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The accumulation of methylamine counteracting solutes in elasmobranchs with differing levels of urea: a comparison of marine and freshwater species

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Summary

We compared levels of the major organic osmolytes in the muscle of elasmobranchs, including the methylamines trimethylamine oxide (TMAO), betaine and sarcosine as well as the β-amino acids taurine and β-alanine, and the activities of enzymes of methylamine synthesis (betaine and TMAO) in species with a wide range of urea contents. Four marine, a euryhaline in freshwater (*Dasyatis sabina*), and two freshwater species, one that accumulates urea (*Himantura signifer*) and one that does not (*Potamotrygon motoro*), were analyzed. Urea contents in muscle ranged from 229–352 μmol g^{-1} in marine species to 2.0 μmol g^{-1} in *P. motoro*. Marine elasmobranchs preferentially accumulate methylamines, possibly to counteract urea effects on macromolecules, whereas the freshwater species with lower urea levels accumulate the β-amino acid taurine as the major non-urea osmolyte. A strong correlation (r^2=0.84, P<0.001) with a slope of 0.40 was found between muscle urea content and the combined total methylamines plus total β-amino acids, supporting the hypothesis that 'non-urea' osmolytes are specifically maintained at an approximately 2:1 ratio with urea in the muscle of elasmobranchs. All species examined had measurable synthetic capacity for betaine in the liver but only one species had detectable TMAO synthetic capacity. We propose a phylogenetic explanation for the distribution of TMAO synthesis in elasmobranchs and suggest that activation of liver betaine aldehyde dehydrogenase, relative to choline dehydrogenase, coincides with betaine accumulation in elasmobranchs. The latter relationship may be important in maintaining methylamine levels during periods of low dietary TMAO intake for species lacking TMAO synthesis.

Key words: trimethylamine oxide (TMAO), betaine, trimethylamine oxidase, choline dehydrogenase, betaine aldehyde dehydrogenase, organic osmolyte, elasmobranch.

Introduction

Urea is a small organic compound, often considered a metabolic waste product, which at high concentrations is known to have deleterious effects on protein structure and function; however, there are several examples of vertebrate tissues and cells that accumulate urea to high concentrations (reviewed by Yancey, 1994). For example, as part of their osmoconforming osmoregulatory strategy, marine elasmobranchs accumulate urea to concentrations as high as 300–500 mmol l^{-1} (Yancey et al., 1982; Yancey, 1994; Ballantyne, 1997). Urea can also reach levels greater than 1000 mmol l^{-1} in the inner medullary region of the kidney of some dehydration tolerant mammals (Yancey, 1988). It has been hypothesized that methylamine organic osmolytes are accumulated in concert with urea to counteract the perturbing effects of urea on protein structure (Yancey et al., 1982; Yancey, 1994). Based on *in vitro* experiments, a urea to 'counteracting' solutes concentration ratio of about 2:1 may be optimal for preserving proper protein function (Yancey, 1994), but few data on *in vivo* experimental tests of this ratio are available.

In a study of three rodent species (Yancey, 1988) the urea gradient found from the mammalian renal cortex to the inner medulla was exploited as a test of the hypothesis that counteracting solutes protect against urea-induced protein denaturation. Consistent with the hypothesis, Yancey found that (i) the total methylamine content of the kidney increases along the urea gradient, and (ii) there was a significant correlation between total methylamine and urea content in the outer and inner medulla across mammalian species (Yancey,

1988). However, the cortex of all three species had much lower total methylamine content than would be expected based on the regression analysis of the medullary regions, despite having significant urea content (30–44 μmol g^{-1}). Furthermore, the cortex and medulla (outer and inner) are anatomically distinct tissues with different functions and intracellular volumes, which complicates interpretations because the accumulation of methylamines in the kidney is largely an intracellular phenomenon (discussed by Yancey, 1988).

Similar to the mammalian kidney, the muscle of marine elasmobranchs also has high non-urea organic osmolyte content. The methylamines trimethylamine oxide (TMAO), betaine and sarcosine (Withers et al., 1994; Steele et al., 2005), as well as β-amino acids, taurine and β-alanine, make up most of the non-urea organic osmolyte component in marine elasmobranch muscle (Yancey, 2001). As is the case in the mammalian kidney, the muscle intracellular fluid of elasmobranchs accumulates methylamines to a higher concentration than is found in the extracellular fluid. It should be noted that methylamines are generally viewed as counteracting solutes whereas β-amino acids are considered compatible solutes; that is, high concentrations of the latter have relatively little effect on protein structure (Yancey, 1994). While the bulk of the non-urea organic osmolytes are typically methylamines (see reviews by Yancey et al., 1982; Yancey, 1994; Yancey, 2001), some marine elasmobranch species require the inclusion of β-amino acids to achieve or approach the 2:1 ratio between urea and other solutes (Steele et al., 2005).

Elasmobranchs are not strictly a marine group of fishes. Stingrays of the family Potamotrygonidae, which have been isolated from their marine ancestors in the Amazon basin for about 15–23 million years (Lovejoy et al., 1998), have entirely lost the physiological capacity to accumulate urea in their tissues (Thorson et al., 1967; Thorson, 1970) and are considered stenohaline freshwater species, i.e. they are unable to survive in salinities of more than about 40% seawater (Thorson, 1970; Tam et al., 2003). In contrast to stenohaline elasmobranchs that are restricted to either marine or freshwater environments, there are also euryhaline elasmobranchs capable of living in both marine and freshwater habitats. Under marine conditions, euryhaline elasmobranchs osmoregulate in a similar manner as strictly marine species (Piermarini and Evans, 1998; Pillans et al., 2005), but acclimation of a euryhaline species to freshwater is associated with a marked decrease of body fluid osmolality that is largely attributed to a reduction in the concentration of urea. Even once fully adapted to freshwater, euryhaline elasmobranchs maintain levels of urea that are quite high (approximately 140–200 mmol l^{-1}) (Thorson et al., 1973; Piermarini and Evans, 1998: Pillans and Franklin, 2004). Recently, a third 'intermediate' form of freshwater elasmobranch has been discovered. *Himantura signifer*, also a stingray, is normally found in freshwater but does venture into brackish water and still has the capacity for urea accumulation (Tam et al., 2003). Plasma urea in *H. signifer* in freshwater is about 45–70 mmol l^{-1}. This species has higher salinity tolerance than potamotrygonid stingrays, partially due to its ability to elevate the osmolality of body fluids *via* further accumulation of urea when acclimated to 60% seawater (Tam et al., 2003; Ip et al., 2005).

In parallel with a pattern of decreased capacity for urea accumulation in freshwater elasmobranchs compared to marine species, there is a diminished capacity for urea synthesis *via* the ornithine–urea cycle. For example, the rate limiting step of urea synthesis in the liver, carbamoylphosphate synthetase III (CPS III) activity, is very low in the stenohaline freshwater *Potamotrygon motoro* compared to euryhaline and marine species (Anderson, 1980; Tam et al., 2003).

