

## ROLE OF UREA AND METHYLAMINES IN BUOYANCY OF ELASMOBRANCHS

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### Summary

The possible role of urea and trimethylamine oxide (TMAO) in providing positive buoyancy has been examined for elasmobranch fishes. TMAO has a considerably lower density than an equimolar solution of urea, and solutions of both TMAO and urea are considerably less dense than equimolar solutions of most other body fluid solutes. The body fluid composition of three elasmobranchs, the whiskery shark *Furgaleus ventralis*, the black whaler shark *Carcharhinus obscurus* and the shovel-nosed ray *Aptychotrema vincentiana*, is typical for marine elasmobranchs, with plasma concentrations of about 260 mmol l<sup>-1</sup> Na<sup>+</sup>, 250 mmol l<sup>-1</sup> Cl<sup>-</sup>, 340 mmol l<sup>-1</sup> urea and 70 mmol l<sup>-1</sup> trimethylamine oxide. A plasma density of 1.015 was calculated for the whaler shark (from the concentrations, relative molecular masses and absolute molal volumes of plasma solutes), which would contribute a positive lift of 8.45 g l<sup>-1</sup>. There is a large positive contribution to buoyancy by urea (3.7 g l<sup>-1</sup>), trimethylamine oxide (1.8 g l<sup>-1</sup>) and Cl<sup>-</sup> (4.0 g l<sup>-1</sup>), whereas slight negative buoyancy is conferred by Na<sup>+</sup> (-0.8 g l<sup>-1</sup>). Divalent cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>) contribute minimal negative buoyancy (about -0.1 g l<sup>-1</sup> each) despite their rather negative partial molal volumes, because of their low concentrations. Muscle fluids contain about 40 mmol l<sup>-1</sup> Cl<sup>-</sup>, 365 mmol l<sup>-1</sup> urea, 160 mmol l<sup>-1</sup> trimethylamine oxide, 16 mmol l<sup>-1</sup> betaine and 69 mmol l<sup>-1</sup> sarcosine. The organic solutes contribute about 12.1 g l<sup>-1</sup> lift. Although urea and TMAO act as balancing osmolytes, and TMAO as a counteracting solute, a positive buoyancy role must be considered as a further adaptive function of urea and TMAO accumulation in chondrichthyan fishes.

### Introduction

Aquatic animals have evolved diverse mechanisms for the regulation of their buoyancy. These include limited calcification of the skeleton, gas-filled bladders, floats or cuttlebones, accumulation of lipid stores, hydrodynamic force generation by lifting

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surfaces and the accumulation of low-density solutes. A buoyancy role for low molecular mass solutes, especially ammonium ( $\text{NH}_4^+$ ), has been described for certain squids (Denton *et al.* 1969; Clarke *et al.* 1979), tunicate eggs (Lambert and Lambert, 1978), protozoans (*Noctiluca miliaris*, Goethard and Heinsius, 1892, cited by Krogh, 1939; *Pyrocystis noctiluca*, Kahn and Swift, 1978) and diatoms (Gross and Zeuthen, 1948). A study of buoyancy control in the deep-sea pelagic shrimp *Notostomus gibbosus* (Sanders and Childress, 1988) has elucidated an important role for trimethylamine (TMA).

Marine elasmobranchs and chimaeras have a high concentration of urea in their extracellular and intracellular body fluids (Smith, 1936; see Holmes and Donaldson, 1969, for a review). The urea is an important osmolyte; it partly fills the approximately  $500 \text{ mosmol l}^{-1}$  'osmotic gap' between the osmotic concentration contributed by ions (about  $600 \text{ mosmol l}^{-1}$ ) and the total osmotic concentration (about  $1100 \text{ mosmol l}^{-1}$ ). Other organic solutes, such as trimethylamine oxide [TMAO;  $(\text{CH}_3)_3\text{NO}$ ], glycine betaine, hereafter referred to as betaine [ $(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$ ] and sarcosine ( $\text{CH}_3\text{N}^+\text{H}_2\text{CH}_2\text{COO}^-$ ), are also accumulated at significant levels, but TMA [ $(\text{CH}_3)_3\text{N}$ ] is not. There has recently been considerable biochemical interest in the perturbing effects of urea on enzyme catalytic function, and the counteracting effects of TMAO and betaine (Yancey and Somero, 1979, 1980; Yancey, 1985; Somero, 1986; Ballantyne and Moon, 1986; Yancey and Burg, 1989, 1990), and on the widespread role of betaine as an osmolyte and cryoprotectant (see Anthoni *et al.* 1991). Whether there is a significant buoyancy role in elasmobranchs for these organic solutes has never been demonstrated. Until now, the study of buoyancy in elasmobranch fishes has concentrated on the roles of skeletal calcification, liver lipid content and hydrodynamic lift from the fins (Bone and Roberts, 1969; Corner *et al.* 1969; Alexander, 1982). The role of particular solutes as buoyancy regulators can best be addressed from a theoretical viewpoint *via* solution physical chemistry. This study first addresses the theoretical background then quantitatively examines the possible role of body fluid osmolytes in buoyancy regulation for elasmobranchs.

