982). Non-muscle tissues in marine teleosts typically have very low TMAO (<15 mmol/kg).

1.2.2 Elasmobranchs

The marine elasmobranchs (sharks, skates and rays) have high tissue and plasma TMAO. Levels in plasma typically range from 30 to 90 mM, while muscle content varies from approximately 35 to 180 mmol/kg. These fish are osmoconformers and TMAO, along with urea and amino acids, are important osmolytes for maintaining water/solute balance.

1.3 TMAO metabolism

The metabolism of methylamines is poorly understood in fishes. TMAO appears to be involved in choline and betaine catabolism but, as explained below, it is unclear if most species actively synthesize TMAO. Unfortunately, measurements of the enzymes involved and of the products are relatively difficult and time consuming, further complicating research in this field.

1.3.1 Precursors of TMAO

TMAO is formed by the oxidation of trimethylamine (TMA) by trimethylamine oxidase (TMAoxi). TMA is thought to be toxic while TMAO is not; thus TMAO synthesis was thought to be a mechanism to detoxify TMA (Yamada 1967). It has become clear that the TMA content of the typical marine fish diet is low enough to be easily excreted, creating doubt in the detoxification theory.

Based on the occurrence of TMAoxi, TMAO could be synthesized from TMA by a number of marine organisms, summarized in appendix 1 (Baker et al. 1963, Goldstein and DeWitt Harley 1973, Augustsson and Strom 1981, Raymond 1998, Raymond and

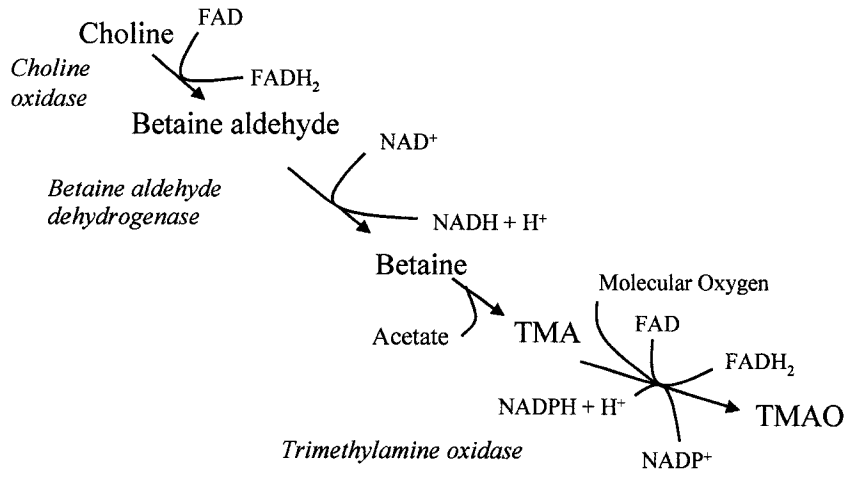


Figure 1.2 Proposed pathway for synthesis of TMAO from choline in fish

DeVries 1998) or from other potential substrates, see appendix 2. In fish, the liver and kidney seem to be the likely sites of synthesis as no other tissues show appreciable TMAoxi activity (Baker et al. 1963, Augustsson and Strom 1981).

The breakdown of choline appears (Fig. 1.2) to be a source of TMAO (Bilinski 1964, Goldstein and Funkhouser 1972, Charest et al. 1988), but presumably catabolism of any trimethylamine-containing compound can result in the liberation of TMA, the immediate precursor to TMAO. In support of this, butyrobetaine is a potentially viable precursor to TMAO, even though not a commonly occurring molecule in fish (Bilinski 1964). Dietary choline is likely in the greatest abundance in the form of phosphatidylcholine, a major phospholipid. The breakdown of phosphatidylcholine results in the formation of choline, which may enter into TMAO synthesis, and diacylglycerol. Recently, a very strong correlation between diacylglycerol content, and TMAO has been found in some molluscs and it has been hypothesized that a similar trend may exist in fish (see Seibel and Walsh 2002 for review). Conversely, bacterial breakdown of choline, but not phosphatidylcholine, in the gut of tilapia has been linked to increased muscle TMAO content (Niizeki et al. 2002).

1.3.2 TMA oxidase

TMAoxi has only been partially characterized and has not been isolated in fish. As in mammals, it is located in the microsomes, uses NADPH as a cofactor and is also FAD linked (Fig. 1.2). This enzyme cannot use NADH as an electron donor and requires dissolved oxygen, but is not inhibited by carbon monoxide (Baker and Chaykin 1962, Baker et al. 1963, Augustsson and Strom 1981). The lack of inhibition by carbon

monoxide indicates that it does not involve cytochrome p450. Based on similar effects of inhibitors, and a broad substrate specificity, it has been suggested that the TMAoxi of fish could be classified as a flavin containing monooxygenase (FMO) (Goldstein and DeWitt Harley 1973, Schlenk and Li-Schlenk 1994). FMO activity has been associated with the oxidation of tertiary amines as well as the breakdown of many nitrogen and sulphur-containing xenobiotics (Ziegler 1988).

An important point is that the presence of TMAoxi is required for endogenous synthesis. However, due to the broad substrate specificity, the possibility that this enzyme may have another or several functions means that the presence of TMAoxi indicates the capacity for TMAO synthesis but is insufficient to conclude that a species is actively synthesizing TMAO. Since TMAO would be readily available in the diet of most marine fish determining if a species actively supplements its TMAO pool by synthesis is difficult and time consuming to determine (see below).

1.3.4 Teleost TMA oxidase

There have been conflicting data on the occurrence of TMAoxi activity, and thus the potential for TMAO synthesis, in various fish groups. In teleosts there appears to be little consistency even within a taxonomic group (Appendix 1). Some pleuronectiformes (flatfishes) appear to have appreciable TMAoxi while others do not. Of note, Baker et al. (1963) could not find detectable amounts of TMAoxi in *Platyichtys stellatus*, while Bilinski (1964) did find appreciable conversion of injected [^{14}C] TMA to [^{14}C] TMAO. This discrepancy has yet to be resolved. Typically, gadiformes (cods) have quite high TMAoxi, flatfishes may have high to undetectable levels and perciformes (perch-like

fishes) have moderate to high activities. Other taxonomic groups have not been adequately surveyed. It is important to note that the number of species tested to date is very low and thus only vague generalizations may be made about the occurrence of TMAoxi in teleosts. Furthermore, different assays are often used, making comparisons between studies difficult.

1.3.5 Elasmobranch TMA oxidase

Some elasmobranch species have quite high activity (Appendix 1) while others lack detectable TMAoxi or have questionable levels of activity. This is best shown by experiments with *Squalus acanthias*. Baker et al. (1963) and Goldstein et al. (1967) were unable to detect TMAoxi activity; however, Goldstein and DeWitt-Harley (1973) did find levels of TMA incorporation into TMAO, which likely bordered on the limits of detection. Schlenk and Li-Schlenk (1994) found that FMO activity, with dimethylaniline as substrate, was competitively inhibited by TMA in microsomes isolated from *S. acanthias*. This suggests that there is TMAoxi activity, albeit non-specific, in *S. acanthias* liver.

Schlenk and Li-Schlenk (1994) suggest that the lack of appreciable activity in *S. acanthias* by previous authors may have been due to interference from the use of whole homogenates or loss of activity during preparation, possibly from temperature-related inactivation. More recent studies have found detectable TMAoxi activity, when measured spectrophometrically at 22°C, in a number of fish species (Raymond 1998, Raymond and DeVries 1998). These assays were linear for an hour, negating the likelihood of temperature inactivation. Furthermore, the previous studies on *S. acanthias*

in question used radiolabelled TMA, not spectrophotometric assays, and should not have appreciable interference from the use of whole homogenates.

1.4 Endogenous or exogenous source for TMAO

There are two potential sources of TMAO in fish, exogenous or dietary, and endogenous synthesis. Ultimately TMA must be oxidized to TMAO enzymatically, via trimethylamine oxidase activity, at some point in the food chain. The lack of detectable enzyme activity indicates a solely exogenous source for TMAO in a given species. Similarily, there are experimental data showing that some fishes require dietary TMAO, or at least TMA, for the accumulation of TMAO. As stated above, the presence of TMAoxi activity does not in itself indicate active TMAO synthesis. Until the enzyme, or enzymes, responsible for tertiary amine oxidation is isolated and properly characterized this will continue to make interpretation of crude tissue enzyme activities difficult.

1.4.1 Teleosts

A number of studies have attempted to determine if TMAO is synthesized by given species or obtained through the diet. However, many studies utilized freshwater species that do not normally accumulate TMAO to appreciable levels (Hebard et al. 1982) and these data are not included. Those studies involving species found in marine waters typically involved euryhaline species that also have low levels of TMAO. Atlantic salmon, *Salmo salar*, have no appreciable TMAO while developing in freshwater where dietary TMAO would be essentially absent, but those caught in marine waters where dietary TMAO would be available do have appreciable muscle TMAO

(Norris and Benoit 1945, Cowey and Parry 1963). Benoit and Norris (1945) found that pink salmon (*Oncorhynchus gorbuscha*) raised in a marine environment only accumulated TMAO when fed a TMAO containing diet. Charest et al. (1988) found that adult pink salmon can convert choline to TMAO *in vivo* suggesting that synthetic capability may have an ontogenic aspect as well.

Work by Okaichi et al. (1959) found that for the globe fish (*Diodon nicthemerus*) and filefish (*Sebastisus marmoratus*), muscle TMAO increased only in fish fed a TMAO-containing diet. This was in contrast to the jack mackerel (*Tarchurus japonicus*) which maintained TMAO levels even if the diet contained no TMAO, suggesting active TMAO synthesis in this species. To my knowledge, no such experiments have been done on species known to have high TMAoxi.

1.4.2 Elasmobranchs

Elasmobranchs maintain high tissue levels of TMAO and thus may present a good model system to study the source of TMAO in fish. Unfortunately, other than the work on TMAoxi in elasmobranchs, very little has been done in this area. Only one study has examined the long-term effects of fasting on TMAO in an elasmobranch (Cohen et al. 1958). These investigators held *S. acanthias* for up to 41 days without food and found that plasma TMAO levels did not decrease over the experiment. The authors attributed this to renal reabsorption of TMAO and slow release from the much larger muscle TMAO pool.

Goldstein and colleagues have made attempts to determine the source of TMAO in elasmobranchs. Goldstein et al. (1967) found that *S. acanthias* could not convert

labeled choline or TMA to TMAO *in vivo*. As stated above, this species seems to lack the capacity to convert TMA to TMAO *in vitro* as well. Another elasmobranch species, *Ginglymostoma cirratium*, has been shown to convert TMA to TMAO *in vivo* from choline and TMA and this species has also shown *in vitro* TMAoxi activity (Goldstein and Funkhowser 1972). Although this is strong evidence for a link between TMAoxi and *in vivo* synthesis, a follow-up paper developed a link between elasmobranch TMAoxi and mammalian FMO activity (Goldstein and DeWitt Harley 1973). Thus, it is still unanswered if this is a case of conversion by a non-specific enzyme that may serve another physiological role or if in fact these fish will actively synthesize TMAO from precursors during periods of dietary limitation.

1.5 Physiological Roles of TMAO

A number of physiological roles have been proposed for TMAO in fish. These include osmoregulation, macromolecular stabilization against various perturbants and colligative antifreeze. It is important to point out that these potential physiological roles are based on the occurrence or levels of TMAO in certain species compared to 'typical' species or environments or on very 'unnatural' *in vitro* experiments. Though this provides circumstantial evidence for some of these roles, there is still much work to do to confirm the many potential functions of TMAO in fish.

