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Dogmas and controversies in the handling of nitrogenous wastes: Osmoregulation during early embryonic development in the marine little skate *Raja erinacea*; response to changes in external salinity

S. L. Steele[1], P. H. Yancey[2] and P. A. Wright[1,*]

[1]*Department of Zoology, University of Guelph, Guelph, ON N1G 2W1, Canada* and [2]*Biology Department, Whitman College, Walla Walla, WA 99362, USA*

*Author for correspondence (e-mail: patwrigh@uoguelph.ca)

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Summary

Marine elasmobranchs retain relatively high levels of urea to counterbalance the osmotic strength of seawater. Oviparous species, such as the little skate *Raja erinacea*, release encapsulated embryos that hatch after about 9 months on the seafloor. To study the ureosmotic capability of skate embryos, we measured a variety of possible osmolytes and ornithine–urea cycle (OUC) enzyme activities in little skate embryos, and determined their physiological response to dilute seawater (75% SW) exposure relative to controls (100% SW). The urea:trimethylamine oxide (TMAO) + other osmolytes ratio was 2.3–2.7:1. At the earliest stage of development investigated (4 months), there were significant levels of the key OUC enzyme, carbamoyl phosphate synthetase III, as well as ornithine transcarbamoylase, arginase and glutamine synthetase, providing evidence for a functional OUC. Embryos (4 and 8 months) survived and recovered from exposure to 5 days of 75% SW. There was a significant increase in the rate of urea excretion (five- to tenfold), no change in OUC enzyme activities, and significant decreases in the tissue content of urea, TMAO and other osmolytes in embryos exposed to 75% SW compared to 100% SW. Taken together, the data indicate that little skate embryos synthesize and retain urea, as well as a suite of other osmolytes, in order to regulate osmotic balance with the external environment. Interestingly, these ureosmotic mechanisms are in place as early as 4 months, around the time at which the egg capsule opens and the embryo is in more direct contact with the external environment.

Key words: trimethylamine oxide, urea excretion, osmoregulation, elasmobranch, embryo, egg case, urea retention, osmolyte, little skate, *Raja erinacea*.

Introduction

Elasmobranchs mostly inhabit marine environments, except for the strictly freshwater genus *Potomatrygon*, and are therefore faced with the dehydrating force of salt water. Elasmobranch body fluids are actually slightly hyperosmotic to seawater (for a review, see Holmes and Donaldson, 1969), the predominant osmolyte being urea, which reaches concentrations up to 680 mmol l^{-1} in tissues, depending on the species (e.g. Browning, 1978). Urea is toxic at these concentrations; protein side chains become more fluid and normal protein folding patterns are interrupted (for a review, see Yancey, 2001b). In order to counteract this extreme uraemia, other organic osmolytes that have protein-stabilizing properties (primarily methylamines) are present in elasmobranch tissues. Trimethylamine oxide (TMAO) is generally the principle counteracting osmolyte in elasmobranchs. The importance of other organic stabilizers varies depending on species, but typically includes sarcosine (Forster and Goldstein, 1976) and betaine (Robertson, 1975; Bedford et al., 1998). Taken together, the various counteracting osmolytes have additive effects on urea destabilization and have been found to perform optimally *in vitro* when the ratio of urea to methylamines is 2:1 (for a review, see Yancey, 2001a). This ratio is well conserved across species, supporting the hypothesis that it is physiologically important for optimum macromolecule stability.

There is very little information on the tissue organic osmolyte ratio in early life stages. In the big skate *Raja binoculata* embryo, the ratio of urea:TMAO can be calculated as approximately 3.4:1 (from data reported by Read, 1968a). Possibly embryonic elasmobranchs are unable to maintain the typical 2:1 ratio, or other organic osmolytes that have not been measured play an important role, or higher ratios are the norm for early life stages. Thus, fundamental information is missing on the role of compatible and counteracting osmolytes in early life stages.