#### *Roles of molecular mass and partial molal volume in density*

The general concept that the density of body fluids can be reduced through the replacement of high molecular mass solutes by those of lower molecular mass is well appreciated, e.g.  $\text{NH}_4^+$  replacement of  $\text{Na}^+$  reduces solution density in numerous marine animals (Gross and Zeuthen, 1948; Clarke *et al.* 1979; Lambert and Lambert, 1978). However, low density can also be achieved by the replacement of solutes with those having a more positive partial molal volume, as elucidated by Sanders and Childress (1988) in accounting for density reduction in *Notostomus gibbosus*, a caridean shrimp. Notwithstanding these pioneering studies, no rigorous discussion of the role of partial molal volumes of solutes to solution density is available in the biological literature, and so we first address here some relevant aspects of the theoretical background.

The effect of a solute on the density of a solution depends not only on the obvious effect of its molecular mass ( $MW_j$ ), but also on its molal volume ( $V_j$ ;  $\text{cm}^3 \text{ mol}^{-1}$ ). A solute with a negative molal volume ( $V_j < 0$ ) causes the volume of the solution to be less than the initial volume of water, despite the addition of further mass (and volume) from the solute.

A solution with  $V_j=0$  will not alter the water volume, whereas a solute with  $V_j>0$  will increase the solution volume. The molal volume can be expressed either as the partial molal volume  $\bar{V}_j^0$  or as the apparent molal volume  $V_{\phi j}$ . [Note that ‘molal’ in partial (or apparent) molal volume is merely an adjectival form of ‘mole’ and is not related to, or to be confused with, the concentration unit ‘molality’ which has units of  $\text{mol kg}^{-1}$  solvent.]

The partial molal volume ( $\bar{V}_j^0$ ) is the differential change in the volume of an infinite volume of solvent (water for biological systems) upon the addition of an infinitesimally small amount of solute  $j$  to form a solution; it is the molal volume for a solute at zero concentration (Millero, 1972). The apparent molal volume is defined as the change in volume of solvent upon the addition of a mole of solute; it is the molal volume at finite solute concentrations. Clearly, the partial and apparent molal volumes become equal at infinite solute dilution i.e.  $(V_{\phi j})_{c \rightarrow \infty} = V_{\phi j}^\infty = \bar{V}_j^0$ .

The apparent molal volume for a non-electrolyte solute at modest molar concentrations ( $C_j$ ;  $\text{mol l}^{-1}$ ) is related to the partial molal volume (Millero, 1972) as:

$$V_{\phi j} = \bar{V}_j^0 + k\sqrt{C_j}, \quad (1)$$

where  $k$  is an empirical constant reflecting solute–solute interaction.

For an electrolyte, the relationship is:

$$V_{\phi j} = \bar{V}_j^0 + A_V\sqrt{C_j} + B_VC_j, \quad (2)$$

where  $A_V$  is the theoretical (Debye–Hückel) limiting slope and  $B_V$  is an empirical constant reflecting ion–ion interactions. The theoretical value for  $A_V$  is  $1.868 \text{ cm}^3 \text{ l}^{1/2} \text{ mol}^{-3/2}$  for a 1:1 electrolyte at  $25^\circ\text{C}$  (Millero, 1972). In practice, and especially for non-electrolytes, the difference between the partial molal volume and the apparent molal volume is often quite small, or even negligible, providing  $C_j$  is low ( $\leq 1 \text{ mol l}^{-1}$ ).

The effect of molecular mass ( $MW_j$ ) and apparent molal volume ( $V_{\phi j}$ ) on solution density ( $\rho_{\text{solution}}$ ) for an aqueous solution (Millero, 1972) is:

$$\rho_{\text{solution}} = \rho_0 + \frac{C_j MW_j}{1000} - \frac{C_j \rho_0 V_{\phi j}}{1000}, \quad (3)$$

where  $\rho_0$  is the density of pure water ( $997.047 \text{ g l}^{-1}$  at  $25^\circ\text{C}$ ). Since  $\rho_0$  is approximately 1 for water, it follows that  $|\rho_0 V_{\phi j}| \approx |V_{\phi j}|$ . Thus, the addition of a solute with an apparent molal volume (in  $\text{cm}^3 \text{ mol}^{-1}$ ) less than its molecular mass (in  $\text{g mol}^{-1}$ ) will increase the density of the solution compared with water, whereas addition of a solute with  $V_{\phi j} > MW_j$  will reduce the density below that of the solvent. We can substitute partial molal volume for apparent molal volume without introducing a significant error, providing that the solution concentration is low. Using equation 3, it is possible to calculate accurately the density of solutions and body fluids from the solute composition, molecular masses and partial molal volumes of their solutes (Sanders and Childress, 1988). For marine animals, the buoyancy role of solutes is determined by the difference between the density of their body fluids and the density of sea water rather than the density of pure water.

Partial molal volumes are additive for different solutes (Millero, 1972). Apparent molal

volumes are also additive, providing that the concentrations are not too high, i.e. providing solute–solute interactions are not significant. For many purposes (see below) it is more useful to consider the effects of individual ions rather those of whole solutes since the ions are the actual species present. For the salt AB, the partial molal volume  $\bar{V}_{AB}^\circ$  is equal to the sum of the partial molal volumes of its ions,  $\bar{V}_{A^+}^\circ$  and  $\bar{V}_{B^-}^\circ$ . However, partial (or apparent) molal volume is a thermodynamic property that can be measured only for electroneutral combinations of ions, e.g. for whole salts such as NaCl. Partial molal volumes of individual ions can be derived in two ways: (1) by the so-called ‘conventional’ method which arbitrarily assigns a zero partial molal volume to protons (i.e.  $\bar{V}_{H^+}^\circ \equiv 0 \text{ cm}^3 \text{ mol}^{-1}$  at all temperatures and pressures); and (2) by reference to the so-called ‘absolute’ (or ‘true’) partial molal volume of  $H^+$  obtained using appropriate non-thermodynamic assumptions, the best of which gives  $\bar{V}_{H^+}^\circ = -5.4 \text{ cm}^3 \text{ mol}^{-1}$  (Millero, 1972).