In contrast to urea, little is known about the synthesis of methylamines in elasmobranchs. The ability to oxidize trimethylamine (TMA) to TMAO is considered an essential component of TMAO synthesis, and the enzyme that catalyzes this reaction is trimethylamine oxidase (TMAoxi), which occurs sporadically in elasmobranchs (Baker et al., 1963; Goldstein and Dewitt-Harley, 1973). When present, TMAoxi activity is high in the liver of elasmobranchs (Goldstein and Dewitt-Harley, 1973), suggesting that this organ is a major site of TMAO synthesis (Baker et al., 1963; Goldstein and Funkenhouser, 1972; Goldstein and Dewitt-Harley, 1973). To our knowledge betaine synthesis has never been studied in an elasmobranch. In mammals and other vertebrates, betaine is synthesized from choline by the sequential and physiologically irreversible dehydration reactions of choline dehydrogenase (ChoDH) and betaine aldehyde dehydrogenase (BADH). Unlike the sporadic pattern of TMAoxi activity, it is likely that the betaine synthetic pathway is found in most if not all elasmobranchs, because it plays a critical role in the catabolism of dietary choline (Haubrich and Gerber, 1981).

The physiological range of urea accumulation found in elasmobranchs, from several hundred millimolar in marine species to negligible amounts in the potamotrygonid stingrays, provides a unique opportunity to test the hypothesis that counteracting solutes are maintained in a conserved ratio with urea, while avoiding the complicating factors associated with the mammalian kidney (e.g. differing intracellular volumes). Therefore, the first goal of this study was to determine if specific methylamines, the total methylamine content, or the combined total of non-urea osmolytes (methylamines and the β-amino acids taurine and β-alanine) are maintained in a conserved ratio with urea in muscle tissue among elasmobranch species with varying degrees of intracellular urea accumulation.

The second goal of this study was to determine if the activity of enzymes involved with methylamine synthesis (TMAO and betaine) in the liver vary in elasmobranchs according to the degree that a particular methylamine is accumulated in muscle, like the relationship between CPS III and urea (see above). To accomplish this, we again exploited the interspecific variation in methylamine accumulation across marine and freshwater elasmobranchs. We focused on the synthesis of TMAO and betaine because these are the predominant methylamines found in the muscle of elasmobranchs (Withers et al., 1994; Steele et

al., 2005) and these compounds are generally the most effective organic osmolytes for the counteraction of urea effects on proteins (Yancey, 1994). We hypothesized that in species that lack TMAoxi activity, there may be elevated betaine synthetic capacity. If methylamines are required to counteract urea effects, the capacity for betaine synthesis may be important in species lacking TMAoxi activity during periods of reduced dietary TMAO intake, in order to supplement the muscle methylamine pool *via* betaine synthesis.

Materials and methods

Animals

Marine species

Leucoraja ocellata Mitchell were caught by divers in Conception Bay, Newfoundland (Canada), and transported to the Ocean Sciences Centre where they were housed in flow-through seawater (31–31.5‰) tanks maintained at 8–13°C for at least 3 months prior to sampling. During this period, they were fed chopped herring 2–3 times per week to satiation. *Leucoraja erinacea* Mitchill were caught by otter-trawl in Passamaquoddy Bay, New Brunswick, Canada, transported to the Huntsman Marine Science Laboratory and sampled within 5–7 days of capture. Animals were housed in a flow-though seawater (approximately 31‰) tank (11–14°C) without feeding.

Taeniura lymma Forsskål and *Chiloscyllium punctatum* Müller and Henle were purchased at a live fish market in Singapore approximately 6 h after capture from the ocean. They were transported to the National University of Singapore (NUS), where they were held in full-strength (30‰) seawater at 25°C in fibreglass aquaria under a 12 h:12 h photoperiod. *T. lymma* were sampled within 3 days of acquisition; *C. punctatum* were sampled the same day they were obtained. Water was changed daily and the animals were not fed.

Euryhaline species in freshwater

Dasyatis sabina Lesueur were caught on baited trot-lines from the St Johns River system, Florida, USA, and housed in freshwater tanks (<1‰) as described (Piermarini and Evans, 1998).

Freshwater species

Potamotrygon motoro Müller and Henle and *Himantura signifer* Compagno and Roberts were obtained from a fish farm in Singapore, where they were fed bloodworms (Chironomidae). They were transported to NUS in water-filled plastic bags. Holding conditions were as described (Tam et al., 2003). Briefly, animals were held in freshwater at 25°C in plastic aquaria (3 animals per aquarium) under a 12 h:12 h photoperiod. Water was changed daily, the animals were not fed. *P. motoro* were sampled within 3 days of acquisition. Three days after specimen acquisition, a group of *H. signifer* was gradually acclimated to half-strength seawater (15‰) using the following regime: freshwater (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (day 4, held until day 14). A control group was kept in freshwater. Both the control and experimental groups comprised animals in several separate aquaria to avoid tank effects. Animals were not fed for the duration of the experiment. Animals from both groups were sampled after 14 days of acclimation. All fish used in this study were in apparent good condition and were maintained on a natural photoperiod unless otherwise stated.

TMAO determination

Samples were homogenized in 9 volumes of ice-cold TCA (5%) with a motorized tissue homogenizer and left on ice for 10 min to precipitate protein. Following centrifugation, 15 600 ***g*** for 5 min, supernatants were assayed directly for TMAO spectrophotometrically (Wekell and Barnett, 1991), as described previously (Sulikowski et al., 2003; Treberg et al., 2005). Elasmobranch tissues typically have low TMA content compared to TMAO (Treberg and Driedzic, 2002), which was confirmed for muscle samples from *L. ocellata*, *L. erinacea* and *D. sabina* (data not shown); thus samples have not been corrected for endogenous TMA. For samples that were expected to have very low TMAO content (*P. motoro* and *H. signifer*), we further modified the assay to improve the lower limits of detection. Tissues were prepared as described above, and 300 μl of TCA extract was added to a 1.5 ml centrifuge tube followed by 300 μl of both toluene and then the iron–EDTA reducing mixture as described (Wekell and Barnett, 1991). The tube was closed and heated at 50°C for 5 min. Tubes were allowed to cool to room temperature, 600 μl of 45% KOH was added and the tube was vortex mixed for 15 s. Tubes were vortexed twice more, after allowing 3–5 min between mixings for phase separation, to extract TMA into the toluene phase. After the final mixing, 200 μl of the toluene phase was added to another 1.5 ml centrifuge tube containing approximately 10–20 mg of sodium sulphate followed by 500 μl of 0.02% picric acid in toluene. This was mixed and left for at least 2 min to remove any residual water and the absorbance of 650 μl of the toluene phase was read at 410 nm. Trimethylamine oxide content of tissues was determined based on the absorbance at 410 nm compared to a standard curve (0–3 mmol l^{-1} for the standard assay and 0–0.3 mmol l^{-1} for the higher resolution assay describe above).