1.5.1 Osmoregulation

Osmoregulation involves two compartments, the intracellular and extracellular, and has two basic components, the maintenance of water and osmolyte balance under

normal conditions and the adaptation to changes or stresses to water balance. Due to the intimate contact of gills with the external media, and the high permeability of water, fish are highly susceptible to changes in the external media, which makes water balance difficult or energetically expensive in the case of fish that maintain different total solute concentrations than the external media. Depending on the species, TMAO plays important roles in the intracellular and extracellular osmoregulation as well as the adaptation to different osmotic environments.

The essentially ubiquitous occurrence of TMAO in the muscle of marine fishes may be related to its use as an osmolyte. As an osmolyte, TMAO has many benefits including those elaborated on below in addition to the benefit that as a dead-end metabolite it would not be involved or competing with other metabolic processes, whereas the accumulation of amino acids would have to compete with other amino acid metabolizing systems in the cell.

1.5.1.1 Teleosts

Marine teleosts have low to high amounts of muscle TMAO. Certain shallow living gadiformes have as much as 80 mM TMAO (assuming ~ 70% water content) in their white muscle (Hebard et al. 1982). Body fluid osmolality of these fish is approximately 330-400 mOsm/kg (Holmes and Donaldson 1969). Thus TMAO would make up from 20% to almost 25% of the total solute in the muscle, making TMAO a substantial component of the maintenance osmoregulatory system of white muscle in these fish and the most abundant organic osmolyte in the fish.

Although not well examined, what little data are available suggest that TMAO levels in teleosts are generally low in non-muscle tissues including blood (Hebard et al. 1982). This suggests that TMAO has an intracellular role in the maintenance osmoregulation in the muscle of marine teleosts but does not constitute a significant amount to the total extracellular osmotic pressure. Potential exceptions to this are certain deep-sea gadiform fishes and some teleosts that live in subzero temperatures. These fish have been shown to have elevated plasma or serum TMAO (Raymond 1994, Gillette et al. 1997 Raymond and DeVries 1998) as well as high plasma osmolalities relative to typical marine teleost values and TMAO accounts for a significant amount of the difference in plasma osmolality.

When euryhaline teleosts are acclimated to from seawater to freshwater, they change the osmolality of their body fluids by approximately 40-100 mOsm/kg (Holmes and Donaldson 1969). Lange and Fugillie (1965) demonstrated that, for two euryhaline teleosts, the muscle TMAO significantly decreases, along with amino acids, on the acclimation from 100% to 50% seawater. Thus, TMAO plays an integral role in the adjustment of the muscle fluid in some marine and euryhaline teleosts when adapting to dilute seawater.

1.5.1.2 Elasmobranchs

Unlike teleosts, the role that TMAO plays in the osmoregulation of elasmobranchs has received a substantial amount of study. Marine elasmobranchs are osmoconformers and maintain body fluid osmolalities either near to or slightly greater than that of seawater. The osmoregulatory system of elasmobranchs has been reviewed,

especially the role of organic osmolytes such as TMAO (Pang et al. 1977, Perlman and Goldstein 1988). TMAO appears to play significant roles in the osmoregulation of the intracellular and extracellular compartments. In many species it is a major osmolyte in many tissues thus making a significant contribution to the maintenance osmoregulation and it is involved in the osmotic adjustment, both intracellularly and extracellularly, that occurs with dilution of the external media from full strength seawater (see above references and references therein).

1.5.2 Protein stabilization

The stabilization of proteins by TMAO and similar molecules against perturbants of structure and function has been reviewed (Yancey et al. 1982, Yancey 1994, Gilles 1997). A number of destabilizers of proteins are counteracted by TMAO *in vitro* and many of these 'forces' are of physiological relevance to marine fishes. These include high urea concentrations (which can reach > 400 mM in elasmobranchs), elevated salt concentrations (seen in Antarctic fishes) and high pressure (equivalent to that experienced by deep-sea fishes) (Gillett et al. 1997, Yancey et al. 2001). All of these conditions have been shown to negatively effect protein function or stability and can be counteracted to varying degrees by TMAO.

1.5.3 Buoyancy

Recently it has been demonstrated that the large amounts of urea and methylamines accumulated by marine elasmobranchs may serve another physiological role, buoyancy. Solutions containing urea or TMAO are less dense than seawater. Thus

the accumulation of these solutes instead of amino acids, most of which result in a solution more dense than seawater, would decrease the net density of the animal which would result in less energy expenditure for locomotion (Withers et al. 1994a, Withers et al. 1994b). Since elasmobranchs lack a swim bladder and are typically more dense than seawater this may play a significant role in these fish. Of note, benthic species like skates and stingrays have high levels of urea and TMAO indicating that these solutes have other and perhaps more significant roles that just buoyancy.

Most marine teleosts that have moderate to high TMAO also have a swim bladder. Attaining neutral buoyancy may not be as energetically expensive in these fish as in some elasmobranchs suggesting that TMAO likely does not have a significant buoyancy role in teleosts.

1.5.4 Colligative Antifreeze

Teleost fishes living in waters that may have temperatures below –1.0 °C typically develop some means of freeze avoidance (DeVries 1982). While many use antifreeze proteins, which work by noncolligative properties, many also increase the osmolality of their body fluids as colligative antifreeze (Raymond 1992, Raymond and DeVries 1998). Some Antarctic and northern teleosts accumulate serum TMAO to sufficient amounts to significantly depress the freezing point and thus TMAO may act as a colligative antifreeze in these fish (Raymond 1994, Raymond and DeVries 1998).

1.6 Conclusion

The common occurrence of TMAO in marine fishes has yet to be fully explained. The distribution of TMAO in tissues other than muscle is unclear, poorly documented, and may be crucial in understanding the physiological and biochemical significance of TMAO. As such, the following chapters report the intertissue distribution of TMAO in several species of fish. This is especially important in the argument for counteraction of pressure effects in deep-sea fish, where only muscle levels have been documented. Clearly, TMAO should be elevated in other tissues if it plays an important role in counteracting pressure effects on proteins. This is the subject of Chapter 2.

Rainbow smelt (*Osmerus mordax*) accumulate TMAO in their plasma during the winter when ambient water temperatures drop to near, or that of, the freezing point of seawater (Raymond 1994). It has been suggested that this may act colligatively as a small but significant contribution to the smelt's antifreeze strategy. If this is true, the plasma levels of TMAO should oscillate with the seasonal water temperatures. Furthermore, nothing is known of the effects of changing extracellular TMAO concentrations on total tissue (approximating intracellular) TMAO content. The changing seasonal plasma TMAO concentration, representative of the extracellular fluid, makes the smelt an ideal teleost model for examining the relationship between extracellular and intracellular TMAO concentration in various tissues. The third chapter examines the effect of seasonal, or elevated, temperature on TMAO accumulation in smelt as well as the correlation of plasma TMAO concentration with the TMAO content of several tissues.

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Chapter 2: Elevated levels of trimethylamine oxide in deep-sea fish: Evidence for synthesis and intertissue physiological importance.

2.1 Introduction

Trimethylamine oxide (TMAO) is a nitrogenous organic molecule commonly found in the muscle of marine fishes (reviewed by Hebard et al. 1982); however the importance of this organic osmolyte has yet to be completely explained with regards to adaptation to various environments. In marine elasmobranchs TMAO, and other methylamine compounds, are important osmoregulatory compounds (Perlman and Goldstein 1988) and likely have significant roles in the counteraction of urea (Yancey 1994) and buoyancy (Withers et al. 1994).

The function of TMAO in teleosts is somewhat more obscure. In some euryhaline species it has a substantial role in osmoregulation in muscle (Lange and Fugelli 1965) but this does not explain the high occurrence of TMAO in marine teleosts that do not normally experience large fluctuations in salinity. Recently a number of fish species found in two extreme environmental groups have been shown to have elevated levels of TMAO. These are marine teleosts adapted to near- or sub-zero temperatures (Raymond 1998, Raymond and DeVries 1998, Raymond and Hassel 2000) and deep-sea fishes (Gillett et al. 1997, Kelly and Yancey 1999).

Of the near- and sub-zero degree adapted teleosts examined for TMAO content, several have what appear to be elevated levels in the serum and liver, and muscle in the case of certain Notothenioid species, when compared to the values tabulated in Hebard et

al. (1982). In deep-sea gadiforms, some species have been shown to have elevated plasma TMAO, whereas all fish species examined to date show elevated muscle TMAO in comparison to shallow water temperate species (Gillett et al. 1997, Kelly and Yancey 1999). Furthermore, Kelly and Yancey (1999) found that, in rajid skates, muscle urea decreased and TMAO increased with depth of capture.

The accumulation of TMAO may be of physiological importance to deep-sea fishes to counteract high pressure effects on protein function (Gillett et al. 1997, Yancey and Siebenaller 1999, Yancey et al. 2001). This suggests that TMAO would be elevated in all tissues of deep-sea fish. The present study was conducted to determine if the trend of elevated muscle TMAO found in deep-sea fishes is also extended to non-muscle tissues of deep-sea fishes like in the cold-adapted teleosts examined by Raymond and colleagues.

Raymond (1998) has suggested a correlation between plasma TMAO content and liver trimethylamine oxidase (TMAoxi) activity. TMAoxi catalyses the conversion of trimethylamine (TMA) to TMAO and has been identified in a number of fish species (Baker et al. 1963, Goldstein and Dewitt-Harley 1973, Agustsson and Strom 1981, Raymond 1998, Raymond and DeVries 1998). A logical progression of this hypothesis is that, for elevated tissue levels of TMAO, there would be an expected increase in TMAoxi activity. Furthermore, if TMAO does counteract high-pressure effects on protein function, deep-sea fishes may have an increased synthetic capacity to produce TMAO compared to related shallow living species. I determined if deep-sea fish may have

increased TMAO synthesis by measuring the activity of TMAoxi as well as comparing levels of the substrate, trimethylamine, in fishes caught from different depths.

2.2 Materials and Methods

2.2.1 Animals

Specimens were caught by otter trawl either in the Atlantic waters off Newfoundland or in Passamoquoddy Bay, New Brunswick. Six species of gadiform fish were caught in either shallow (<150 m: *Gadus morhua, Urophycis chuss* and *Merluccius bilinearis*), moderate (450-700m: *Gaidropsarus ensis* and *Macrourus berglax*) or deep water (1000-1500m: *Antimora rostrata*). An additional deep-sea teleost (*Synaphobranchus kaupi*), an anguilliform, was also collected below 1000m. Two elasmobranchs, squaliform sharks, were also sampled; S*qualus acanthias* and *Centroscyllium fabricii*, from shallow and deep water respectively. Sampling was conducted in November 1999, August 2000 and December 2000 for the deep, shallow and moderate depths respectively.

Fish were killed by a blow to the head and blood was drawn into heparinized syringes by caudal puncture. Blood was centrifuged for 3 minutes in an Eppendorf bench-top centrifuge and plasma was frozen as described below. Tissues were collected immediately following blood collection, blotted dry, frozen with liquid nitrogen and stored below -65°C until used. Of note, tissues were not rinsed or perfused prior to freezing and will have a small amount of contaminating blood.