Both ‘conventional’ and ‘absolute’ scales for partial molal volume must yield the same overall value for  $\bar{V}^\circ$  for electroneutral combinations of ions (since they are derived from such data). The essential difference between the two scales lies in the relative split for cations and anions. The choice of scale, conventional or ‘absolute’, may be critical depending upon what is being compared. If only overall (and hence necessarily electroneutral) effects are being considered, the choice of scale is irrelevant. This is also true if we wish to compare the overall effects of replacement by ions of like charge, e.g. the replacement of  $Na^+$  by  $NH_4^+$ , since their difference is also electroneutral. However, if we wish to discuss the effects of individual cations and anions, then realistic comparisons can only be made using the absolute scale. We pursue these concepts below.

### Materials and methods

A pelagic shark (the black whaler, or dusky shark *Carcharhinus obscurus* Le Suer 1818; single specimen), a semi-benthic shark (the whiskery shark *Furgaleus ventralis* Whitley 1943; single specimen) and a benthic ray (the shovel-nosed ray *Aptychotremata vincentiana* Haacke 1885; two specimens) were obtained from commercial fish suppliers. They were maintained in flowing seawater tanks at approximately 25 °C.

The densities of the elasmobranchs and of liver and muscle samples were determined by weighing them in air and water. Animal and tissue density were calculated from these weights ( $W$ ) as:

$$\rho = \frac{W_{\text{air}} \rho_{\text{seawater}}}{W_{\text{air}} - W_{\text{seawater}}} \quad (4)$$

Blood samples were obtained by heart puncture of restrained and unanaesthetised fish. The fish were then killed, the liver was removed and a lateral muscle sample obtained. Blood samples were stored on ice until centrifuged at 2500 revs  $\text{min}^{-1}$  for 5 min to obtain plasma samples, which were immediately frozen. Samples of muscle were dissolved in  $10 \text{ mol l}^{-1} \text{ HNO}_3$  (0.5 ml per 200 mg of tissue); the supernatant obtained after centrifugation was stored frozen.

Thawed plasma and muscle supernatants were analysed for  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , urea, TMAO, betaine and sarcosine. Cation concentrations were determined with a Varian atomic absorption spectrophotometer (model 475). A sample of plasma was diluted into caesium chloride (1000 p.p.m.  $\text{Cs}^+$ ) for  $\text{Na}^+$  and  $\text{K}^+$  analyses and into strontium chloride (5000 p.p.m.  $\text{Sr}^{2+}$ ) for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  analyses. Chloride was measured with a Buchler–Cotlove amperometric titration analyser. Ammonia and urea concentrations were measured in plasma (diluted 1:50 with distilled water for urea assay) and muscle homogenates by the alkaline hypochlorite/phenylnitroprusside procedure (modified from Fawcett and Scott, 1960). TMAO was measured by the colorimetric procedure of Barnes and Blackstock (1974). Betaine and sarcosine concentrations were determined by HPLC using a Waters 6000A pump, U6K injector and 410 refractive index detector, with a Waters Sugar Pak I column (Wolff *et al.* 1989).

The apparent molal volume ( $V_{\phi_j}$ ) was calculated from the density of biologically appropriate concentration ranges of urea (50–366  $\text{mmol l}^{-1}$ ), TMAO (36–150  $\text{mmol l}^{-1}$ ), betaine hydrochloride (11–245  $\text{mmol l}^{-1}$ ) and sarcosine hydrochloride (23–103  $\text{mmol l}^{-1}$ ). Solutions of solutes of an appropriate biological range were prepared gravimetrically from analytical grade reagents (purchased from Sigma chemicals and freeze-dried prior to use) and distilled degassed water. The presence of hydrates or hydrochlorides was accounted for in subsequent calculations. The density of solutions was measured by vibrating tube densitometry, using a Sodev model 03D densitometer at  $25 \pm 0.001$  °C. The frequency of vibration of the solutions was measured alternately with samples of degassed distilled water, used as a standard of known density.

Apparent molal volumes (in  $\text{cm}^3 \text{mol}^{-1}$ ) were calculated as:

$$V_{\phi_j} = \frac{MW_j}{\rho_0} - \frac{1000(\rho_{\text{solution}} - \rho_0)}{\rho_0 C_j} \quad (5)$$

The partial molal volume ( $\bar{V}_j^\circ$ ) was determined by linearly extrapolating the apparent molal volumes to zero solute concentration (see equations 1 and 2).

## Results

All the elasmobranchs had a positive mass in water (Table 1) and were denser than standard sea water ( $\rho = 1.024 \text{ g cm}^{-3}$  at 25 °C). *C. obscurus* was the least dense and *A. vincentiana* the most dense elasmobranch. All tissue samples were denser than sea water, except for the livers of *F. ventralis* and *C. obscurus*, which were positively buoyant.