As in mammals, urea in elasmobranchs is synthesized *via* the hepatic ornithine urea cycle (OUC), which is composed of the enzymes carbamoyl phosphate synthetase III (CPSase III), ornithine transcarbamoylase (OTCase), argininosuccinate

synthase (ASS), argininosuccinate lyase (ASL) and arginase. Unlike the mammalian CPSase I that prefers ammonia as a nitrogen-donating substrate, elasmobranch CPSase III requires glutamine, and therefore glutamine synthetase (GSase) is a necessary accessory enzyme for this pathway. Is the OUC functional in embryonic elasmobranchs? Read (1968a) discovered that total urea content increased in the oviparous *R. binoculata* with embryonic mass (and presumably age) and that they maintain relatively constant concentrations of urea and TMAO in both the embryonic body and yolk throughout embryonic development. Evans and Kormanik (1985) removed embryos prematurely from the uterus of the viviparous spiny dogfish *Squalus acanthias* and reported that they maintain physiological concentrations of blood urea for at least 3 days. This very early ureosmotic capability may be due to the presence of a functional OUC in embryonic elasmobranchs. Read (1968b) did indeed establish the presence of two OUC enzymes, OTCase and arginase, in the embryonic bodies (but not the yolks) of the spiny dogfish *S. suckleyi* and *R. binoculata*. CPSase III is considered the rate limiting enzyme of the OUC (Anderson, 1995) and GSase is an important auxillary enzyme that supplies the nitrogen donating substrate glutamine. To our knowledge, these enzymes have never been measured in the embryos of any species of elasmobranch. Thus, the co-expression of these four enzymes (GSase, CPSase III, OTCase and arginase) would provide support for the possibility of a functional OUC early in development. In the absence of CPSase III, urea synthesis *via* arginolysis or uricolysis occurs (Mommsen and Walsh, 1992).

Marine elasmobranchs downregulate tissue and/or blood levels of urea and/or TMAO when acclimated to dilute (50%) seawater (Price and Creaser, 1967; Goldstein et al., 1968; Goldstein and Forster, 1971; Cooper and Morris, 1998). Lower tissue osmolytes can be achieved by a short term increase in total (Goldstein et al., 1968) or renal (Goldstein and Forster, 1971; Payan et al., 1973) urea excretion and/or a sustained reduction in the rate of urea synthesis. Changes in urea production or OUC enzyme activities, however, have not been investigated in marine elasmobranchs exposed to dilute seawater.

Our understanding of the development of physiological regulations, such as osmoregulation, in non-mammalian species has been described as ‘embryonic’ (Spicer and Gaston, 1999). While previous studies have been conducted on mature individuals, dilute seawater challenge has never been studied in embryonic elasmobranchs. Smith (1936) proposed that the embryos of elasmobranchs are afforded early protection from osmotic stress either by encapsulation in a sealed egg case (in the case of oviparity) or by incubation in osmotically stable intrauterine fluids (in the case of viviparity). Embryos of oviparous species, however, are exposed directly to seawater well before hatching (Wourms, 1977) and may be able to independently ureosmoregulate by altering the rate of urea excretion and/or the rate of urea synthesis.

The first objective of this study was to determine the full suite of organic osmolytes retained in embryonic elasmobranchs and the ratios of urea:TMAO and other osmolytes. Secondly, we wanted to establish the presence (or absence) of the key regulatory OUC enzyme, CPSase III, as well as related OUC enzymes, in early life stages. Finally, we wanted to determine the ability of elasmobranch embryos to adjust their ureosmotic parameters under dilute water stress. We predict that if embryos have the capacity to acclimate to a lower external salinity, they may increase the rate of urea excretion and/or decrease the activities of OUC enzymes in order to downregulate tissue urea levels. Captive little skates *Raja erinacea* at the Hagen Aqualab, University of Guelph, Canada, release multiple egg cases each year. The embryos develop for approximately 9 months before hatching (at 10°C). In contrast to Read’s studies (Read, 1968a,b) where increasing age was assumed by increasing embryonic mass, the precise dates of oviposition were recorded for individual embryos used in the present experiment. To address our first objective, urea, TMAO and 12 other potential osmolytes (glucose, myo-inositol, taurine, glycerophosphorycholine (GPC), serine, betaine, glycine, proline, creatine, β-alanine, glutamine and sarcosine) were measured in 4- and 8-month post-oviposition embryonic body and yolk, and the ratio of urea to other osmolytes was calculated. The activity of the OUC enzymes CPSase III, OTCase and arginase, as well as the accessory enzyme GSase were measured at both stages. Finally, osmotic stress was induced by exposing the embryos to 100% (control) or 75% seawater for 5 days, and the embryos were subsequently recovered in 100% seawater for 3 days. Urea and ammonia excretion, tissue osmolytes and urea cycle enzymes were measured in both groups.