2.2.2 Osmolyte assays

TMA and TMAO were measured by a modification of the ferrous sulfate/EDTA method of Wekell and Barnett (1991) for use with small tissue samples. Tissue samples were weighed and homogenized in 9 volumes of 5% TCA (wt/v) with a Polytron homogenizer. Plasma samples were thawed on ice and diluted 1:10 (v/v) with 5% TCA, mixed briefly and allowed to stand on ice for approximately 1-2 minutes to allow proteins to precipitate. All samples were centrifuged for 5 minutes at 4°C to remove precipitated proteins and cellular debris. Deproteinized samples were then assayed for both TMAO, via reduction by ferrous sulfate and EDTA, and TMA with no reduction step. TMA is extracted into toluene and quantified spectrophotometrically at 410 nm by its colourimetric reaction with picric acid, 0.02% (w/v) in toluene. TMAO is determined by subtracting the TMA value from the value obtained with the reduction mixture (TMA plus TMAO).

Initially, plastic cuvettes were utilized. However this was found to give very unsatisfactory results for low TMA or TMAO samples, although reproducibility was acceptable in samples with relatively high TMAO content. Due to this, the use of plastic cuvettes was abandoned for a quartz cuvette.

Urea was determined spectrophotometrically at 535 nm with a Sigma BUN-535 kit (Sigma chemical Co., St. Louis, MO, U.S.A.) on the deproteinized samples prepared for TMA/TMAO determination.

2.2.3 TMA oxidase assay

TMAoxi activity was measured by a modification of the spectrophotometric assay given by Agustsson and Strom (1981). Liver or kidney samples were homogenized in 9 volumes of ice cold 40% glycerol in 50 mM imidazole (v/v), pH 7.8 at 20°C. Agustsson and Strom (1981) homogenized in 250 mM sucrose; however preliminary experiments with captive cod (*Gadus morhua*) indicated that the glycerol buffer gave somewhat higher and more consistent activities (data not shown). Homogenates were centrifuged for 10 minutes in an Eppendorf centrifuge (10000 rpm) at 4°C to remove cellular debris. Assay conditions were as follows: 50 mM pyrophosphate buffer (pH 8.2 at 20°C), 6.0 mM $MgCl_2$, 0.15 mM NADPH. For reaction rates, 1.0 mM TMA was also added and gave apparent saturating kinetics, TMA was omitted for control rates. The oxidation of NADPH was determined at 340 nm at 25°C with a Beckman DU640 spectrophotometer.

2.2.4 Osmolality

Osmolality for teleost plasma samples was determined with a Fiske one-ten freezing point osmometer.

2.2.5 Statistics

All means were compared with a oneway ANOVA. $p<0.05$ was considered to be statistically significant.

2.3 Results

2.3.1 Teleosts

TMAO content of tissues from the deep-sea teleosts was significantly elevated above moderate and shallow species in all cases except liver in *A. rostrata* (Table 2.1). Typically, *A. rostrata* tissues had approximately 50-100 mmol/kg more TMAO than shallow and moderate fish. *S. kaupi* had TMAO contents of approximately 100-150 mmol/kg greater than other fishes. Other than white muscle in *M. berglax*, fish caught at moderate depths had TMAO contents within the range of the three shallow species. White muscle had substantially more TMAO than all other tissues and the heart had slightly higher levels than other tissues in the species caught in shallow and moderate waters.

Plasma TMAO was very high in the deep caught teleosts, as was osmolality (Table 2.2). The increase in TMAO, relative to shallow and moderate species, accounts for most of the increased osmolality.

Levels of TMA in the white muscle, brain and heart were generally low (less than 1.4 mmol/kg, data not shown). The kidney, and in some cases liver, did have elevated TMA content (Table 2.3). *A. rostrata* had very high TMA in the liver and kidney, whereas *S. kaupi* had high liver but not kidney TMA.

Table 2.1. TMAO content of tissues and plasma osmolality from teleost fishes caught from depths as described in Material and Methods. Values are means±SD, n value is in parenthesis. Units are mmol/kg. Capture depths are S, shallow (<150 m) M, moderate (500-700 m) and D, deep (<1000 m). Letters indicate no significant difference ($p>0.05$) from *a G. morhua*, b *M. bilinearis*, c *U. chuss*, d *G. ensis*, e *M. berglax*, f *A. rostrata* and g *S. kaupi*. na = not analysed.

Species	Depth	**White Muscle**	**Heart**	**Brain**	**Liver**	**Kidney**
Gadus morhua	S	48.0±5.21 (6)^{abd}	5.59±1.48 (6)^{abc}	1.14±0.85 (6)^{ab}	0.74±0.44 (6)^{abde}	0.79±0.40 (6)^{ace}
Merluccius bilinearis	S	40.8±1.32 (4)^{ab}	8.79±2.85 (4)^{abcde}	0.61±0.20 (4)^{ab}	0.71±0.90 (4)^{abdef}	na
Urophycis chuss	S	64.7±7.09 (6)^{cd}	5.36±1.01 (5)^{abc}	2.91±1.16 (6)^{cde}	4.32±0.88 (6)^{cef}	0.43±0.85 (6)^{ace}
Gaidiosaurus ensis	M	46.3±17.5 (4)^{abcd}	9.51±2.62 (4)^{bde}	3.05±0.32 (4)^{cde}	0.97±1.01 (4)^{abdef}	3.71±1.07 (4)^{de}
Macrurous berglax	M	95.4±4.77 (3)	11.61±1.0 3 (3)^{bde}	3.62±0.71 (3)^{cde}	1.98±1.27 (3)^{cef}	1.84±1.07 (3)^{acde}
Antimoria rostrata	D	159±12.8 (4)	59.5±9.02 (3)	48.0±4.59 (4)	4.38±3.73 (7)^{bcdef}	46.3± 15.5 (6)
Synaphobranchus kaupi	D	206±22.3 (3)	115±10.5 (3)	98.2±5.79 (3)	92.6± 9.34 (3)	111±11.5 (3)

Urea was below 6 mmol/kg in all tissues from all teleost species (data not shown). TMAoxi activity did not increase with depth of capture (Table 2.3). Shallow teleosts had very high activity in the liver, and in the case of *G. morhua*, kidney as well. The moderate and deep caught fish had no significant difference between them. Liver activity was significantly less than all shallow species. Kidney activity in *G. morhua* was significantly greater than all other species.

Table 2.2. Plasma TMAO concentration and osmolality in teleost fishes caught in shallow, moderate and deep water. [1] significant difference from *A. rostrata*, [2] significant difference from *S. kaupi* ($p < 0.05$). Values are means±SD, n value is in parenthesis

Species	Plasma TMAO (mM)	Osmolality (mOsm/kg)
Gadus morhua	1.06±0.48 (5)[12]	368±30.9 (4)[2]
Merluccius bilinearis	0.75±1.0 (5)[12]	377±24.2 (5)[2]
Urophycis chuss	8.67±4.02 (6)[12]	373±16.0 (4)[2]
Gaidiosaurus ensis	0.45±0.57 (4)[12]	351±8.51 (3)[12]
Macrurous berglax	0.26±0.22 (3)[12]	369±13.8 (3)[2]
Antimoria rostrata	91.2±21.8 (4)	449±76.8 (5)
Synaphobranchus kaupi	114±10.3 (3)	569, 608

Table 2.3. TMA content and activity of TMAoxi in the liver and kidney of teleost fishes. Values are means ± SD, n value is in parenthesis. TMA in mmol/kg, TMAoxidase activity in U/hr. Species depth of capture as in Materials and Methods. [1] significantly different from *A. rostrata* and [2] significantly different from *S. kaupi*. [*] significantly different from liver ($p < 0.05$). na = not analysed

	Liver		Kidney	
Species	**TMA**	**TMAoxi**	**TMA**	**TMAoxi**
Gadus morhua	1.47±1.19 (6)[1]	5.37 ± 1.80 (6)[12]	2.93±1.28 (6)[12]	6.95 ± 0.85 (5)[12]
Merluccius bilinearis	0.16±0.12 (4)[12]	9.05 ± 3.93 (7)[12]	na	na
Urophycis chuss	1.01±.045 (6)[1]	9.39 ± 6.74 (5)[12]	3.95±1.37 (6)[12*]	1.33 ± 0.66 (4)*
Gaidiosaurus ensis	0.27±0.18 (4)[12]	1.28 ± 1.35 (4)	3.34±0.47 (4)[12*]	1.51 ± 0.65 (4)
Macurous berglax	0.91±0.28 (3)[12]	1.71 ± 0.79 (3)	3.01±0.49 (3)[12*]	0.80 ± 0.29 (3)
Antimoria rostrata	4.94±1.18 (7)[2]	0.95±0.45 (5)	9.65±2.87 (6)[2*]	2.28 ± 1.11 (6)
Synaphobranchus kaupi	2.20±0.69 (3)[1]	0.80 ± 0.42 (6)	0.34±0.09 (3)[1*]	1.20 ± 0.66 (3)

2.3.2 Elasmobranchs

Levels of TMAO were significantly greater in all tissues sampled from *C. fabricii* than in *S. acanthias* (Table 2.4). Furthermore, urea was significantly less in *C. fabricii* in all cases except liver. The ratio of urea: TMAO was significantly less for *C. fabricii* in all tissues. TMA was very low in all tissues (< 2.1 mmol/kg or L), data not shown. TMAoxi was below levels of detection in liver and kidney of both species.

Table 2.4. TMAO and urea content and ratio of urea: TMAO in tissues from a shallow, *Squalus acanthias*, and deep-sea Squaliform shark, *Centroscyllium fabricii*. Values are means ± SD. n = 6 for *S. acanthias*, for *C. fabricii* n = 4 for plasma, 6 for rectal gland and 5 for all other tissues. Units are mmol/L for plasma and mmol/kg for all tissues. * significantly different from *S. acanthias*.

	Squalus acanthias			*Centroscyllium fabricii*		
	TMAO	**Urea**	**Urea: TMAO**	**TMAO**	**Urea**	**Urea: TMAO**
White muscle	167±11.8	300±6.60	1.80±0.13	248±14.7*	241±8.1*	0.97±0.08*
Red muscle	130±7.15	306±21.4	2.35±0.16	196±12.1*	213±13.3*	1.08±0.02*
Heart	79.1±7.79	295±15.4	3.75±0.30	180±6.2*	252±19.8*	1.40±0.09*
Brain	58.2±7.10	327±16.0	5.70±0.88	130±4.5*	241±0.2*	1.84±0.13*
Rectal Gland	56.2±7.05	334±21.4	6.02±0.77	150±6.0*	228±4.6*	1.52±0.06*
Kidney	63.2±4.35	319±14.5	5.08±0.54	160±11.5*	242±9.2*	1.52±0.11*
Liver	21.2±5.73	105±27.6	5.17±1.59	80.8±24.9*	115±53.0	1.42±0.33*
Plasma	79.8±3.63	366±17.6	4.58±0.19	223±48.8*	259±66.6*	1.46±0.06*

2.4 Discussion

2.4.1 Teleosts

The TMAO content of the deep caught species was elevated over the moderate and shallow caught species. The single exception to this, the liver of *A. rostrata* where TMAO content appears to be the result of blood 'contamination' within the tissue, will be discussed below. The increased TMAO accounts for much of the change in plasma osmolality (Table 2.2) and, in most tissues, intracellular TMAO would accommodate for the increased solute in the extracellular fluid. These data strongly suggest that TMAO accumulation is of physiological importance to these deep-sea fish because of elevated levels in a variety of tissues. Lange and Fugelli (1965) have shown that TMAO has a role in the osmotic adaptation of euryhaline teleosts; however the species examined in the current study would not likely experience environmental dilution negating this as a likely function. See Gillett et al. (1997), Kelly and Yancey (1999) and Yancey et al. (2001) for detailed discussion of potential benefits of TMAO accumulation in deep-sea organisms.