The plasma of the elasmobranchs had typical solute concentrations for marine species (Table 2). The total  $[\text{Na}^+ + \text{Cl}^-]$  concentration was about 550  $\text{mmol l}^{-1}$ ; high urea concentrations (340–350  $\text{mmol l}^{-1}$ ) and TMAO concentrations (70–75  $\text{mmol l}^{-1}$ ) accounted for much of the osmotic gap of about 550  $\text{mosmol l}^{-1}$ . Other ions ( $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{NH}_4^+$ ) and organic solutes (TMA, betaine, sarcosine) were only present at relatively low concentrations ( $< 10 \text{ mmol l}^{-1}$ ).

The solute composition of whole muscle was substantially different from that of plasma (Table 2). The whole-muscle  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations were lower and the  $\text{K}^+$

Table 1. *Body mass (g) in air and water, and density ( $\rho$ , g cm<sup>-3</sup>) of the whole fish, of a muscle sample and of the entire liver for three elasmobranch fishes*

	<i>Furgaleus ventralis</i>	<i>Carcharhinus obscurus</i>	<i>Aptychotrema vincentiana</i>
Air mass	7100	6750	340
Water mass	279	93.5	16.9
$\rho_{\text{total}}$	1.069	1.041	1.080
$\rho_{\text{muscle}}$	1.049	1.082	1.071
$\rho_{\text{liver}}$	0.974	0.997	1.073

Table 2. *Plasma and whole-muscle solute concentrations (mmol l<sup>-1</sup>) for the elasmobranch fishes measured in this study compared with values for other elasmobranchs, for plasma (from Holmes and Donaldson, 1969;  $\pm$  standard error; the values in parentheses are the numbers of species) and for whole muscle samples (from Robertson, 1975, 1989)*

	<i>Furgaleus ventralis</i>	<i>Carcharhinus obscurus</i>	<i>Aptychotrema vincentiana</i>	Other elasmobranchs
<b>Plasma</b>				
Na <sup>+</sup>	260	279	264	249 $\pm$ 8 (28)
K <sup>+</sup>	6.9	14.4	7.3	8.0 $\pm$ 0.8 (29)
Cl <sup>-</sup>	238	275	287	245 $\pm$ 6 (37)
Ca <sup>2+</sup>	4.2	3.4	3.6	5.6 $\pm$ 0.4 (24)
Mg <sup>2+</sup>	2.0	2.8	1.6	2.9 $\pm$ 0.2 (18)
Urea	342	346	337	352 $\pm$ 12 (19)
NH <sub>4</sub> <sup>+</sup>	0.74	0.70	0.70	
TMAO	70.0	75.4	71.1	84 (2)
TMA	0.92	1.04	0.70	
<b>Muscle</b>				
Na <sup>+</sup>	39.3	40.8	33.6	42–54
K <sup>+</sup>	97.7	106	98.2	119–165
Cl <sup>-</sup>	31.7	43.0	28.7	36–44
Ca <sup>2+</sup>	5.5	2.4	2.0	2.1–2.7
Mg <sup>2+</sup>	1.09	0.92	5.28	12.9–15.1
Urea	357	365	357	333–411
NH <sub>4</sub> <sup>+</sup>	4.2	3.9	3.6	3.3–4.7
TMAO	166	160	162	180–182
TMA	9.0	8.2	6.6	
Betaine	56.8	15.8	56.9	47–101
Sarcosine	0.5	69	11.2	

TMA, trimethylamine; TMAO, trimethylamine oxide.

concentration was higher than for plasma. The urea concentration was similar for whole muscle and plasma. There were higher concentrations of TMAO, betaine and sarcosine in whole muscle than in plasma. The remaining solute gap for whole muscle presumably

Table 3. Molecular mass and partial molal volumes ( $\bar{V}_j^\circ$ ) of nitrogenous solutes at 25 °C

Solute	Molecular mass	Partial molal volume (cm <sup>3</sup> mol <sup>-1</sup> )
Urea	60.1	44.34±0.27 (0.10)
TMAO	75.1	72.67±0.26 (0.07)
Betaine	117.2	101.23±0.56 (0.27)
Sarcosine	89.2	69.00±6.02 (0.38)

Values of  $\bar{V}_j^\circ$  are the intercept ± 95 % confidence limits (standard error of estimate).

reflects the high amino acid and protein concentrations of intracellular fluid in elasmobranchs.

The apparent molal volumes, calculated as an average value for the ranges of solute concentrations used, were positive for urea (44.35±0.04 cm<sup>3</sup> mol<sup>-1</sup>,  $N=5$ ), TMAO (71.34±0.29 cm<sup>3</sup> mol<sup>-1</sup>,  $N=5$ ), betaine hydrochloride (120.5±0.42 cm<sup>3</sup> mol<sup>-1</sup>,  $N=6$ ) and sarcosine hydrochloride (87.0±0.17 cm<sup>3</sup> mol<sup>-1</sup>,  $N=3$ ). There was a significant effect of concentration on apparent molal volume for TMAO ( $\Delta V_{dj}/\Delta C = -12.7 \pm 0.7$ ,  $t_3=18.1$ ,  $P<0.001$ ) and betaine (+11.4±1.4,  $t_4=8.3$ ,  $P<0.002$ ).

The partial molal volumes  $\bar{V}_j^\circ$ , extrapolated by linear regression to zero solute concentration, were all positive, ranging from 44.3 to 101.2 cm<sup>3</sup> mol<sup>-1</sup> (Table 3). The partial molal volume was calculated for betaine and sarcosine from the value for the hydrochloride salt, assuming a molal volume of 17.83 cm<sup>3</sup> mol<sup>-1</sup> for HCl at 25 °C (Millero, 1972). The  $\bar{V}_j^\circ$  for urea determined here was very similar to a previously reported value of 44.23 cm<sup>3</sup> mol<sup>-1</sup> (Höiland, 1986).