HPLC analysis of organic osmolytes

The determination of all other organic osmolytes was done by HPLC using a Waters SugarPak column and refractive index detector. Muscle samples were homogenized in 9 volumes of 6% PCA and then prepared as described (Wolff et al., 1989), where the mobile phase is 50 mg l^{-1} Ca-EDTA. While this provided suitable resolution of most osmolytes, we found that there was poor resolution between betaine and alanine (Fig. 1). Tam et al. (Tam et al., 2003) found that alanine content is significant (2 μmol g^{-1}) in the muscle of *H. signifer*. Since alanine would potentially complicate betaine determinations in this species, as well as *P. motoro*, we

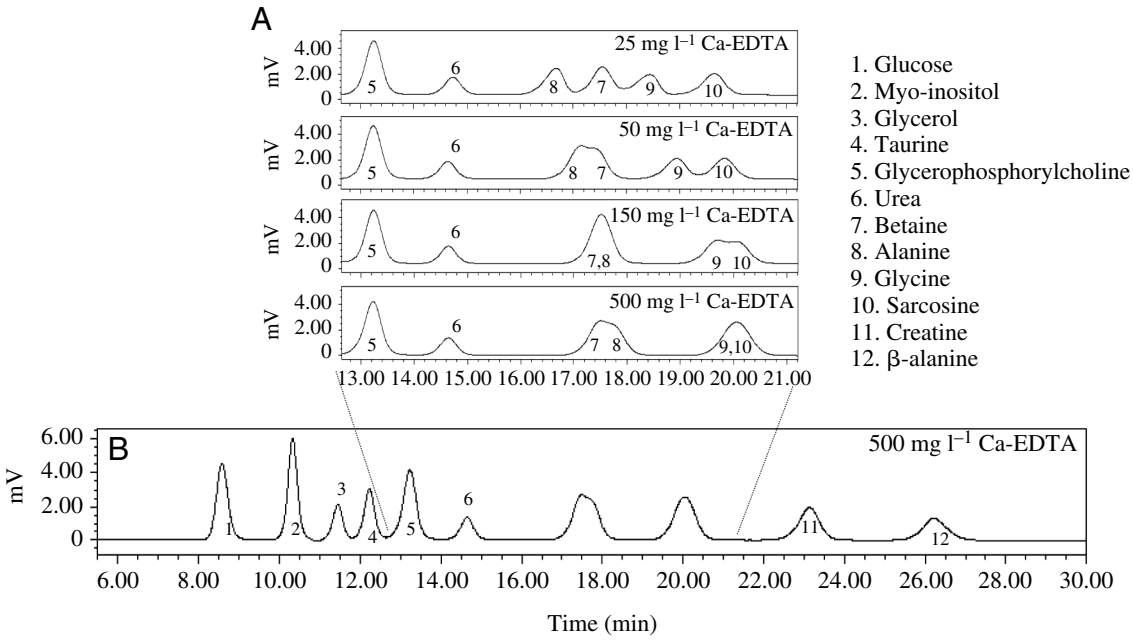

Fig. 1. The separation of common organic osmolytes using a Waters SugarPak column, as described in the Materials and methods. (B) Separation of most major osmolytes common in elasmobranch muscle, as well as several other common organic osmolytes, using a mobile phase of 500 mg l^{-1} Ca-EDTA. (A) Enlargement of the region of response from the approximate retention times of 12.5 to 21 min, using different concentrations of Ca-EDTA as the mobile phase. Note the retention times of glycine and alanine are reduced as the concentration of Ca-EDTA decreases.

modified the method of Wolff et al. (Wolff et al., 1989) to improve the resolution of alanine and betaine.

Using a flow rate of 0.6 ml min^{-1}, at a column temperature of 90°C, we tried a number of concentrations of Ca-EDTA in the mobile phase and found that a mobile phase of 500 mg ml^{-1} was suitable for the determination of all solutes in the present study (data not shown), except betaine and sarcosine because of little to no resolution from alanine and glycine, respectively (Fig. 1). However, a mobile phase of 25 mg l^{-1} Ca-EDTA allowed for suitable resolution of betaine, alanine, glycine and sarcosine (Fig. 1) if the column is fully equilibrated with calcium, which is a requirement to maintain resolution. By running a set of samples (4–6) at 500 mg l^{-1} followed by alternating to the 25 mg l^{-1} Ca-EDTA mobile phase and re-running the same samples, all of the major organic osmolytes (other than TMAO) could be assessed.

Enzyme activities

Liver samples were homogenized in 5 or 9 volumes of ice-cold buffer (50 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA, pH 7.5 at 20°C). For ChoDH and BADH, Triton X-100 was added to homogenates (0.2% final concentration). Homogenates were used without centrifugation for ChoDH and TMAoxi. For BADH, homogenates needed to be cleared by centrifuging (15 600 ***g*** for 10 min at 4°C) to reduce the light scattering effects of cellular debris. All assays were conducted at 20°C and were linear with time and protein.

Choline dehydrogenase, which catalyzes the conversion of choline to betaine aldehyde, was assayed by a modification of the method used by Haubrich and Gerber (Haubrich and Gerber, 1981). The assay mixture was composed of 50 mmol l^{-1} phosphate buffer (pH 8.0), 2 mmol l^{-1} phenazine methosulphate (PMS) and 5 mmol l^{-1} choline, including sufficient [^{14}C-methyl]choline-HCl to give 0.125 μCi ml^{-1} (1 Ci=3.7×10^{10} Bq) in the final assay volume of 300 μl. The reaction was terminated by adding an equal volume (300 μl) of ice-cold 6% PCA followed by centrifugation (15 600 ***g*** for 5 min). 500 μl of the supernatant was collected and mixed with 45 μl of 6 mol l^{-1} KOH to remove most of the perchlorate. This was centrifuged as above, the supernatant collected and frozen (–20°C) for later analysis.

To determine the amount of [^{14}C-methyl]betaine aldehyde produced, samples were thawed and duplicate 100 μl portions mixed with 20 μl of 1 mol l^{-1} NaOH and 20 μl of 30% hydrogen peroxide and incubated at room temperature for 60 min to chemically oxidize betaine aldehyde to betaine. To separate [^{14}C-methyl]choline from [^{14}C-methyl]betaine, a small ion-exchange column was made by adding 1 ml of a 1:1 slurry of BioRad AG-50W resin (Hercules, CA, USA; Li^+ form, 200–400 mesh) and water to a Pasteur pipet with the tip blocked with a small piece of cotton. The column was packed with 2 ml of deionized water and the entire oxidized sample was added to the column. Labeled choline is retained by the resin while betaine was washed off with 2 ml of deionized water and collected into a 20 ml scintillation vial. The elutant was mixed with 10 ml of Ecolume (ICN, Irvine, CA, USA) followed by scintillation counting. The increase in

radioactivity, relative to that found when PCA was added before initiating the reaction, was used as the measure of ChoDH activity. The formation of product was entirely dependent on the inclusion of the electron acceptor PMS (there was no difference in values obtained without PMS and when the reaction was immediately terminated after adding substrate).