The increased plasma, and presumably all body fluid, osmolalities in the deep-sea species are of intrinsic interest and are consistent with data from Gillett et al. (1997). As explained in Gillett et al. (1997), if there was a strong selective force towards increased osmolality in teleosts, shallow species would also have increased osmolality. However, the accumulation of a particular osmolyte far above 'normal' concentrations suggests a selective benefit in doing so. Furthermore, high dietary intake of TMAO could account for higher than 'typical' levels of this single solute but would not explain an increased osmolality of body fluids, again suggesting some physiological role in deep-sea fish.

In all species examined, the white muscle had substantially more, 32-110 mmol/kg, TMAO than all other tissues. There appears to be a disequilibrium between muscle and other tissues, as well as plasma, suggesting active transport. However, it is important to note that the possibility that high plasma TMAO may be a result of 'leakage' from muscle was not eliminated. If this is the case, the gradient between plasma and muscle would be even greater. To my knowledge, there has been no work involving a potential active TMAO transporter in teleosts, although TMAO transport in elasmobranch erythrocytes has received some attention (Wilson et al. 1999, Koomoa et al. 2001). Similar to the trend between non-muscle tissues in deep-sea and shallow fish, this disequilibrium suggests a physiological role of TMAO in muscle.

The consistently higher muscle TMAO content may shed some light on this question. As explained in Yancey et al. (2001), TMAO has the tendency to compress protein conformation and increase protien-protein interactions that result in a net decrease in the total hydration shell of the protein. The accumulation in muscle may be important in maintaining the integrity of the muscle fibers or potentially counteracting general ionic destabilization of contractile proteins (Nosek et al. 1998). For example, the polymerization of G-actin to f-actin results in an unfavorable volume increase and the actin from a deep-sea fish has adapted to have an approximately 6 fold decrease in this volume change relative to a shallower living congeneric species (Swezey and Somero 1985). TMAO increases the polymerization of G-actin to f-actin at 1 and 500 atm and thus could help in the formation and maintenance of f-actin in shallow and deep-sea teleosts (Yancey et al. 2001). Since the effects of pressure on actin polymerization in

shallow-living teleosts may be several fold higher, especially in the 1-100 atm range (Swezey and Somero 1985), TMAO may be of substantial importance in marine deep-sea and shallow-living teleosts, alike.

The liver of *A. rostrata* and kidney of *U. chuss* have very low TMAO, especially compared to plasma content. Some portion of the total tissue TMAO content would be from extracellular fluid, presumably 10-20% of total water content, which the plasma values would be representative of. With experimental error taken into account the levels of TMAO in these two tissues would be almost entirely accounted for by the extracellular fluid. This suggests that there is a strong TMAO concentration gradient into the cells of these tissues and potentially a mechanism to remove TMAO from the intracellular fluid or means of preventing the entry of TMAO into the cell.

Tissue TMA content was relatively low in most tissues and the plasma of all teleosts (typically < 1.4 mmol/kg or mM). The kidney, and in the case of *A. rostrata* and *S. kaupi* the liver, had substantially higher TMA content. This could be significant with respect to TMAO synthesis because the liver and kidney are the sites of TMAoxi activity in fish (Baker et al. 1963; Augustsson and Strom 1981). The TMA in the above tissues could either be sequestered from the plasma or come from the catabolism of trimethylamine containing compounds such as choline or betaine (Bilinski 1964, Charest et al. 1988). As TMA is typically low in organisms whereas choline and derivatives such as phosphatidylcholine are quite common, the breakdown of trimethylamine-containing compounds would appear to be more likely. However, we cannot discount that the high kidney TMA is not due to accumulation prior to excretion.

Levels of liver TMAoxi activity in the present study show a negative correlation with increasing depth, with the shallow species being significantly greater than the moderate and deep caught fish. Although this may reflect the established trend of decreased activity in several enzymes with increasing depth of capture (Hochachka and Somero 1984), it also indicates that there does not appear to be increased metabolic capacity for TMAO synthesis with increased TMAO content in these fish. Furthermore, the potential for synthesis of TMAO via a yet uncharacterized pathway can not be eliminated. The kidney activity was not significantly different from the liver, the only exception being *U. chuss* where the kidney was significantly less than the liver. Thus, this study does not support the hypothesis that elevated tissue TMAO content correlates with elevated liver, or kidney, TMAoxi activity. High kidney, or liver, TMA content along with appreciable amounts of TMAoxi activity does suggest an active synthesis of TMAO. This is best demonstrated in the liver of *A. rostrata* and kidney of *U. chuss* where TMA is actually higher than TMAO, creating an excellent substrate to product relationship for TMAO synthesis.

2.4.2 Elasmobranchs

The increased TMAO and decreased urea content of all tissues in *C. fabricii* compared with *S. acanthias* is consistent with, and expands upon the data from, Kelly and Yancey (1999). It appears that TMAO is elevated in most, if not all, tissues in deep-sea elasmobranchs, accompanied by a decrease in urea content. The inability to demonstrate measurable TMAoxi activity in either elasmobranch species is significant because it indicates that the TMAO accumulation is not likely via endogenous synthesis.

The lack of appreciable activity in *S. acanthias* is consistent with previous work involving the metabolism of TMAO in this species (Goldstein et al. 1967, Goldstein and DeWitt-Harley 1973).

The accumulation of TMAO in deep-sea elasmobranchs could have many roles. As in teleosts it may act to counteract pressure. In addition the decrease in urea strongly suggests that deep-sea elasmobranchs may minimize disruptive protein effectors, such as urea, while increasing stabilizing agents such as TMAO.

The ratio of urea to TMAO is a useful measure for comparing the relative intracellular concentrations for the same tissue between species. If urea is in approximate equilibrium between extracellular and intracellular fluid, as suggested by Sulikowski and Maginniss (2001), the ratios between any other solute and urea can be used to give relative solute content regardless of the water content of a tissue. That is, for a given animal, although the values expressed in mmol/kg may differ between tissues, the difference will be proportional and mostly due to difference in water contents. TMAO is not in equilibrium between the extracellular and intracellular fluid. However, if tissues vary over an approximate range of 10 to 20% extracellular fluid, the TMAO content (mmol/kg) will approximate the intracellular TMAO concentration (mM) much more closely than the extracellular concentration due to the low contribution of the extracellular fluid to total tissue fluid. Although this ratio will not account for potential contamination by the extracellular fluid, it estimates the concentration of TMAO relative to urea and is likely representative of the relative intracellular ratios. This accommodates for tissues that have very high lipid content such as the liver.

Furthermore, although caution should be used with comparisions between different tissues, the ratio of urea to TMAO in the white and red muscle of both elasmobranch species is substantially lower than all other tissues. This indicates that, as in teleosts, there is substantially more TMAO in the muscle tissues of elasmobranchs than other non-muscle tissues and that there is a substantial disequilibrium between the muscle intracellular TMAO and the extracellular TMAO represented by the plasma. Thus the above hypotheses of protein stabilization by TMAO in the muscle of marine teleosts may also apply to elasmobranchs.

Another potential role of TMAO in elasmobranchs is that it contributes to buoyancy by decreasing the density of a solution relative to salt water (Withers et al. 1994). Although urea also decreases the density of a solution, an equilmolar amount of TMAO will result in a greater change. Thus, deep-sea elasmobranchs may accumulate TMAO to become nearer to neutral buoyancy and would then require less net lift, which would result in decreased drag and decreased energy expenditure. *C. fabricii* appears to be an active predator and such an adaptation may be quite beneficial during periods of low food availability. Withers et al. (1994) also found a trend of decreasing methylamines with elasmobranchs that are more benthic. This may explain why the deep-sea shark I have examined had approximately 45 mmol/kg more white muscle TMAO than a deep-sea skate caught from between 1800-2000 m in Kelly and Yancey (1999), although this previous study found a similar ratio of urea: TMAO as I did.

2.5 Conclusions

The data strongly suggest a physiological importance of TMAO in teleost fishes and especially in deep-sea fishes. The accumulation of TMAO in various tissues, as well as high activity of TMAoxi with concurrently high TMA content indicates that some of the fish examined are capable of synthesizing and retaining TMAO. It has already been established that TMAO may have several important physiological roles in elasmobranchs. The elevated TMAO content is suggestive of increased importance in all tissues examined from a deep-sea elasmobranch, with a subsequent decrease in urea relative to a shallow water species. This study has also opened questions on why muscle accumulates more TMAO than other tissues, as well as to the potential of TMAO transporters in the muscle of teleosts and elasmobranchs.

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Chapter 3: The accumulation of trimethylamine oxide in smelt (*Osmerus mordax*): Temperature effects, synthesis and correlation between plasma concentrations and tissue contents.

3.1 Introduction

Trimethylamine oxide (TMAO) is a commonly occurring nitrogenous solute in marine teleost fishes (reviewed by Hebard et al. 1982). Typically, TMAO is only found to any great degree in the swimming muscle. A few exceptions to this have been found, namely polar and subarctic fishes adapted to near or below 0°C (Raymond 1994, Raymond and DeVries 1998, Raymond and Hassel 2000) and teleosts from the deep-sea (Gillett et al. 1997, previous chapter) have been shown to have elevated plasma TMAO concentrations. Of teleosts known to have elevated plasma TMAO the smelt, *Osmerus mordax*, has received the most study. Raymond (1994, 1998) has shown that winter-caught smelt have increased plasma and liver TMAO levels and that TMAO concentrations are affected by acclimating fish to temperatures different from the temperature they were caught in. That is, fish caught from subzero temperatures decrease TMAO when warm-acclimated and fish from 5°C water increase TMAO when acclimated to –1°C (Raymond 1994). This sets the framework for several experiments which were undertaken to elucidate potential triggers and mechanisms of TMAO accumulation in smelt.

Although wild-caught smelt accumulate TMAO in the winter, little is known about the temporal accumulation of TMAO or what threshold temperature, if any,

triggers the accumulation or decrease in TMAO. The acclimation study mentioned above demonstrates that temperature is a major effector of TMAO in smelt, but it gives only rudimentary understanding about what temperatures actually will result in the increase in TMAO. An aspect of the current study was to determine the effect of temperature on the levels of TMAO in smelt. This was done by holding smelt either at ambient temperature (ranging from 11°C to –1.2°C) or by maintaining them at an elevated temperature well above the freezing point of seawater (approximately 5°C or 9°C) from fall to spring.

Raymond (1998) found detectable levels of trimethylamine oxidase (TMAoxi) in smelt; thus they appear to have the metabolic machinery for the synthesis of TMAO. As explained elsewhere (see chapter 1) the presence of TMAoxi is not conclusive evidence of active TMAO synthesis, only that if TMA is formed it could be oxidized to TMAO. Since smelt are known to accumulate TMAO during winter conditions, they present an opportunity to examine if there is an increase in the capacity for TMAO synthesis in parallel with increased TMAO levels. This will also test the hypothesis that has been put forth that there is a correlation between plasma TMAO concentration and TMAoxi levels in teleost fishes (Raymond 1998).