### Discussion

The buoyancy of elasmobranchs can vary widely from negative to positive depending on their habits (Bone and Roberts, 1969). All of the elasmobranchs examined here were more dense than sea water; the pelagic black whaler shark *Carcharhinus obscurus* had the lowest overall density, that of the semibenthic *Furgaleus ventralis* was intermediate, and the benthic shovel-nosed ray *Aptychotremata vincentiana* had the highest density. The primary organ contributing to positive buoyancy in elasmobranchs is often the liver, because of its high lipid content and correspondingly low density (Bone and Roberts, 1969; Corner *et al.* 1969). The density of the liver was lowest for the whiskery shark, intermediate for the black whaler shark and highest (greater than that of sea water) for the shovel-nosed ray. Consistent with these density measurements, the liver of the whiskery shark had a higher lipid content (11.5 % of wet mass) compared with those of the black whaler (8.9 %) and the shovel-nosed ray (0.68 %).

Although the data reported here for plasma and whole-muscle solute concentrations of the whiskery shark, black whaler shark and shovel-nosed ray represent only one or two measurements per species, they are consistent with previous measurements for a variety of other elasmobranchs (e.g. Smith, 1936; Holmes and Donaldson, 1969; Robertson, 1975, 1989; Table 2). The solute compositions for the species that we studied are,

therefore, appropriate for our primary objective of evaluating the potential significance of urea and methylamines to the buoyancy of these elasmobranchs.

#### *Calculation of solution density*

The density of a simple solution can be estimated from the molecular mass, the molar concentration and the partial molal volume of the solute. Consider, for example, the density of  $1 \text{ mol l}^{-1}$  NaCl (molecular mass 58.54 Da; partial molal volume  $16.62 \text{ cm}^3 \text{ mol}^{-1}$ ) at  $25^\circ\text{C}$ . One litre of solution contains 58.54 g of NaCl and the partial molal volume contributes  $16.62 \text{ cm}^3$  of volume, so the volume of water is  $983.38 \text{ cm}^3$  ( $1000.00 - 16.62$ ) and the mass of water is 980.48 g ( $983.38 \text{ cm}^3 \times 0.997047 \text{ g cm}^{-3}$  at  $25^\circ\text{C}$ ). The density of the solution is total mass/total volume =  $(980.48 + 58.54) / 1000.00 = 1.039 \text{ g cm}^{-3}$ . Densities of  $1 \text{ mol l}^{-1}$  solutions of relevant biological solutes (Table 4) indicate a range from  $1.0 \text{ g cm}^{-3}$  (TMAO) to  $1.14 \text{ g cm}^{-3}$  ( $\text{K}_2\text{SO}_4$ ). Compared with standard sea water (density  $1.024 \text{ g cm}^{-3}$ ),  $1 \text{ mol l}^{-1}$  solutions of TMAO,  $\text{NH}_4\text{Cl}$ , urea, betaine and sarcosine confer positive buoyancy, whereas NaCl, KCl,  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{CaSO}_4$  and  $\text{K}_2\text{SO}_4$  confer increasingly negative buoyancy. Note that the accuracy of these calculations does not depend on the choice of conventional or absolute partial molal volume, since solutes or salts are electroneutral.

The density of hypothetical 'solutions' of individual ions can be calculated from the absolute molal volume of the ion in the same manner as for solutes or salts. It can, therefore, be shown that hypothetical  $1 \text{ mol l}^{-1}$  'solutions' of both  $\text{NH}_4^+$  and  $\text{Cl}^-$  have substantial positive buoyancy compared with sea water, whereas  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{SO}_4^{2-}$  confer negative buoyancy (Table 4). Amino acids have a partial molal volume of about  $60\text{--}150 \text{ cm}^3 \text{ mol}^{-1}$  (Cohn and Edsall, 1943), and their effect is to increase solution density. For protein, the partial molal volume is expressed on a mass rather than a mole basis; the partial specific volume is about  $0.73 \text{ cm}^3 \text{ g}^{-1}$  (Hunter, 1966). For the relatively high protein concentration of  $100 \text{ g l}^{-1}$ , the density is about the same as for sea water at  $25^\circ\text{C}$ . This simple approach to pure solutions generally indicates the most favourable solutes/ions for minimising the density of a solution, although the actual density effect is, of course, concentration-dependent.

The density of a complex solution, such as animal body fluids, can be estimated from the concentrations and partial molal volumes of its dissolved solutes (see Sanders and Childress, 1988), providing that the solute concentrations are not too high (see above, *Roles of molecular mass and partial molal volume in density*). Consider, for example, sea water. Summing the mass and volume contributed by each solute allows the density to be calculated as  $1.0237 \text{ g cm}^{-3}$  (Table 5). For example,  $10.81 \text{ g l}^{-1}$   $\text{Na}^+$  effectively decreases the solvent volume by 3.11 ml, whereas  $10.35 \text{ g l}^{-1}$   $\text{Cl}^-$  increases the volume by 12.73 ml. The sum of the solute masses in 1 l of sea water is 34.98 g, and the overall volume change is  $+8.37 \text{ ml l}^{-1}$ . The volume of water in 1 l of sea water is thus  $991.63 \text{ ml}$  ( $1000.00 - 8.37$ ) and this weighs 988.71 g ( $\rho_{\text{H}_2\text{O}} = 0.997047 \text{ g cm}^{-3}$  at  $25^\circ\text{C}$ ). Thus, the density of sea water is calculated from total mass (1023.69 g)/total volume (1000.00 cm) to be  $1.0237 \text{ g cm}^{-3}$ . This is in excellent agreement with the tabulated density of standard sea water (Riley and Skirrow, 1975) of  $1.024 \text{ g cm}^{-3}$ .