A spectrophotometric assay (Perrino and Pierce, 2000) was used to determine the BADH activity at 340 nm on centrifuged homogenates. Control rates of absorbance change in the absence of substrate were determined and subtracted in all cases.

A radioisotopic assay was developed, based on the assay of Baker and Chaykin (Baker and Chaykin, 1962), to determine the TMAoxi activity of liver homogenates. The assay conditions were as follows: 50 mmol l^{-1} Tris (pH 8.5), 50 mmol l^{-1} KCl, 0.3 mmol l^{-1} NADPH, 1.0 mmol l^{-1} TMA including [^{14}C]TMA at a final concentration of 0.1 μCi ml^{-1}. Control values were obtained by the omission of NADPH from the assay medium – product formation is entirely dependent on NADPH (data not shown). The reaction was stopped by combining the reaction mixture with an equal volume of ice-cold 5% TCA. After 10 min, to allow complete precipitation of proteins, the mixture was centrifuged at 15 600 ***g*** for 5 min at room temperature and 150 μl of the supernatant loaded onto a small column of BioRad AG-50W resin (Na^+ form, 200-400 mesh). Columns were made by adding 1 ml of a 1:1 slurry of resin and deionized water (0.5 ml bed volume) to a Pasteur pipet with the tip blocked by a small amount of cotton. The resin was washed and packed by two 1 ml volumes of deionized water prior to the addition of the TCA extracted assay mixture. The TCA extract was loaded into the column and non-exchangeable compounds removed by two 1 ml volumes of deionized water. Radiolabelled TMA and TMAO were eluted with 2 ml of 0.1 mol l^{-1} NaOH and the elutant collected into a 20 ml scintillation vial. The elutant was heated to 70–80°C and evaporated to dryness in a fumehood. Under alkaline conditions TMA is volatile whereas TMAO is not, thus by evaporating the 0.1 mol l^{-1} NaOH elutant under moderate heat the TMA is removed while the [^{14}C]TMAO remains. The residue was redissolved in 1 ml of deionized water and mixed with 10 ml of scintillation cocktail (Ecolume) to determine the amount of TMAO produced by scintillation counting.

The protein concentration of homogenates was determined using a commercial kit (BioRad D_c protein assay) using BSA as a standard.

Statistical analysis

When appropriate, means were compared by one-way analysis of variance (ANOVA; $P<0.05$ was considered significant) and patterns across species were assessed by linear regression.

Results

Methylamines and β-amino acids in the muscle of marine and freshwater elasmobranchs

Marine species

The total methylamine content (summed values for TMAO, betaine and sarcosine) in marine elasmobranch muscle ranged from approximately 100 to 160 μmol g^{-1} (Fig. 2). There was substantial interspecific variation in the distribution of methylamines in the marine specimens. In the shark *C. punctatum*, the predominant methylamine was TMAO. The skate *L. ocellata* accumulates mostly TMAO but also has notable amounts of sarcosine, whereas the closely related skate *L. erinacea* has roughly equivalent levels of TMAO, betaine and sarcosine. Finally, the stingray *T. lymma* accumulates similar levels of TMAO and betaine and negligible amounts of sarcosine (Fig. 2). The distribution of β-amino acids was also highly species dependent in marine animals. A modest amount of taurine was found in *C. punctatum* along with negligible β-alanine. All three marine species of the order Rajiformes (skates and rays) have substantial total β-amino acid content (sum of taurine and β-alanine) but the skates predominantly accumulated β-alanine while the stingray, *T. lymma*, preferentially accumulates taurine (Fig. 2).

Of note, glycerophosphorylcholine and polyol (glycerol, sorbitol, myo-inositol) content was low (<1 μmol g^{-1}) in all species examined (data not shown). Creatine values ranged from 38 to 52 μmol g^{-1} in muscle and did not relate to urea accumulation.

Euryhaline species in freshwater

The non-urea osmolytes in the muscle of *D. sabina* are

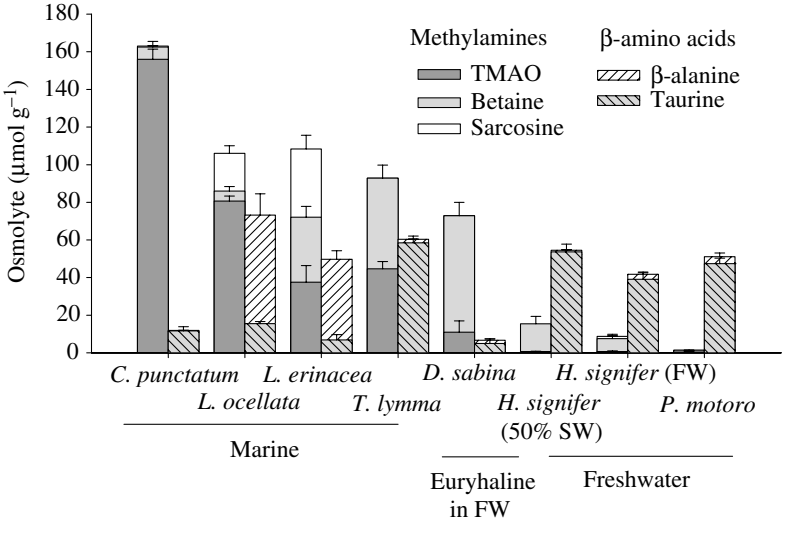

Fig. 2. Muscle organic osmolyte contents (μmol g^{-1}) in marine, euryhaline and freshwater elasmobranchs. Values are mean ± s.e.m. and N=6 (*C. punctatum*), 4 (*L. ocellata*), 4 (*L. erinacea*), 6 (*T. lymma*), 5 (*D. Sabina*), 7 (all compounds except TMAO, where N=5) in *H. signifer* acclimated to 50% seawater, 10 (all compounds except TMAO, where N=9) in *H. signifer* acclimated to freshwater (FW), 7 (all compounds except TMAO, where N=5) in *P. motoro*.

predominantly methylamines, mostly betaine, with β-amino acids making only a modest contribution.