Finally, little if anything is known about the interplay between the extracellular concentration of TMAO and the intracellular compartment. Smelt are an excellent model organism for probing the relationship between extracellular and intracellular concentrations because they are known to change their plasma TMAO concentrations, representative of the extracellular compartment. This allows for comparison with tissue

contents, representative of the intracellular compartment, over a range of extracellular concentrations.

3.2 Materials and Methods

3.2.1 Experimental animals

The experiments were conducted over 2 separate winter seasons, the first from 1999 to 2000 (99/00) and second in 2000 to 2001 (00/01). Approximately 180 smelt for the 99/00 experiments were caught by beach seine in Conception Bay Newfoundland in late October. Animals for the 00/01 experiments (approximately 350) were caught by beach seine in Long Harbour, Placentia Bay Newfoundland on October 9, 2000. In both cases fish were transported to the Ocean Sciences Centre in an aerated live-well filled with water from the capture site. Upon arrival at the OSC smelt were transferred to two 4000 litre indoor ambient temperature flow-through seawater tanks with approximately equal numbers of fish in each tank. They were kept on a natural photoperiod with fluorescent lights set on an outdoor photocell. Smelt for the 99/00 experiment were fed frozen brine shrimp 3 times a week while the 00/01 fish were fed chopped herring. In both cases feeding was visually confirmed and evidence of food consumption was observed in sampled fish.

3.2.3 Experimental protocol

In both experiments one tank of fish was allowed to track ambient seawater temperatures (ambient group) while another tank was maintained at a constant and

elevated temperature relative to seasonal winter temperatures (warm group). The temperature profiles are shown in Figures 3.1 and 3.2 for the 99/00 and 00/01 experiments respectively. For the 99/00 experiment this was accomplished by maintaining one tank at ambient temperature while another was held at approximately 5°C. In the case of the 00/01 experiment, fish were allowed to track ambient temperatures until they dropped to 4°C (on Dec. 11, 2000). At this point one tank was maintained with heated seawater at approximately 9°C for the duration of the experiment.

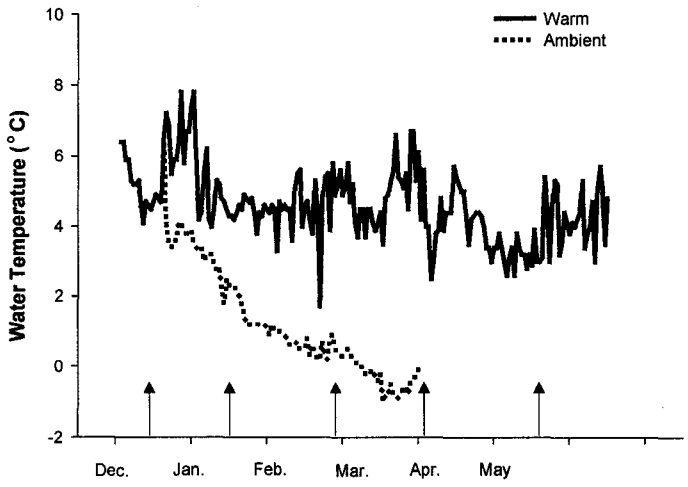

Figure 3.1. Temperature profiles for 99/00 warm and ambient smelt groups, arrow indicate sample times.

During the 99/00 experiment fish from both tanks were sampled on December 15, January 11, February 29 and March 30. Due to lack of experimental animals in the ambient group, fish were only sampled on May 15 from the warm group. On the initial

sample period, December 15, 3 fish from each tank were sampled. There was no difference between any measured characteristic and data from both tanks were pooled into an initial data point. For the 00/01 experiment, 3 fish were taken from each tank on October 19 and the data pooled. Fish were subsequently sampled on November 1, 15 and 28, December 11 and 20, January 23, February 20, March 23, April 6 and May 14.

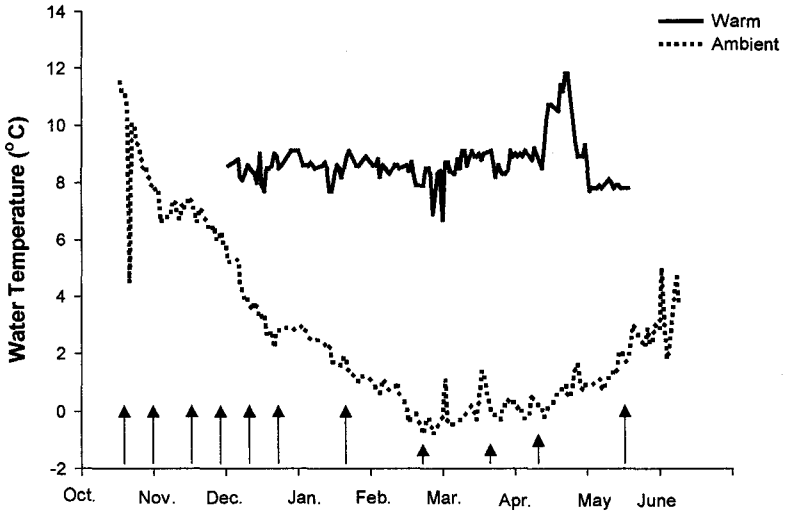

Figure 3.2. Temperature profiles for 00/01 warm and ambient smelt groups, arrows indicate sample times

Fish were bled by caudal puncture with heparinized syringes and then killed by a blow to the head. Tissues were dissected out and frozen in liquid nitrogen dry-shippers. Whole blood was centrifuged at approximately 5000g for 5 minutes; plasma was collected and frozen as above. Samples were maintained at or below –60°C until analyzed.

3.2.3 TMAO determinations

TMAO was determined by the method of Wekell and Barnett (1991), modified for small samples. Briefly, tissues were homogenized in 9 volumes of cold 5% (w/v) tricholoacetic acid (TCA) while plasma was mixed with 9 volumes of 5% TCA and allowed to stand for several minutes on ice. Precipitated proteins were removed by centrifuging at 10000g at 4°C for 5 minutes. The supernatant was used directly for analysis. Toluene is added to the samples and TMAO is reduced to TMA by the addition of a ferrous sulphate (in 0.1 M HCl)-EDTA mixture and incubation at 50°C for 5 minutes followed by the addition of 45% KOH. TMA is extracted into the toluene phase by frequent mixing. An aliquot of the toluene phase is mixed with 0.02% (w/v) picric acid in toluene. TMA reacts colourimetrically with picric acid and the absorbance was read at 410 nm. Preliminary studies found endogenous TMA contents to be very low relative to TMAO and thus no correction for TMA was made.

For samples from the 99/00 experiment, disposable plastic cuvettes were used as in Raymond (1994) and Raymond and DeVries (1998). This had satisfactory results on moderate to high TMAO content samples, but gave poor reproducibility on very low TMAO content samples. As such, samples from the 00/01 experiment were analyzed using a quartz cuvette.

3.2.4 TMA oxidase activity

TMAoxi was determined by a modified version of the spectrophotometric assay used by Augustsson and Strom (1981). Augustsson and Strom (1981) homogenized tissues in a 250 mM sucrose based buffer. Preliminary data from cod (*Gadus morhua*) tissues found that homogenizing in 9 volumes of a 50 mM imidazole and 40% (v/v) glycerol buffer, pH 7.8, gave somewhat higher and more consistent activities. Thus, the Imidazole/glycerol buffer was used for this study. For kidney samples, homogenates were centrifuged for 5 minutes at 10000g at 4°C to remove cellular debris. Initially this was also the case for liver samples, however no detectable levels of activity could be found above the control rate. Further steps were used in an attempt to improve the resolution of the assay (see below in results section).

The standard assay conditions were as follows: 50 mM potassium pyrophosphate buffer (pH 8.2), 6.0 mM $MgCl_2$ and 0.15 mM NADPH. The reduction of NADPH was followed at 340 nm on a Beckman DU640 spectrophotometer at 25°C. For reaction rates, the assay also included 3.0 mM TMA while control rates were determined in separate cuvettes in the absence of TMA. All assays were run in duplicate or triplicate. For true reaction rates, the control decrease at 340 nm was subtracted from the decrease at 340 nm in the presence of TMA.

Although 25°C is well above the normal physiological temperature range of smelt, other studies have found that the enzyme reaction is linear from cold water fishes for an hour at 22°C (Raymond 1998, Raymond and DeVries 1998). This demonstrates that despite this non-physiological temperature, the enzyme is likely stable, allowing for

better resolution of this typically low activity enzyme than would be found at a lower temperature within the physiological range.

To date, there is no procedure for isolating TMAoxi from fish, although it is known that the enzyme is found in the microsomal fraction (Augustsson and Strom 1981). Insufficient amounts of kidney tissues could be collected from these relatively small fishes to attempt a partial purification or even to isolate kidney microsomes. To determine the apparent K_m of smelt kidney TMAoxi for TMA, homogenates from 3 fish were pooled and assayed at varying TMA concentrations, with a fixed NADPH concentration (0.15 mM).

3.2.5 Statistical analysis

All means were compared by one way ANOVA. Correlations between plasma TMAO concentration and tissue contents were done by linear regression analysis on individual animals or the means from each sample period in the case of the 99/00 experiment.

3.3 Results

3.3.1 99/00 experiment

The mean plasma TMAO concentration was 13.5 mM initially and increased in both groups in January (Fig. 3.3a). In February, when ambient temperature fell below O°C, the ambient group reached its peak TMAO concentration at 19.2 mM. Plasma

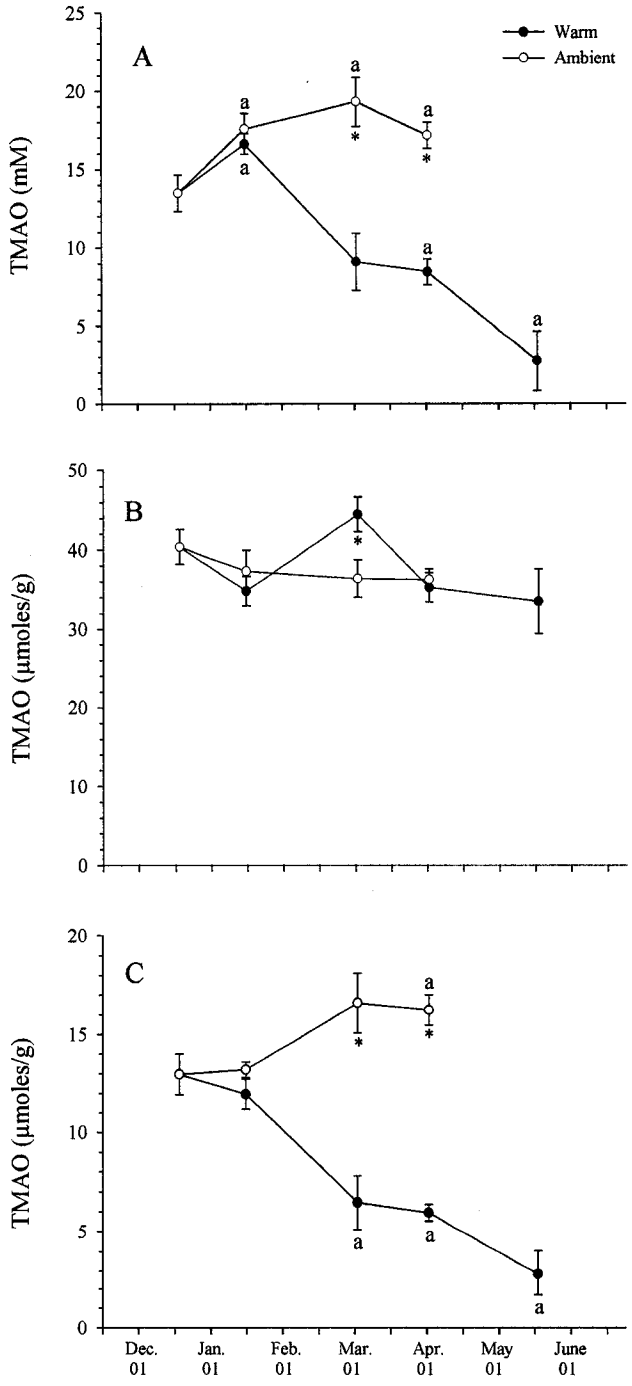

Figure 3.3. TMAO levels in plasma (A), white muscle (B) and liver (C) in warm and ambient smelt groups from the 99/00 experiment. Values are mean ± S.E.M, n = 6 for initial point and 5 for all other points. a, significant difference from initial point; * significant difference between groups.