In a similar manner, the density of *C. obscurus* plasma is estimated from its solute

Table 4. Molecular mass, partial molal volume and calculated density (using equation 3) for 1 mol l<sup>-1</sup> solutions of solutes, salts and individual ionic species at 25 °C compared with the density of standard sea water

	MW <sub>j</sub>	$\bar{V}_j^\circ$	MW <sub>j</sub> / $\bar{V}_j^\circ$	$\rho_{\text{solution}}$
TMAO	75.1	72.67	0.967	1.000
NH <sub>4</sub> <sup>+</sup>	18.0	12.46	0.692	1.003
NH <sub>4</sub> Cl	53.3	35.69	0.669	1.006
Cl <sup>-</sup>	35.3	23.23	0.658	1.009
Urea	60.1	44.34	0.738	1.013
Betaine	117.1	102.51	0.875	1.013
Sarcosine	89.2	69.21	0.776	1.017
<b>Standard sea water</b>				<b>1.024</b>
Protein (100 g l <sup>-1</sup> )				1.024
Alanine	89.1	60.6	0.680	1.026
Na <sup>+</sup>	23.0	-6.61	-0.287	1.027
Glycine	75.1	43.5	0.579	1.029
K <sup>+</sup>	39.1	3.62	0.093	1.033
Lysine	146.2	108.5	0.742	1.035
NaCl	58.5	16.62	0.284	1.039
KCl	74.4	26.85	0.361	1.045
Mg <sup>2+</sup>	24.3	-31.97	-1.316	1.053
Tryptophan	204.2	144.1	0.706	1.058
Ca <sup>2+</sup>	40.1	-28.65	-0.714	1.066
SO <sub>4</sub> <sup>2-</sup>	96.1	24.8	0.258	1.068
MgCl <sub>2</sub>	94.9	14.49	0.152	1.078
CaCl <sub>2</sub>	110.7	17.81	0.161	1.090
MgSO <sub>4</sub>	120.7	-7.19	-0.060	1.125
Na <sub>2</sub> SO <sub>4</sub>	142.1	11.56	0.081	1.128
CaSO <sub>4</sub>	136.5	-3.87	-0.028	1.137
K <sub>2</sub> SO <sub>4</sub>	174.6	32.02	0.183	1.140

Note that values for ions are hypothetical (see text).

composition (Table 2) to be 1.015 g cm<sup>-3</sup> (Table 5). The plasma would thus provide a positive buoyancy force in sea water of about 8.45 g l<sup>-1</sup>. There would be some further unaccounted minor solutes (e.g. SO<sub>4</sub><sup>2-</sup>, amino acids) that would slightly reduce the calculated lift, but the actual plasma density should be reasonably close to the calculated value for these elasmobranchs. The plasma density is calculated to be 1.014 g cm<sup>-3</sup> for *F. ventralis* and 1.015 g cm<sup>-3</sup> for *A. vincentiana*. Their positive buoyant forces would be 13.0 and 9.13 g l<sup>-1</sup> respectively.

#### Estimation of individual solute contributions to lift

It is not intuitively obvious how the lift contribution can be calculated for individual solutes or ions in a solution. We have adopted the following procedure. The relative contribution of each solute in a solution to buoyancy can be calculated from its concentration, molecular mass and absolute partial molal volume if we conceptually

Table 5. Calculation of density at 25 °C for sea water, and for the plasma and whole-muscle fluid of the black whaler shark *Carcharhinus obscurus*, from solute concentrations and partial molal volumes

	Sea water			Plasma			Muscle		
	$C_{\text{solute}}$ (g l <sup>-1</sup> )	$\Delta V_{\text{solute}}$ (ml l <sup>-1</sup> )	$\Delta V_{\text{solute}}$ (ml l <sup>-1</sup> )	$C_{\text{solute}}$ (g l <sup>-1</sup> )	$\Delta V_{\text{solute}}$ (ml l <sup>-1</sup> )	$\text{Lift}_{\text{solute}}$ (g l <sup>-1</sup> )	$C_{\text{solute}}$ (g l <sup>-1</sup> )	$\Delta V_{\text{solute}}$ (ml l <sup>-1</sup> )	$\text{Lift}_{\text{solute}}$ (g l <sup>-1</sup> )
Na <sup>+</sup>	10.81	-3.11	-1.84	6.42	-1.84	-0.81	0.94	-0.27	+0.13
K <sup>+</sup>	0.39	0.04	0.05	0.56	0.05	-0.13	4.14	0.39	-0.30
NH <sub>4</sub> <sup>+</sup>	0	0	0.009	0.01	0.009	+0.01	0.07	0.05	+0.11
Mg <sup>2+</sup>	1.30	-1.71	0.068	0.068	-0.090	-0.08	0.02	-0.03	-0.02
Ca <sup>2+</sup>	0.41	-0.29	0.14	0.14	-0.097	-0.14	0.10	-0.07	-0.09
Cl <sup>-</sup>	10.35	12.73	6.39	9.71	6.39	+4.00	1.52	1.00	+0.88
SO <sub>4</sub> <sup>2-</sup>	2.71	0.70							
Urea	0	0	15.34	20.79	15.34	+3.74	21.94	16.18	+6.13
TMAO	0	0	5.47	5.66	5.47	+1.81	12.02	11.63	+4.81
TMA	0	0	0.07	0.06	0.07	+0.04	0.49	0.59	+0.36
Betaine	0	0					1.85	1.60	+0.26
Sarcosine	0	0					6.15	4.76	+0.85
$\Sigma$ solute	34.98	8.37	25.31	43.42	25.31	+8.45	49.24	35.82	+13.11
Water	988.71	991.63	974.69	971.80	974.69		961.33	964.18	
Total	1023.69	1000.00	1000.00	1015.23	1000.00		1010.57	1000.00	
Density	1.0237			1.0152			1.0106		
Lift	-			+8.45			+13.11		