Freshwater species

The most striking pattern in muscle of the two freshwater species examined is that β-amino acids are accumulated to a much greater extent than methylamines, regardless of whether the species accumulates urea (Fig. 2). Taurine is the predominate β-amino acid in both *H. signifer* and *P. motoro*. Betaine was found at levels of 14.7±4.0 μmol g^{-1} (mean ± s.e.m.) and 6.8±2.3 μmol g^{-1} in the muscle of *H. signifer* acclimated to 50% seawater and freshwater, respectively. Sarcosine was low in all freshwater species (~1.0 μmol g^{-1} or less), as was TMAO: 0.67±0.15 μmol g^{-1} for *H. signifer* in 50% seawater, 0.71±0.28 μmol g^{-1} *H. signifer* in freshwater and 0.39±0.16 μmol g^{-1} in the muscle of *P. motoro.*

Plasma methylamines in urea accumulating elasmobranchs in freshwater

Plasma concentrations of TMAO and betaine were 5.7±0.82 mmol l^{-1} and 3.9±1.0 mmol l^{-1}, respectively, in *D. sabina* and were below the limits of detection in *H. signifer*. This indicates that, like marine elasmobranchs (see Withers et al., 1994), there is a much lower methylamine concentration in the extracellular fluid than in the intracellular fluid of the muscle in freshwater elasmobranchs.

Total methylamines and β-amino acids in comparison to muscle urea content

Values for muscle urea content followed the expected decrease from marine to freshwater species. Urea content ranged from 230 to 350 μmol g^{-1} for marine species, was 146±7.0 μmol g^{-1} in the euryhaline freshwater *D. sabina*, and was 96±6.5 and 75±3.1 μmol g^{-1} for 50% seawater and freshwater acclimated *H. signifer*, respectively. Urea accumulation in *P. motoro* was negligible (2.0±0.4 μmol g^{-1}) (Fig. 3). No single methylamine or β-amino acid correlated with urea across species (data not shown). Total methylamines show a qualitatively similar trend across species, as seen with urea, while total β-amino acids do not. Although total β-amino acids appear to have a random distribution among marine and euryhaline species, when a species has high total methylamines (for example *C. punctatum* and *D. sabina*) it also has a proportionally lower total β-amino acids (Fig. 3).

The qualitative trend between muscle urea and total methylamine content (Fig. 3) is statistically supported ($P<0.01$) by linear regression analysis (Fig. 4), with a slope approaching that which would be expected for a 2:1 ratio between intracellular urea and total methylamines (see below). However, the fit of the data is modest ($r^2=0.65$) compared to the regression of total methylamines plus total β-amino acids (discussed below). Also, there are some species far off the overall trend (e.g. *C. punctatum*), and despite having much lower urea content, *D. sabina* has a total methylamine content similar to the marine skates and stingray. These observations do not support the notion of a highly conserved ratio between total methylamines and urea in elasmobranch muscle in all species. The data do illustrate that species with high levels of urea (i.e. marine species and *D. sabina* in freshwater), preferentially accumulate methylamines over β-amino acids.

The apparent inverse relationship between total methylamines and β-amino acids is explained when compared across all species relative to the urea content (Fig. 4). There is a highly significant ($P<0.001$) correlation between urea and the combined total methylamines and total β-amino acids. The total 'non-urea' osmolyte levels are linked to the degree of urea accumulation and the slope of the regression (0.40) is very close to what would be expected if a 2:1 ratio was maintained intracellularly. It is important to appreciate that methylamines and β-amino acids are accumulated intracellularly in marine species (between 2- and >10-fold) and based on the difference in muscle and plasma values this is also the case in euryhaline and freshwater species. Because of this discrepancy between extra and intracellular fluids, if the 2:1 ratio is only achieved in the intracellular space, dilution of the intracellular pool by extracellular fluid, which is approximately 10% of total muscle tissue fluid (Forster and Goldstein, 1976), would result in a slope of less than 0.5. Urea concentration in the intracellular and extracellular fluid is similar and would not be influenced in the same manner (see review by Yancey, 2001).

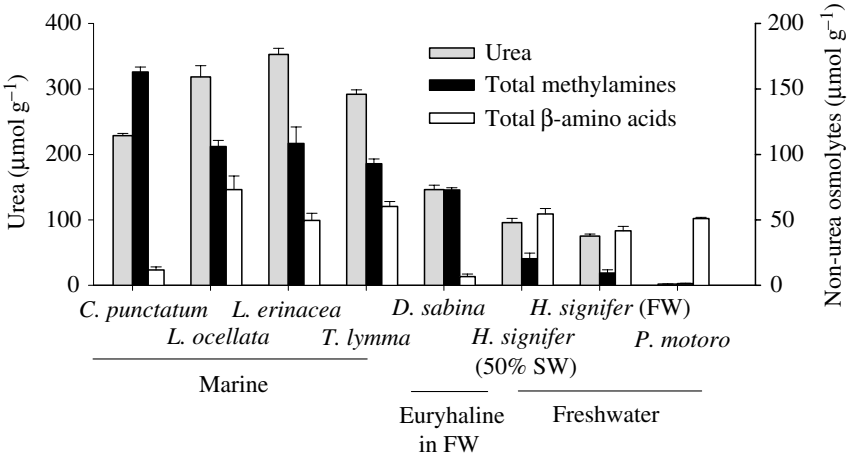

Fig. 3. Muscle urea content (μmol g^{-1}) with the total methylamine and β-amino acid (non-urea osmolytes) contents (μmol g^{-1}) in marine, euryhaline species in freshwater (FW) and freshwater elasmobranchs. Values are mean ± s.e.m. and N=6 (*C. punctatum*), 4 (*L. ocellata*), 4 (*L. erincea*), 6 (*T. lymma*), 5 (*D. sabina*), 5 (methylamines) and 7 (β-amino acids) in *H. signifer* acclimated to 50% seawater, 9 (methylamines) and 10 (β-amino acids) in *H. signifer* acclimated to freshwater, 5 (methylamines) and 7 (β-amino acids) in *P. motoro*.

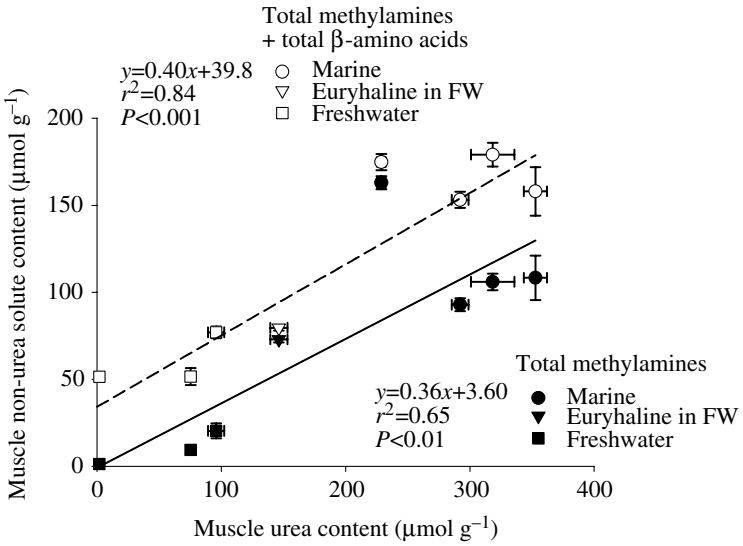

Fig. 4. Relationship of muscle urea content with total methylamine (closed symbols, solid line) or combined total of methylamine and β-amino acid (open symbols, broken line) content in marine (circles), euryhaline species in freshwater (FW) (triangles) and freshwater elasmobranchs (squares). Values are mean ± s.e.m. and *N* values are the same those for total methylamine contents in Fig. 4. The regressions are statistically significant (total methylamines, $P<0.01$; total methylamines plus β-amino acids, $P<0.001$).