TMAO had significantly decreased to 9.07 mM by February 29 in the warm-acclimated group and continued to decrease throughout the experiment. Interestingly, plasma TMAO concentrations in the warm smelt remained elevated in March.

Muscle TMAO contents did not significantly change from the initial sample group in either treatment at any time (Fig. 3.3b). There was a significant ($p < 0.05$) difference between warm and ambient groups in February, however neither values were significantly different compared to initial values.

Liver TMAO content mirrored the trend seen in plasma where levels significantly increased from initial, 12.7 mmol/kg, to 16.5 mmol/kg in the ambient group whereas TMAO continually decreased from the initial content in the warm group (Fig.3c).

It was observed that while mean liver TMAO content increases linearly ($r^2 = 0.874$, $p < 0.01$) as plasma TMAO concentration increases (Fig. 3.4a), mean white muscle TMAO remains relatively constant (Fig. 3.4b).

3.3.2 00/01 experiment

As the initiation of the TMAO accumulation in smelt plasma appeared to be 'missed' in the first experiment a second experiment was required to determine at what temperature TMAO begins to accumulate. Plasma TMAO concentrations were elevated by November 1 when ambient temperatures were approximately 7°C (Fig. 3.5). Thus, if there is a thermal threshold for the accumulation of TMAO in smelt during the fall

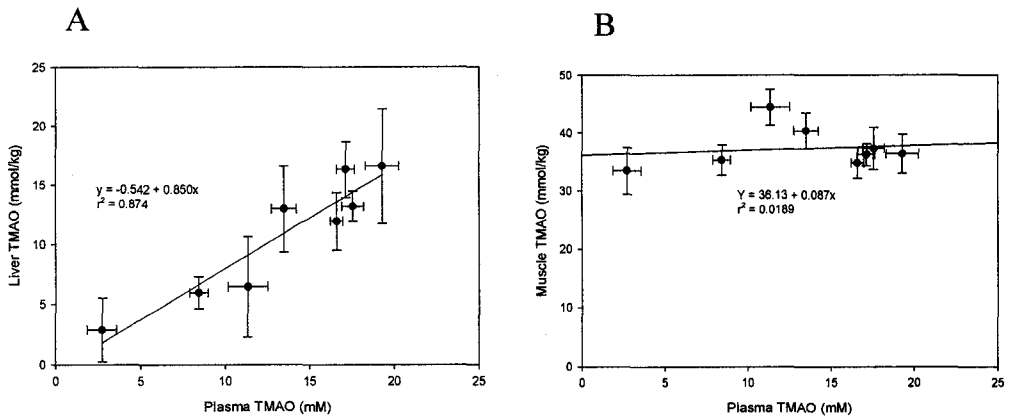

Figure 3.4. Relationship between mean plasma TMAO concentration and tissue contents for A, liver and B, white muscle in smelt from the 99/00 experiment; n = 6 or 5 for all measurements.

season, it occurs between 9 and 7°C. The results of the 00/01 experiment parallel the 99/00 experiment very well. In the 00/01 ambient group plasma TMAO essentially plateaued from December to April with a maximum of 20.2 mM in January. As in the 99/00 experiment, although plasma TMAO was elevated in December, to approximately 75% of the highest levels in the experiment, holding the animals at an elevated temperature caused a decrease in plasma TMAO to levels not different from the initial concentration by December 20. TMAO concentrations remained low for the rest of the experiment in the warm group. The elevated temperature was approximately 4°C higher than that of the 99/00 experiment resulting in what appears to be a more marked decrease in TMAO. A final improvement in the 00/01 experiment was extending the final sample

period to May when ambient temperatures have begun to increase. In the ambient group the TMAO significantly fell from 18.9 in April to 8.9 mM in May. This was accompanied by an increase in temperature from -1°C to approximately 2-3°C.

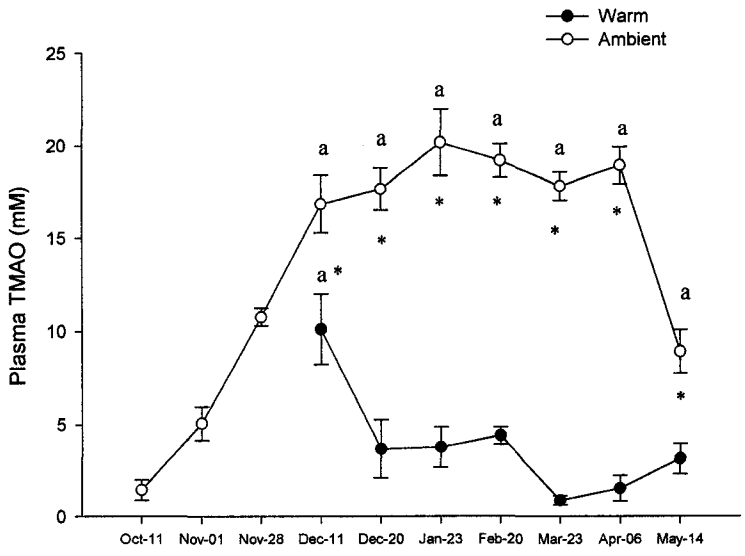

Figure 3.5. Plasma TMAO levels, mean values ± S.E.M., in warm and ambient smelt from 00/01 experiment n= 6 initially and 3-5 for all other points; a, significant difference from initial value, * significant difference between warm and ambient fish ($P<0.05$).

Preliminary studies found levels of liver TMAoxi activity to be bordering on the limits of detection and no appreciable activity in the muscle, heart, brain, intestine, gill or spleen. When run in triplicate, many liver samples did not have consistently elevated rates of NADPH oxidation with added TMA when compared with control rates (data not shown). This appeared to be potentially due to background 'noise' caused by the homogenate. In an attempt to improve the resolution of the assay, liver samples were centrifuged for 10 minutes at 25000g at 4°C and the supernatant collected. The

supernatant was again centrifuged at 25000g for 10 minutes at 4°C and the second supernatant was collected, taking care not to disturb the loosely sedimented particulate matter at the bottom of the tube. Despite the extra steps, this procedure resulted in TMAoxi levels that were not consistently above the control rates of the decrease in absorbence at 340 nm. As such, it was determined that liver TMAoxi activity was below the limits of detection for the assay protocol being used.

Kidney TMAoxi was found to be quite high in smelt, an order of magnitude higher than liver levels reported by Raymond (1998), and thus only kidney TMAoxi was measured over the season in smelt held at different temperatures. There was no significant difference between groups at any sample period, nor were there any seasonal or temperature trends during the experiment with levels ranging from approximately 10.8 to 21.0 μmol of substrate converted (g wet tissue weight)$^{-1}$ hr^{-1} (U/hr) (Fig. 3.6).

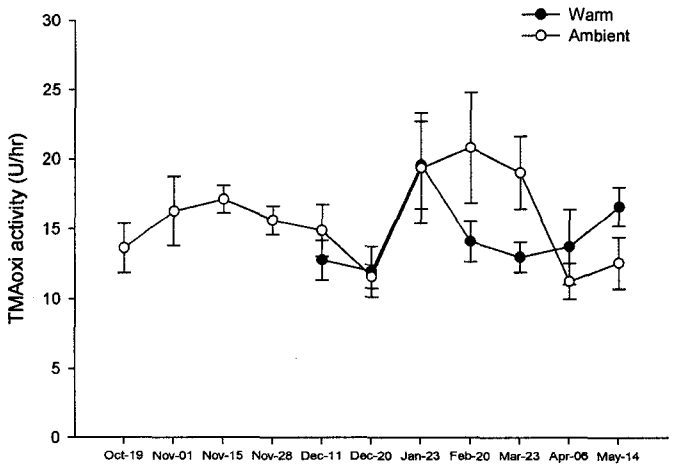

Figure 3.6. Kidney TMAoxi activity (U/hr), mean values ± S.E.M., in warm and ambient smelt from 00/01 experiment n= 6 initially and 5 for all other points; no significant difference between groups or from the intial point.

The apparent K_m for TMA was 0.2 mM (Fig. 3.7a,b). Of note, preliminary examinations of the kidney TMA content from samples of varying TMAO content was 0.15±0.08 (mean±SD) mmol/kg (range of 0.089 to 0.29 mmol/kg). These samples were the same fish used in Fig 3.8b and represent fish with a wide range of plasma TMAO concentrations, suggesting that it is unlikely that kidney TMA is elevated as plasma TMAO levels increase. Furthermore, there was no trend between TMA content and TMAO or temperature (data not shown)

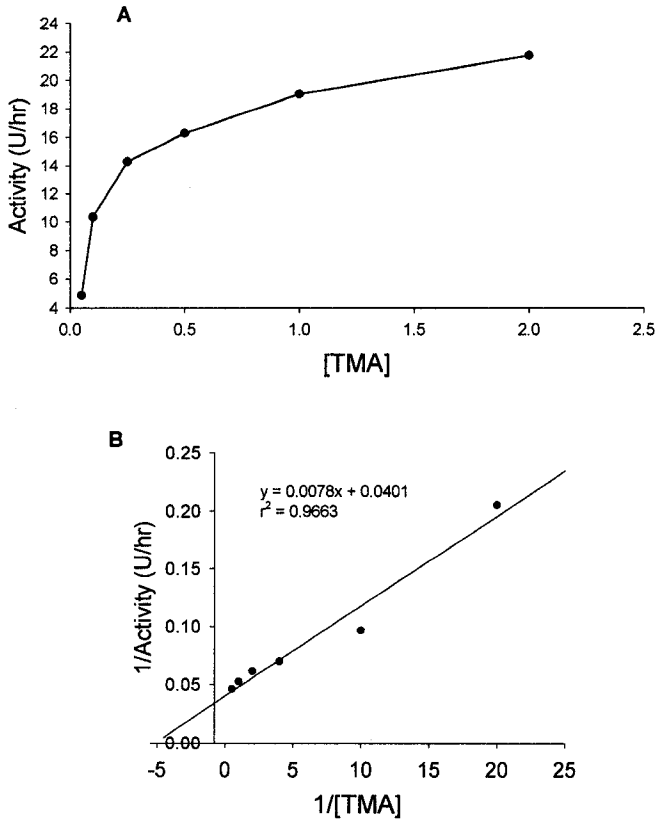

Figure 3.7. Michaelis-Menton plot (A) and Lineweaver-Burke transformation (B) for TMAoxi actvity, fixed NADPH concentration (0.15 mM), from pooled smelt kidney homogenates.