The relative contributions of each solute to buoyancy are also shown (see text for method of calculation).

Solute concentrations and partial molal volume contributions are expressed per litre of solution.

The solute composition for sea water is from Sanders and Childress (1988).

$C_{\text{solute}}$  is the solute mass per litre of solution.

$\Delta V_{\text{solute}}$  is the change in solution volume due the presence of that solute.

$\text{Lift}_{\text{solute}}$  is the calculated lift due to the presence of that solute in plasma, compared with sea water.

divide the solution into a number of fluid compartments, each of which contains only one solute species (e.g.  $\text{Na}^+$ ) and has a volume in proportion to its molar concentration relative to the total molar concentration, i.e. has the same molar concentration as the solution. Let us consider, for example, 1 l of plasma of *C. obscurus*, for which the total molar concentration of solutes (ions and non-electrolytes) that are accounted for is  $997 \text{ mmol l}^{-1}$ . The  $\text{Na}^+$  concentration of plasma is  $279 \text{ mmol l}^{-1}$ , so the  $\text{Na}^+$  'compartment' with a  $\text{Na}^+$  concentration of  $997 \text{ mmol l}^{-1}$  has a volume of 279.98 ml ( $279/997$ ). The density of a  $997 \text{ mmol l}^{-1}$   $\text{Na}^+$  solution, calculated from the partial molal volume using equation 3, is  $1.026571 \text{ g l}^{-1}$ . The lift of 1 l of  $997 \text{ mmol l}^{-1}$   $\text{Na}^+$ , relative to sea water ( $\rho=1.024 \text{ g cm}^{-3}$ ) is  $-2.7068 \text{ g}$ , and the lift due to the  $\text{Na}^+$  compartment of 1 l of plasma is  $-0.80635 \text{ g}$  ( $-2.7068 \times 279/997$ ). The usefulness of this calculation of lift contribution of an individual ion depends on the absolute partial molal volume being a realistic estimate of molal volume for an ion; a very different lift contribution of  $\text{Na}^+$  ( $+0.84 \text{ g l}^{-1}$ ) would be calculated from the conventional partial molal volume. The calculation of lift contribution for non-electrolytes or electroneutral salts is not subject to these considerations of absolute *versus* conventional molal volume.

The total lift calculated for plasma of *C. obscurus* is  $8.45 \text{ g l}^{-1}$  (Table 5), but part of this arises because the total concentration of solutes measured for *C. obscurus* plasma is lower than that for sea water. The calculated lift contribution of urea ( $3.7 \text{ g l}^{-1}$ ) is about 44 % of the overall lift. Lift due to TMAO ( $1.81 \text{ g l}^{-1}$ ) is also a significant fraction (21 %) of the total. Of the other main solutes, only  $\text{Cl}^-$  contributes positively to the overall lift ( $4.0 \text{ g l}^{-1}$  or 47 %).  $\text{Na}^+$  contributes significant negative lift ( $-0.81 \text{ g l}^{-1}$ , or  $-10 \%$ ) because of its substantial concentration and negative absolute molal volume. The other solutes contribute little buoyancy because they are present at low concentrations. Analysis of the plasma densities for *F. ventralis* and *A. vincentiana* (not shown) yields similar results, because of their similar solute compositions. Solute not accounted for, such as amino acids and protein, might affect the calculation of the overall buoyancy of plasma because of the low partial molal volume of amino acids and partial specific volume of protein, but they would not alter our interpretations of the substantial positive contributions of urea and TMAO to buoyancy. Muscle fluid of the elasmobranchs has a substantially different ion composition from plasma (Table 2), but similar urea and TMAO concentrations. In addition, betaine and sarcosine are present in significant amounts. These intracellular organic solutes are calculated to contribute substantial positive buoyancy to the black whaler shark (Table 5).

#### *Role of urea and TMAO in buoyancy*

How significant are urea and TMAO to the overall buoyancy of these elasmobranchs? To answer this fully, we would need to account for the buoyancy effect of all solutes, in all body fluid compartments, and for all other tissues (cartilage, lipid, etc.). Such a detailed analysis is not at present possible, but we can estimate the relative buoyancy roles of urea and TMAO as follows.