Muscle organic osmolyte accumulation in H. signifer *in response to salinity challenge*

Although not the primary goal of this study, by examining the muscle solutes in *H. signifer* that have been acclimated to 50% seawater in a fasted state, it is possible to determine which solutes they can synthesize and accumulate strictly from endogenous resources. When acclimated to 50% seawater, muscle urea and taurine content significantly increased in *H. signifer*. Mean betaine content more than doubled compared to freshwater control animals (15±4.0 and 6.8±2.3 μmol g^{-1}, respectively). While this increase in betaine only approached statistical significance ($P=0.084$), there are a number of considerations that are suggestive of endogenous synthesis of a methylamine solute in response to salinity challenge. This has not yet been demonstrated in an elasmobranch in the absence of feeding. None of the other measured methylamines increased in response to salinity challenge; however, the summed total methylamines did significantly increase in *H. signifer* acclimated to brackish water (Fig. 3). Betaine is the major constituent of the muscle methylamine pool and the difference between total methylamines and betaine are very similar, supporting the notion of betaine accumulation. Moreover, one of the freshwater animals had unusually high muscle betaine content (22.2 μmol g^{-1}; based on the analysis of the other nine freshwater specimens, this value is more than 3 s.d. higher than the mean) while all other solutes for this individual were well within the 'normal' range found in this species. Exclusion of this individual does result in a significant difference in betaine content between freshwater and 50% seawater groups.

Methylamine synthesizing enzyme activities

TMAO synthesis

Trimethylamine oxidase activity was below the limits of detection (approximately 0.05 μmol h^{-1} g^{-1}) for all seven Rajiformes species (skates and stingrays, including members of the families Rajidae, Dasyatidae and Potamotrygonidae) in the present study, showing no trend between TMAO accumulation and synthetic capacity in these taxa. The shark *C. punctatum* had high liver TMAoxi activity (1.27±0.07 nmol min^{-1} mg $protein^{-1}$ or 5.1± 0.87 μmol h^{-1} g^{-1}) and also had the highest level of TMAO in muscle (Fig. 2 and Table 1).

Betaine synthesis

All species examined had measurable liver ChoDH and BADH activity. Thus, all species have a significant capacity for betaine synthesis. There is a qualitative trend of increasing ChoDH and BADH activity with increasing betaine content in those species able to tolerate full strength seawater (marine and euryhaline species) but neither trend extends to the freshwater species. Both freshwater species have high enzyme activity and relatively low muscle betaine content (Fig. 5). Quantitatively, the relationship between liver ChoDH activity and betaine accumulation across the species that can survive in full strength seawater is not significant if analyzed on a species mean basis ($P>0.15$, Fig. 5A) or on data for individual animals ($P=0.09$; data not shown). The trend with liver BADH activity and muscle betaine content approaches significance using species means ($P=0.08$, Fig. 5B), and when analyzed on an individual basis is highly significant across these five species ($P<0.001$, $r^2=0.49$; data not shown). Liver BADH activity appears to be a better indicator than ChoDH of muscle betaine content in elasmobranchs analyzed, and the above suggests that BADH activity is elevated to a greater degree than ChoDH in betaine-accumulating elasmobranchs. When expressed as a ratio of BADH/ChoDH, there is a significant relationship with muscle betaine content on either a species mean or individual basis with all species including the freshwater *H. signifer* and *P. motoro* (Fig. 5C). Thus, across species, as muscle betaine content increases, the enzymatic potential for conversion of betaine aldehyde to betaine (BADH) significantly increases relative to the capacity for the production of betaine aldehyde (ChoDH activity). The only species with measurable TMAoxi activity, *C. punctatum*, had low muscle betaine content and very low liver BADH activity.

Discussion

Muscle organic osmolytes: from marine to freshwater elasmobranchs

The present study is the first to provide a detailed and broad comparison of muscle organic osmolytes across several species of marine and freshwater elasmobranchs. In marine species,

Table 1. *The activities of enzymes involved with trimethylamine oxide (trimethylamine oxidase) and betaine synthesis (choline dehydrogenase and betaine aldehyde dehydrogenase) in the liver of marine, euryhaline species in freshwater and freshwater elasmobranchs*

		Enzyme activity (nmol min^{-1} mg $protein^{-1}$)		
Species	*N*	TMAoxi	ChoDH	BADH
Marine				
Chiloscyllium punctatum	3[1]	1.3±0.07[2]	0.34±0.12	0.37±0.06
Leucoraja ocellata	4	bld	0.18±0.05	1.3±0.13
Leucoraja erinacea	4	bld	0.21±0.08	4.0±1.0
Taeniura lymma	6	bld	0.60±0.11	16±1.4
Euryhaline in freshwater				
Dasyatis sabina	2[3]	bld	0.53±0.01	10±1.0
Freshwater				
Himantura signifer (50% SW)	6	bld	1.1±0.23	4.8±1.6
Himantura signifer (FW)	10	bld	1.1±0.15	11±4.1
Potamotrygon motoro	4	bld	0.90±0.36	5.1±1.9

TMAoxi, trimethylamine oxidase; ChoDH, choline dehydrogenase; BADH, betaine aldehyde dehydrogenase.

Values are mean ± s.e.m. bld, activity below the limits of detection: less than 0.05 μmol h^{-1} g^{-1} or equivalent to less than approximately 0.01 nmol min^{-1} mg $protein^{-1}$ (*N*=2–3).

[1]*N*=5 for TMAoxi.

[2]On a wet mass basis; 5.1±0.87 μmol h^{-1} g^{-1}.

[3]As only two liver samples were available for this species, values are mean ± range.