The strong correlation from the previous experiment between mean liver content and the plasma concentration of TMAO, while white muscle was unaffected, prompted the investigation of other tissues. The high degree of linearity found seasonally in liver was also found when tissues of individual fish with varying plasma TMAO were plotted against each other. In this case liver, kidney, brain, intestine and heart had an r^2 of 0.97, 0.85, 0.98, 0.93 and 0.85, respectively (Fig. 3.8 a, b, c, d and e). Of note, the regression line from individual fish plotted in Fig. 3.8a is not significantly different from the regression of the mean data (taken over an entire season) in Fig. 3.4a. The slopes of the regressions in tissues showing a linear trend were similar with the exception of heart, which was approximately 3 fold higher than other tissues (Fig. 3.8e).

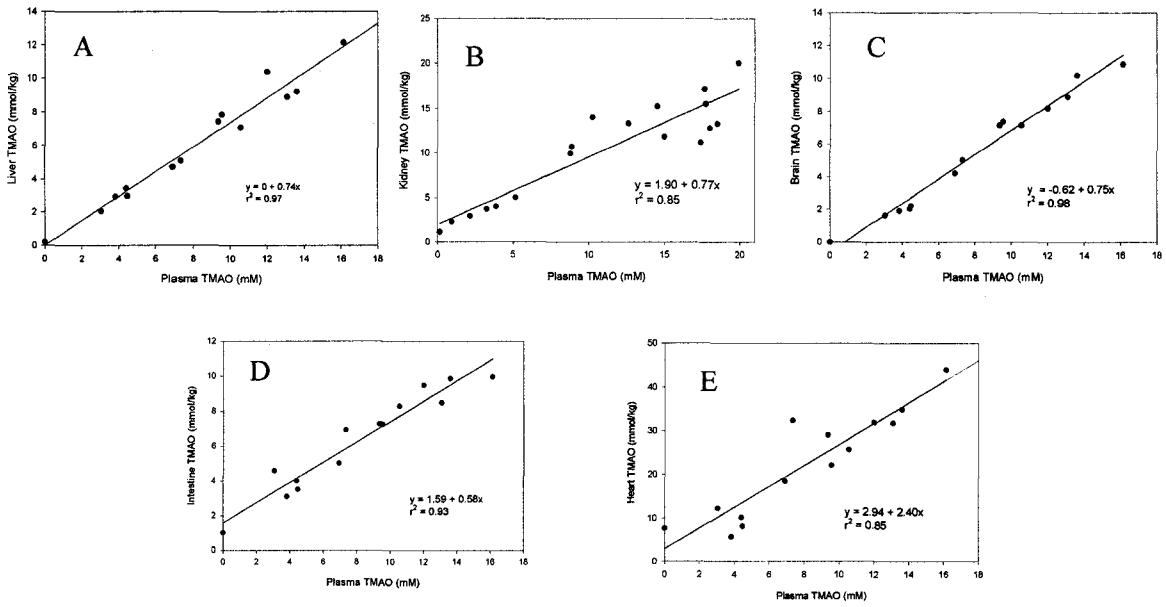

Figure 3.8. Relationship between plasma TMAO concentration and tissue contents in smelt; A, liver B, kidney C, brain D, intestine and E, heart.

3.4 Discussion

3.4.1 Relation between temperature and the accumulation of TMAO in Smelt

If there is a threshold temperature for the accumulation of TMAO in smelt, it appears to be above 5°C and likely between 9 and 7°C. Although there may be a low temperature activated switch that results in TMAO accumulation in smelt, it is premature to conclude that other environmental cues such as photoperiod are not key triggers. Interestingly, TMAO began accumulating in the 00/01 experiment by 7°C but the post-winter decrease in plasma TMAO concentration occurred below 3°C. Thus, if temperature is the major trigger for TMAO fluctuations, there are different threshold temperatures for accumulation and decrease.

In both experiments the warm group of fish decreased levels of TMAO. Whatever mechanism is involved with TMAO accumulation, maintaining fish at 5°C will cause the process to be inhibited; however, TMAO levels do not drop to pre-accumulation levels until several months later (Fig. 3.3a) when it would be expected that TMAO would also drop in the ambient group (Fig. 3.5). While it appears that the mechanism is inhibited at 5°C , it may shut off when fish are held at 9°C (nearing or above the temperature when TMAO begins to accumulate). TMAO levels had dropped after only 9 days at 9°C in the warm group during the 00/01 experiment. Raymond (1994) estimated TMAO excretion in cold acclimated smelt at 9 μmol 100 g^{-1} hr^{-1}. Although this number must be used cautiously, due to the levels of TMAO being at the limits of detection, this would mean a 100 g smelt could excrete 216 μmol of TMAO per

day. This rate would be sufficient for the observed initial decrease in the 00/01 experiment.

3.4.2 Potential role of TMA oxidase in TMAO accumulation in smelt

Based on the tissue distribution of TMAoxi activity found by Baker et al. (1963), the liver is typically viewed as the likely site of TMAO synthesis in fish (Goldstein and DeWitt-Harley 1973, Augustsson and Strom 1981, Raymond 1998, Raymond and DeVries 1998). Although equivalent or greater activity can also be found in the kidney (Baker et al. 1963, Raymond 1998) relative to the whole animal, the liver is a much larger organ than the kidney supporting the notion that the liver is the major contribution for synthesis, if TMAO is being synthesized. Contrary to this, no appreciable TMAoxi activity could be found in smelt liver, although Raymond (1998) reported an activity of 0.18 to 0.24 U/hr. In the same study, kidney activity was found to be slightly higher at 0.73 U/hr (Raymond 1998). In the present study, the lowest mean TMAoxi activity for any sample group was 17 fold higher than previously reported for kidney and over 20 fold higher than liver (Raymond 1998). Furthermore, the levels of TMAoxi activity found in this study are among the highest reported in fish. Despite the relatively small size of the kidney (19.1% ± 9.4 of the liver mass, n = 10 Treberg unpublished observation), the exceptionally high TMAoxi found in this present study in the kidney strongly suggests that if smelt are actively synthesizing TMAO, synthesis occurs in the kidney and not in the liver as believed in other fishes.

Although the kidney appears to be the likely site of TMAO synthesis, there was no apparent trend between temperature and levels of TMAoxi activity, although there

was a great deal of variability between groups. Furthermore, there was no significant trend between kidney TMAoxi activity and plasma TMAO concentrations. Thus, although the metabolic machinery for TMAO synthesis is present in the smelt kidney, there is not an increased capacity for synthesis in conjunction with increased TMAO levels. Interestingly, there was no difference between the ambient and warm groups. As the fish held at 9°C would have a higher relative TMAoxi activity, and capacity for TMAO synthesis, than fish at lower temperatures, absolute levels of kidney TMAoxi activity do not relate with TMAO accumulation.

The above raises the question, if TMAO is being synthesized, could regulation occur by some other means? This may happen by increased flux through to the production of TMA; however, the preliminary data found that tissue levels of TMA were very low in the kidney (< 0.2 mmol/kg) indicating that enhanced TMA production does not appear to have a key role in TMAO accumulation.

TMAoxi in fish is believed to be, or similar to, the flavin containing monooxygenase, E.C. 1.14.13.8 (FMO) of mammals (Schlenk and Li-Schlenk 1994). To my knowledge, there are no known allosteric modulators of FMO; however, any potential role of direct or indirect (ie. competetive) activation or inhibition of TMAoxi in relation to the regulation of TMAO synthesis in fish has yet to be examined.

The Michaelis-Menton plot in figure 3.7a gives the best evidence so far how TMAO synthesis could increase while TMAoxi remains at constitutive levels. With an apparent K_m of 0.2 mM for TMA (Fig. 3.7b) and relatively large changes in activity at lower TMA concentrations while subsequently 'trailing' off at higher TMA

concentrations, smelt kidney TMAoxi appears to be well adapted for regulation simply by shifts in the intracellular TMA concentration. The apparent K_m for TMA is slightly higher than physiological concentrations and if production of TMA were to increase from the catabolism of trimethylamine-containing compounds, the rate of oxidation to TMAO would increase rapidly by the affinity of the enzyme for this substrate. Thus, there may be no need for further regulation at this locus.

Alternately, the accumulation of TMAO may not represent increased synthesis, but rather an increase in retention. Along with TMAO, glycerol is accumulated by smelt and can reach 400 mM (Raymond 1992). It is hypothesized that these solutes act as colligative antifreezes to enhance the smelt's freeze-avoidance response. As the solutes that are accumulated by smelt as colligative antifreeze are all relatively small and permeable to biomembranes, especially glycerol, it seems likely that smelt have physiological mechanisms for curtailing solute loss. This may be important because glycerol synthesis and accumulation would be metabolically costly, diverting carbon from use as an energy source. The most like sites of solute loss are the gills and the kidney, and TMAO accumulation may simply reflect reduced removal of dietary TMAO. This is an area yet to be explored.

3.4.3 Intertissue differences in levels of TMAO

Smelt present a unique opportunity not only to examine the intertissue distribution of TMAO, but also how changing extracellular TMAO concentrations, represented by plasma concentrations, affect tissue levels. This is the first time such observations have

been looked at in detail in a teleost fish. In a general sense, the tissue distribution of TMAO follows the trend of white muscle > heart > liver ≅ kidney ≅ brain > intestine. Of note, the lower levels in the intestine may be a result of low water content relative to total mass due to the high amount of connective tissue. The intertissue distribution found in smelt is consistent with data on other teleosts (reviewed by Hebard et al. 1982, see also chapter 2).

The data in figures 3.4 and 3.8 illustrate several key trends, the most striking of which is that levels of TMAO in the liver, kidney, brain, intestine and heart are directly proportional to plasma concentrations. Furthermore, with the exception of heart, the slope of these regressions range from 0.58 to 0.77 which is close to what may be expected assuming intracellular water content of approximately 70% and equilibration of TMAO between the intra and extracellular compartments.

Heart TMAO content increases with increasing plasma TMAO over three times more than the other tissues that show a linear trend. Although heart TMAO content is proportional to plasma concentration, the intracellular concentration is much higher than that of the extracellular compartment. This may be due to some role of TMAO in relation to cardiac muscle function and implies active uptake as discussed below.

Data in Figure 3.4a represent the trend in liver over an entire season while the liver data in Figure 3.8a are from fish selected from February when the different smelt groups were expected to have very different plasma TMAO concentrations. These regression equations are not significantly different and suggest that the trends seen in

figure 3.8 are representative of trends that will be seen regardless of the season, that is they are not an artifact of the sample period.

While the TMAO content of the other tissues was proportional to plasma concentration, TMAO in the white muscle is essentially unchanged by the extracellular concentrations seen in the 99/00 experiment (Fig. 3.4b). This leads to two hypotheses; first that white muscle in smelt may function optimally at a particular intracellular TMAO concentration and second there is an active and regulated uptake of TMAO into the muscle.

White muscle from many marine fishes has high TMAO content (Hebard et al. 1982) although the near ubiquitousness of TMAO in the muscle of marine fishes has yet to be clearly explained. Yancey et al. (2001) have shown that TMAO counteracts the breakdown of f-actin into g-actin by high pressure in a deep-sea teleost. TMAO favours protein:protein interactions (Yancey 1994) and accumulation in the striated muscle of marine fish (where f-actin is a major functional and structural component) may aid in the maintaining the structure of f-actin or favour the polymerization of g-actin to f-actin (see discussion Chapter 2). Alternately, TMAO has been shown to counteract the negative effects of high ionic strength on isolated muscle fibre contractility *in vitro* (Nosek et al. 1998) and the accumulation of TMAO in marine fishes may play a similar role *in vivo*.