First, what would be the effect on buoyancy if urea and TMAO were not present in the body fluids? The most realistic way to consider this is to calculate solute replacement effects on the lift at constant body fluid osmolality. If plasma urea and TMAO were

replaced by NaCl, then the lift calculated from the contributions of all solutes would be reduced to about  $5.4 \text{ g l}^{-1}$ , compared with  $8.5 \text{ g l}^{-1}$  when urea is present. Note that the validity of this calculation does not depend on whether conventional or absolute partial molal volumes are used, since urea has been replaced by an electroneutral combination of  $\text{Na}^+$  and  $\text{Cl}^-$ . There is no marked effect of urea and TMAO replacement by NaCl because  $\text{Cl}^-$  contributes favourably to positive buoyancy. This plasma composition would be similar to that of the hagfish *Myxine glutinosus*, which is an iono-osmoconformer with a high NaCl concentration rather than a ureo-osmoconformer (Robertson, 1976). However, a more pronounced effect on density of muscle fluid would be expected if urea and TMAO were replaced by electrolytes such as  $\text{K}^+$ ,  $\text{SO}_4^{2-}$  and protein<sup>-</sup> (negatively charged protein), since these do not contribute as positive a molal volume as does  $\text{Cl}^-$ . Thus, the density of body fluid compartments is significantly reduced by the high concentrations of urea and TMAO. Another ion replacement strategy would be to replace  $\text{Na}^+$  with  $\text{NH}_4^+$ , as occurs in some marine animals. Retaining urea and TMAO but replacing all  $\text{Na}^+$  with  $\text{NH}_4^+$  increases the total solute contributions to about  $15.2 \text{ g l}^{-1}$  lift from  $8.5 \text{ g l}^{-1}$ ; this substantial increase in lift requires an  $\text{NH}_4^+$  concentration of  $279 \text{ mmol l}^{-1}$ , which far exceeds the toxicity level in vertebrates (about  $1 \text{ mmol l}^{-1}$ ; see Withers, 1992). Replacing all  $\text{Na}^+$ , urea and TMAO with  $\text{NH}_4^+$  and  $\text{Cl}^-$  increases the lift to  $17.1 \text{ g l}^{-1}$ , but requires an even higher  $\text{NH}_4^+$  concentration of  $490 \text{ mmol l}^{-1}$ .

Second, how significant is the lift contribution of urea and TMAO in body fluids to the overall buoyancy of elasmobranchs? If we assume a combined lift contribution of about  $5.7 \text{ g l}^{-1}$  for urea and TMAO, an extracellular space of 12% and an intracellular space of 57% (Holmes and Donaldson, 1969), we can estimate their contributions to buoyancy as a total lift of 26.6 g for *C. obscurus*, compared with 13.3 g of lift for the liver and a total overall negative buoyancy of 93.5 g (Table 6). Similarly significant contributions of body fluid urea and TMAO are also calculated for *F. ventralis* and *A. vincentiana*. Body fluid buoyancy is estimated to exceed that of the liver for all of the species examined here, although the contribution of the liver to overall buoyancy was generally low for all of these elasmobranchs, reflecting the small size of their liver. Thus, the buoyant force of the

Table 6. *Estimated lift contributions of the extracellular and intracellular fluids (calculated from plasma and muscle values) and liver of elasmobranch fishes compared with their overall negative buoyancy (mass in water)*

	Body fluid	Liver*	Overall mass in water (g)
<i>Furgaleus ventralis</i>	29.25 (10.5%)	24.1 g (8.6%)	279
<i>Carcharhinus obscurus</i>	26.60 (28.4%)	13.3 g (14.2%)	93.5
<i>Aptychotrema vincentiana</i>	1.30 (7.7%)	-0.3 g (-1.8%)	16.9

Values are expressed in grams (and as a percentage of total mass in water in parentheses).

\*The lift contribution of the liver includes the positive buoyancy of its extracellular and intracellular fluids as well as that of lipids and other solids.

urea and TMAO in the body fluids is substantial compared with, for example, that of the fatty liver.

Freshwater elasmobranchs do not accumulate high levels of urea or TMAO (Smith, 1931; Thorson *et al.* 1967). Could there, nevertheless, be a potential role for urea or TMAO in buoyancy regulation for freshwater elasmobranchs? Both solutes have  $MW_j > \bar{V}_j^0$ , so solutions would be as dense as or more dense than fresh water (see equation 3; Table 4). Accumulation of neither solute can therefore confer positive buoyancy in fresh water, although they would be suitable in freshwater animals as replacements for solutes with more negative  $\bar{V}_j^0$ .

The above analyses indicate that urea and TMAO have substantial effects on the buoyancy of marine elasmobranchs, contributing about  $5\text{--}6\text{ g l}^{-1}$  of lift. A solution of TMAO has a lower density than an equimolar solution of urea and therefore confers more lift. However, the ratio of TMAO to urea is generally about 1:2, rather than being predominantly TMAO. This low proportion of TMAO may reflect a higher metabolic cost of synthesis for TMAO compared with urea, or the dietary content of TMAO may limit its accumulation in body fluids (Kirschner, 1993). The presence of high concentrations of urea in marine elasmobranchs and chimaeras is generally ascribed to the ureo-osmoconforming strategy of these fishes (Griffith and Pang, 1969; Griffith, 1985, 1991). The high concentrations of methylamines, especially TMAO, and the 1:2 ratio of TMAO to urea, are ascribed to the counteracting solute effect of TMAO (Yancey and Somero, 1980; Yancey, 1985; Somero, 1986; Ballantyne and Moon, 1986; Yancey and Burg, 1989, 1990). Nevertheless, both urea and TMAO have substantial buoyancy effects, and this must surely be considered as a further adaptive advantage for their accumulation in the chondrichthyan fishes, which lack swimbladders.

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