TMAO is a major component of the muscle methylamine pool, but in the transition from euryhaline to freshwater species, betaine becomes the major methylamine. Marine elasmobranchs accumulate more methylamines than β-amino acids while freshwater species preferentially accumulate β-amino acids. The accumulation of methylamines over β-amino acids in species with high urea content is consistent with the hypothesis that marine elasmobranchs specifically accumulate methylamines to counteract urea's effects on macromolecules. However, if this is true, it appears that at some lower urea concentration, perhaps between 100–150 mmol l^{-1}, the need to specifically accumulate methylamines to counteract urea decreases in favour of β-amino acids, which are generally viewed as 'compatible' rather than counteracting osmolytes (Yancey, 1994). Consistent with our observed shift from the accumulation of counteracting methylamines to the compatible β-amino acids at lower urea levels, Yancey and Burg (Yancey and Burg, 1990) found that the growth of cultured mammalian cells, both renal and non-renal, was not inhibited by urea concentrations below 100–150 mmol l^{-1}.

Despite the shift from methylamines to β-amino acids as the major non-urea osmolytes, there is still a strong correlation between the total non-urea osmolytes (methylamines and β-amino acids) and urea in the muscle of elasmobranchs (Fig. 4). This relationship is close to the expected 2:1 ratio when either the total methylamines or the combined values for methylamines and β-amino acids are regressed with urea content. Overall, the combined total methylamines and β-amino acids provides a substantially better fit than the total methylamines. We caution against overinterpreting the significance of the slope of methylamine regression because data for *C. punctatum* indicate that there is not a highly conserved ratio between urea and methylamines in the muscle of some marine elasmobranchs. That being said, it is clear that total methylamine content is higher in urea-accumulating elasmobranchs, even if there is not a tightly regulated ratio between methylamines and urea. Both of the above trends between urea and other organic osmolytes are consistent from marine species to the Potamotrygonid stingrays, with the intercept at negligible urea content being remarkably similar to the measured value for *P. motoro* (Fig. 4).

The distribution of TMAO synthesis in elasmobranchs

The lack of detectable TMAoxi activity in all of the skates and stingrays examined in the present study is consistent with past surveys on TMAO synthesis in elasmobranchs (Fig. 6 and references therein). The sporadic distribution of TMAoxi has been known, but unexplained, for several decades (Baker et al., 1963; Goldstein and DeWitt-Harley, 1973). It was speculated by Goldstein and Palatt (Goldstein and Palatt, 1974) that 'random deletions' may give an explanation for TMAoxi distribution, and they drew an analogous comparison with ascorbic acid biosynthesis in mammals where some higher taxa have lost the capacity for physiologically sufficient endogenous synthesis. Fig. 6 illustrates how the available data on the presence or absence of detectable capacity for TMA oxidation (*in vitro* or *in vivo*) is directly linked to phylogeny in chondrichthyans (elasmobranchs and the chimaeras or holocephalans). While further species need to be studied to confirm the distribution postulated in Fig. 6, as well as to fill in 'gaps' such as missing orders and families, we propose that

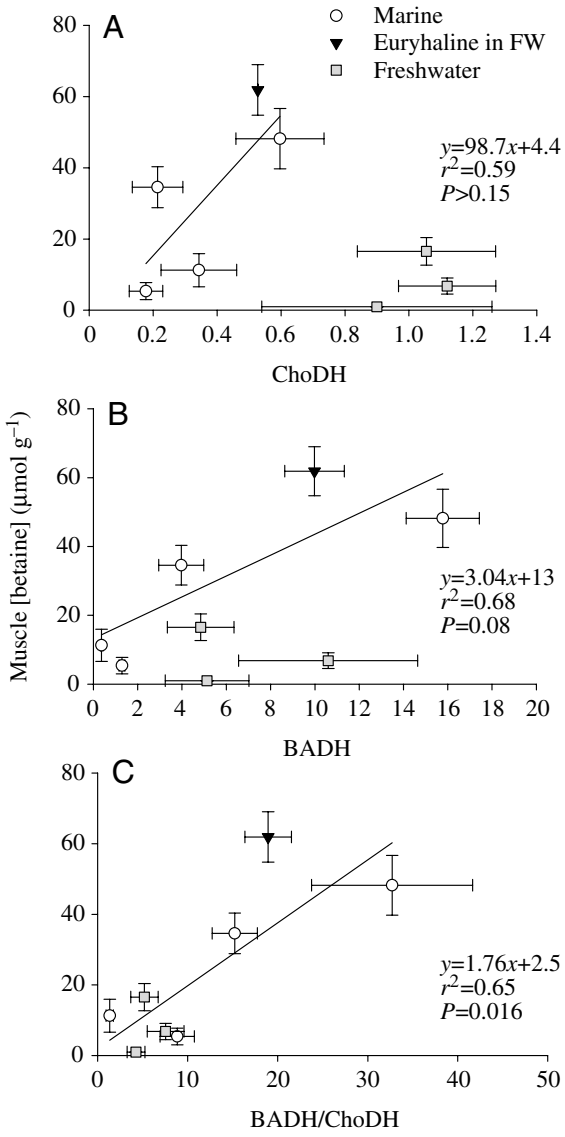

Fig. 5. Relationship between liver enzyme activities of betaine synthesis (nmol min^{-1} mg $protein^{-1}$) and the accumulation of betaine in the muscle (μmol g^{-1}) of marine, euryhaline and freshwater elasmobranchs. Figures are for muscle betaine and (A) liver choline dehydrogenase activity (ChoDH), (B) liver betaine aldehyde dehydrogenase (BADH), (C) ratio of BADH/ChoDH. The regression equations for A and B are only for species able to survive in full-strength seawater (marine and euryhaline) while the equation in C is for all species; for C, the equation (not shown) for those species able to survive under marine conditions is not significantly different from the regression equation including all species. Data are taken from Table 1 and Fig. 3.

the distribution of TMAoxi in elasmobranchs can be explained when grouped taxonomically.

Capacity for betaine synthesis and muscle accumulation

The present data indicate that an increase in the activity of liver BADH, relative to ChoDH, coincides with the accumulation of betaine in the muscle of elasmobranchs (Fig. 5C), suggesting that BADH plays a regulatory role in betaine synthesis. The uptake of choline by rat liver mitochondria, and thus the exogenous supply of choline, is believed to be the major control site of betaine synthesis (Kaplan et al., 1993). However, rat liver cells do not synthesize betaine in response to hyperosmotic challenge (Wettstein et al., 1998). Rabbit kidney cells (TALH) do synthesize and accumulate betaine when exposed to hyperosmotic conditions (Grunewald and Eckstein, 1995) and are found in a 'high urea' environment, making them a better analogy to elasmobranch betaine synthesis. Kidney cells under high osmolality conditions exhibit little or no activation of ChoDH, whereas BADH activity increases to 3 times that found in cells in isoosmotic (300 mOsm) medium. Grunewald and Eckstein (Grunewald and Eckstein, 1995) surmized that BADH may be the rate limiting enzyme of betaine synthesis in mammalian kidney cells under these conditions. Our observation that BADH activity relative to ChoDH (the initial enzymatic step of betaine synthesis) increases with betaine accumulation in elasmobranchs is consistent with the notion that BADH activity may be rate limiting in elasmobranchs; however, the supply of choline to the liver as a precursor to betaine may still be of critical importance in the control of actual *in vivo* betaine synthesis.