The consistency of white muscle TMAO content in smelt strongly suggests a regulated intracellular concentration. This is because the intracellular concentration is much higher than the extracellular compartment and is also unaffected by changes in the intracellular to extracellular concentration gradient. For muscle TMAO levels to be

regulated, there would have to be some means of active transport, or alternately a novel and undescribed TMAO synthetic pathway, because the small size of TMAO would likely result in some 'leakage' out of the muscle. Interestingly, the data on heart discussed above is suggestive of active transport because TMAO is again moved up a substantial concentration gradient. In heart, levels do not appear to be directly regulated, but are instead modulated by plasma concentrations. To date, no work has been done on the transport of TMAO in fish muscle; however, in light of this data (see also chapter 2) such investigations appear to be warranted.

3.5 Conclusions

The accumulation of TMAO in smelt may have a thermal trigger at approximately 5°C and can be suppressed by temperatures above this range. TMAO accumulates in the plasma and there is a proportional relationship with plasma concentrations in the liver, kidney, intestine, brain and heart whereas TMAO in the white muscle is maintained at a constant level. The kidney, not the liver, appears to be the most likely source for endogenous synthesis of TMAO in smelt and has exceptionally high levels of TMAoxi, although the accumulation of TMAO does not coincide with an increase in TMAoxi activity. TMAO is likely in, or near, equilibrium between the extracellular and intracellular compartments in the liver, kidney, intestine and brain and is elevated in the heart and white muscle, the latter of which is regulated at a constant level. These experiments also provide data that are evidence for the existence of a TMAO uptake mechanism in smelt heart and white muscle.

3.6 Literature cited

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4: Summary

When approaching physiological problems in a comparative sense, one must do one of two things, examine a number of species to determine if differences occur over the conditions be tested, as in Chapter 2, or subject a single species to changing conditions, as in Chapter 3. In case of the studies involved with this thesis, the experimental approach worked very well, be the approach interspecific or intraspecific. There were numerous fundamental or mechanistic consistencies between the deep-sea study and the smelt experiments.

The most obvious is that both studies found that the adaptation to a particular environment (be it high pressure or subzero temperatures) resulted in the accumulation of TMAO in fish. As both were relatively low temperature environments, it is tempting to speculate on a hypothesis that TMAO plays some role in marine fish at low temperatures. With currently available data, this can only be done with marine teleosts living near the freezing point of seawater as there are insufficient data to support a more general hypothesis on TMAO accumulation in cold water fishes. Yet the data in this thesis do build upon previous studies and go one step further to determining if the accumulation of TMAO may have general adaptive significance at low temperatures.

Raymond and DeVries (1998) have shown a trend of increasing liver TMAoxi activity with increasing serum TMAO. The data in chapters 2 and 3 do not support this hypothesis. The deep-sea fishes with detectable liver TMAoxi actually had much lower levels than the shallow water species that had marginal plasma TMAO concentrations. Furthermore, smelt were found to have no appreciable liver TMAoxi activity while the

kidney had levels of this enzyme an order of magnitude higher than the liver activities presented in Raymond and DeVries (1998). As such, the studies in this thesis do support the hypothesis that certain species of fish have the metabolic machinery to make TMAO but whether or not TMAO is actively synthesized is still an unanswered question.

A major consistency found in all species examined in Chapters 2 and 3 as well as in many species previously examined (see Hebard et al. 1982) is that white muscle has a disproportionately high TMAO content relative to plasma/serum concentrations and the levels in other tissues. This is true not only in teleosts, but also in elasmobranch fishes, which is an observation often ignored. This suggests that there is some active means of TMAO uptake and retention, or an underscribed synthetic pathway for TMAO, in the swimming muscle of marine fishes and that TMAO plays some physiologically important role in the function of striated muscle. Interestingly, the heart also shows a trend of higher TMAO content than would be expected if in equilibrium with the plasma concentration. Again, this suggests an active uptake of TMAO and some role in the functioning of striated muscle.

Taken together, the results in chapters 2 and 3 demonstrate not only that TMAO is accumulated in deep-sea fish and smelt at temperatures approaching 0°C, but also that there is some consistency in the intertissue distribution of TMAO, with striated muscle (especially locomotory muscle) having the highest TMAO contents. Although this has been examined in the past, albeit in less detail, previous works tended to be geared to more descriptive rather than functional interpretation. The present work illustrates that muscle is not representative of other tissues although it appears that some presume that it

is, especially in the case of elasmobranchs (the often touted 2:1 ratio of urea to TMAO or total methylamines is based mostly on data from white muscle).

Finally, two excellent model 'systems' have been found for the study of TMAO, it's metabolism and importance in marine fishes; comparison of shallow living species with deep-sea confamiliar species and the seemingly unique smelt which via experimental manipulation can be used as a whole animal model system that can increase, or decrease, TMAO allowing for the study of how these changes are mediated and regulated. In the future I plan to use the above model systems, as well as others, to further elucidate the metabolism, regulation and importance of TMAO and related methylamines in aquatic animals.

4.1 Literature cited

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Appendix 1.

Occurrence of liver trimethylamine oxidase (TMAoxi) activity in teleost and elasmobranch fishes, activity in μmol $g^{-1}hr^{-1}$. RL, radiolabelled assay, Spec., spectrophotometric assay, BLD, activity below limits of detection.

Species	Common name	TMAoxi	Method	Reference
Teleosts				
Perciformes				
Leptocottus armatus	Bullhead	16.8	RL	Baker et al. 1963
Porichthys notatus	Northern midshipman	16.7	RL	Baker et al. 1963
Ophiodon elongatus	Lingcod	4.2	RL	Baker et al. 1963
Phanerodon furcatus	Perch	BLD	RL	Baker et al. 1963
Anoplopoma fimbria	Sable fish	< 1.0	RL	Baker et al. 1963
Roccus saxatilis	Striped bass	11.5	RL	Baker et al. 1963
Pneumatophuorus diego	Pacific mackerel	BLD	RL	Baker et al. 1963
Nototheniformes				
Dissostichus mawsoni	Antarctic toothfish	0.94	Spec.	Raymond and DeVries 1998
Gymnodraco acuticeps	Antarctic dragonfish	0.72	Spec.	Raymond and DeVries 1998
Notothenia angustata	Cod icefish	BLD	Spec.	Raymond and DeVries 1998
Pleuronectiformes				
Hippoglossus stenolepis	Pacific halibut	10.4	RL	Baker et al. 1963
Atheyesthes stomias	Arrowtooth halibut	< 1.0	RL	Baker et al. 1963
Psettichthys melanostictus	Sand sole	4.2	RL	Baker et al. 1963
Eopsetta jordani	Petral sole	BLD	RL	Baker et al. 1963
Microstomis pacificus	Dover sole	BLD	RL	Baker et al. 1963
Parophyrus ventulus	English sole	< 1.0	RL	Baker et al. 1963
Platichthys stellatus	Starry flounder	BLD	RL	Baker et al. 1963
Limanda limanda	Yellowtail flounder	BLD	RL	Agustsson and Strom 1981
Pleuronectes platessa	Flounder spp.	BLD	RL	Agustsson and Strom 1981
Gadiformes				
Gadus morhua	Cod	2.0-9.0	RL	Agustsson and Strom 1981
Eleginus gracillus	Saffron cod	0.21	Spec.	Raymond 1998
Salmoniformes				
Osmerus mordax	Smelt	0.24	Spec.	Raymond 1998
Onchorhynchus tshawytascha	Chinook salmon	< 1.0	RL	Baker et al. 1963
Onchorhynchus gairdneri (SW)	Rainbow trout	3.5	RL	Baker et al. 1963
Onchorhynchus gairdneri (FW)	Rainbow trout	< 0.2	RL	Baker et al. 1963

Continued ...

Appendix 1 continued:

Species	Common name	TMAoxi	Method	Reference
Elopiformes				
Alosa sapidissima	Shad	2.7	RL	Baker et al. 1963
Clupea harengus	Herring	0.65	Spec.	Raymond 1998
Elasmobranchs				
Rajiformes				
Raja erinacea	Little skate	0.0051	RL	Goldstein and DeWitt-Harley 1973
Raja binoculata	Big skate	< 1.0	RL	Baker et al. 1963
Torpedo californica	Electric ray	BLD	RL	Baker et al. 1963
Squaliformes				
Squalus acanthias	Spiny dogfish	< 1.0	RL	Baker et al. 1963
" " " "		0.0041	RL	Goldstein and DeWitt-Harley 1973
Orectolobiformes				
Ginglymostoma cirritum	Nurse shark	6.3	RL	Goldstein and DeWitt-Harley 1973
Carcharhiniformes				
Mustelus californicus	Smoothhound	17.1	RL	Baker et al. 1963
Negaprion brevirostrus	Lemon shark	8.3	RL	Goldstein and DeWitt-Harley 1973

Note: For full reference citations, see chapter 1.

Appendix 2.

The *in vivo* conversion of injected radiolabelled potential precursors of TMAO in various fishes. + conversion to TMAO, ± marginal conversion to TMAO of questionable physiological significance, - conversion below levels of detection.

Species	Precursor	Conversion to TMAO	Reference
Platichthys stellatus	Methyl-[^{14} C]-Choline	+	Bilinski 1964
	Methyl-[^{14} C]-TMA	+	Bilinski 1964
	Methyl-[^{14} C]-Butyrobetaine	+	Bilinski 1964
	[^{14} C]-Methylamine	±	Bilinski 1964
	Methyl-[^{14} C]-Carnitine	±	Bilinski 1964
	2-[^{14} C]- Glycine	±	Bilinski 1964
	[^{14} C]-Formate	-	Bilinski 1964
Parophrys vetulus	Methyl-[^{14} C]-Betaine	+	Bilinski 1964
	Methyl-[^{14} C]-Methionine	+	Bilinski 1964
	1-[^{14} C]- Acetate	-	Bilinski 1964
	2-[^{14} C]- Acetate	±	Bilinski 1964
	2-[^{14} C]- Bicarbonate	-	Bilinski 1964
Gadus morhua	Methyl-[^{14} C]-TMA	+	Augustsson and Strom 1981
Paralabrax clathratus	Methyl-[^{14} C]-TMA	+	Charest et al. 1988
	Methyl-[^{3} H]-Choline	+	Charest et al. 1988
	Methyl-[^{14} C]-Choline	-	Charest et al. 1988
	Methyl-[^{14} C]-Betaine	+	Charest et al. 1988
Oncorhynchus gorbuscha	Methyl-[^{14} C]-TMA	+	Charest et al. 1988
	Methyl-[^{14} C]-Betaine	+	Charest et al. 1988
Squalus acanthias	Methyl-[^{14} C]-Choline	-	Goldstein et al. 1967
	Methyl-[^{14} C]-Choline	-	Goldstein et al. 1967
Ginglymostoma cirratum	Methyl-[^{14} C]-Choline	+	Goldstein and Funkhouser 1972
	Methyl-[^{14} C]-TMA	+	Goldstein and Funkhouser 1972
Negaprion brevirostrus	Methyl-[^{14} C]-TMA	+	Goldstein and DeWitt-Harley 1973
Dasyatis americana	Methyl-[^{14} C]-TMA	+	Goldstein and DeWitt-Harley 1973
Raja erinacea	Methyl-[^{14} C]-TMA	+	Goldstein and DeWitt-Harley 1973

Note: For full reference citation, see chapter 1.