Curiously, there was no change in the activities of ChoDH or BADH in *H. signifer* acclimated to 50% seawater compared to freshwater acclimated animals. If activation of liver BADH is indicative of betaine synthesis in marine elasmobranchs, it is unclear why this would not also be the case in a freshwater species. It is possible that the high constitutive enzyme activities in this species preclude any need for activation in order to facilitate flux from choline to betaine. Furthermore, the liver supply of choline precursors and endogenous synthesis of choline may provide the requisite materials for any increased synthesis of betaine.

Although the liver is the major site of betaine synthesis in mammals (Haubrich and Gerber, 1981), it is important to note the possibility that other tissues not included in the present study, or alternate metabolic pathways, may be involved with the synthesis of methylamines in elasmobranchs. That stated, substantial constitutive capacity for betaine synthesis *via* choline degradation, and thus potentially sarcosine synthesis as well (King et al., 1980), was found in all elasmobranchs examined.

Exogenous or endogenous origins for methylamines?

The relative importance of endogenous methylamine synthesis to their accumulation in elasmobranchs is still in need of clarification, but our data do offer some preliminary insights on the source of methylamines. The only species in this study that was fully acclimatized to laboratory conditions for several months was *L. ocellata*. Specimens of *L. ocellata* were fed chopped herring, which is a rich source of TMAO (approximately 25–40 μmol g^{-1}) that has very low levels (<1 μmol g^{-1}) of betaine and sarcosine (Carr et al., 1996). When compared to the congeneric *L. erinacea*, which were

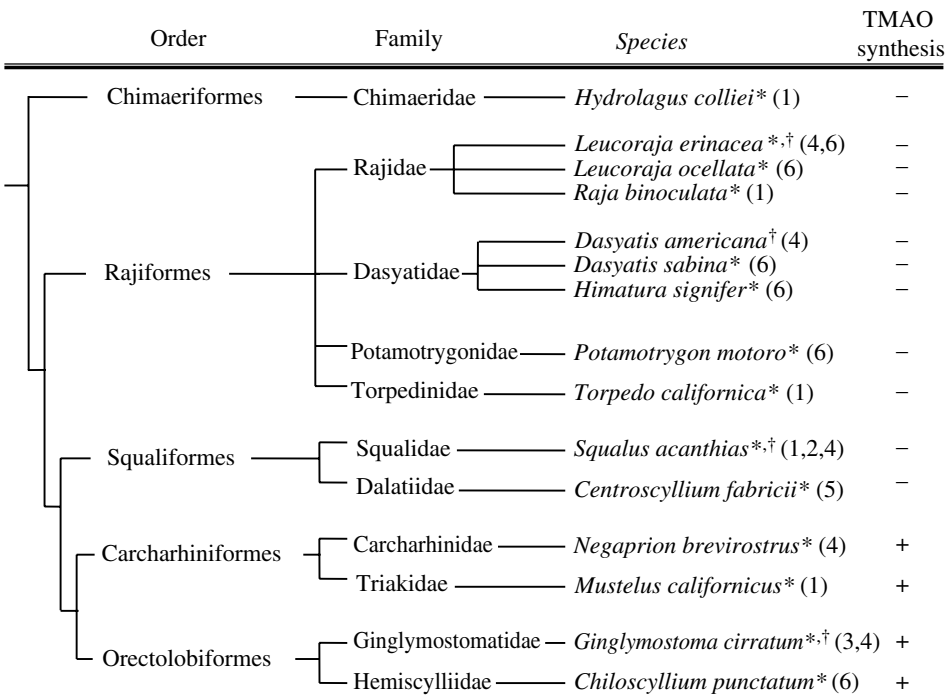

Fig. 6. Summary of the capacity for the oxidation of trimethylamine to trimethylamine oxide (TMAO), indicative of TMAO synthetic capacity, in chondrichthyian fishes (elasmobranchs and chimaeras) arranged phylogenetically. The tree is simplified from Winchell et al. (Winchell et al., 2004) and, for simplicity, orders where no data on the oxidation of trimethylamine are available have not been included. *TMAO synthesis was assessed *in vitro*; †TMAO synthesis was assessed by *in vivo* conversion of labeled precursor; –, below limits of detection or questionable synthetic capacity; +, significant TMAO synthesis present. Values in parentheses indicate the source of data: [1]Baker et al., 1963; [2]Goldstein et al., 1967; [3]Goldstein and Funkenhouser, 1972; [4]Goldstein and Dewitt-Harley, 1973; [5]Treberg and Driedzic, 2002; [6]present study.

sampled shortly after capture and fed on a natural diet of invertebrates that are high in betaine (Carr et al., 1996; Collette et al., 2004), specimens of *L. ocellata* were characterized by higher TMAO and lower betaine and sarcosine levels. No skates examined to date have exhibited demonstrable capacity for the conversion of TMA to TMAO, yet both species have betaine and likely sarcosine synthetic capacity (Table 1; King et al., 1980). It is tempting to interpret these differences between congeneric skates as due to a coupling between dietary derived and endogenously synthesized methylamine accumulation in the muscle of skates. In this scenario, when insufficient amounts of TMAO, or betaine, can be obtained from the diet, the remaining required methylamine solutes are made up by endogenously synthesized betaine and sarcosine; albeit the synthesis of these solutes may require exogenous choline or other precursory metabolites (King et al., 1980). The muscle of *D. sabina* had modest levels of TMAO and high betaine content. This species apparently lacks the capacity for TMAO synthesis, but can synthesize betaine like the skates above (Table 1). Most freshwater fish and invertebrates have low levels of TMAO (reviewed by Hebard et al., 1982), thus these euryhaline stingrays caught in freshwater likely have a diet that is low in TMAO. As with the above skates, it is reasonable to suggest that these stingrays compensate for any 'methylamine-gap' in the muscle by accumulating endogenously synthesized betaine. The above postulations can also be extended and simplified in the case of *C. punctatum*, where the high muscle TMAO content could be reflective of the capacity for endogenous TMAO synthesis and thus maintenance of high levels irrespective of dietary TMAO content. However, while the above scenarios may be logically reasonable, it is important to acknowledge that at present they are still hypothetical and need to be experimentally tested.

List of abbreviations

BADH	betaine aldehyde dehydrogenase
BSA	bovine serum albumin
ChoDH	choline dehydrogenase
PCA	perchloric acid
PMS	phenazine methosulphate
TCA	trichloroacetic acid
TMA	trimethylamine
TMAO	trimethylamine oxide
TMAoxi	trimethylamine oxidase

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