Materials and methods

Animals

Sexually mature little skates *Raja erinacea* Mitchill (mass ~500 g) held in captivity at the Hagen Aqualab, University of Guelph, Canada, regularly produced embryos, which they deposited onto a sand substrate. Upon oviposition the embryos were removed from the adult tank, tagged with the date of oviposition, and placed in separate (100 l, 1.2 m×0.6 m×0.2 m) tanks supplied with recirculating artificial seawater (Crystal Sea, Baltimore, MA, USA; 33 p.p.t., pH 8.2, 10°C) for the duration of their development (approximately 9 months). The embryos were not fed during the experiment as they were feeding endogenously before hatching. The embryo mass was approximately 0.35 g (at 4 months) and 4.13 g (at 8 months).

Experimental protocol

Prior to exposure to dilute seawater (SW), embryos (4 and 8 months) were removed from their egg cases by making a small incision at one end of the case and gently tipping the animal into the experimental chamber. By removing the egg case, nitrogen excretion rates to the external environment could be measured directly rather than by sampling both inside and outside the egg case in intact individuals. In preliminary experiments on 3–4-month embryos, urea and TMAO embryonic tissue and yolk contents were measured in intact

embryos. There were no differences between these embryos and those in the present study (N=4–6); for example, urea in the embryonic tissue (382±26.2 mmol kg^{-1} tissue water) and yolk (448±45.2 mmol kg^{-1} tissue water) of the intact embryos was not significantly different from the values in decapsulated 4-month embryos presented in this study. Furthermore, Leonard et al. (1999) compared metabolic rate (i.e. oxygen consumption) in late term intact *versus* decapsulated embryos and found no significant differences. Embryos were placed individually into 250 ml plastic beakers with perforations, to allow for water renewal, and were then placed in individual 12 l chambers of either 100% (33 p.p.t., control) or 75% (25 p.p.t., dilute) aerated SW (10°C). Dilute SW was made by mixing seawater from the system with deionized water to a salinity of 25 p.p.t. (this dilution did not affect the pH or temperature of the water). Water in all chambers was changed every 48 h to ensure that excreted ammonia or urea was less than 20 μmol l^{-1}.

Excretion rates were measured by placing the beaker in which the animal was housed in a new, unperforated beaker containing either 60 ml (4-month) or 70 ml (8-month) of fresh 100% or 75% SW for a 3 h flux period. Water samples (15 ml) were collected at 0 and 3 h and stored at –20°C (for up to 6 weeks) for later analysis of ammonia and urea content. Urea and ammonia excretion rates were calculated as previously described by Wright and Wood (1985). This procedure for measuring nitrogen excretion rates was repeated every 24 h up to and including 120 h after the onset of exposure to 100% or 75% SW. It should be mentioned that preliminary trials using various volumes of water and flux periods were conducted, and the volumes and times chosen were those that produced the best representation of excreted ammonia and urea at both developmental stages. After the final flux period, embryos were removed from their chambers, briefly blotted dry and weighed. In the case of 4-month embryos, the embryonic body was carefully dissected from the yolk sac. The embryonic body was then quickly weighed, placed in a cryovial and flash frozen in liquid nitrogen. For 8-month animals, whole embryos were first weighed and then killed by cervical section. Yolk was quickly sampled from both 4- and 8-month stages (approximately 0.5–1 ml) by draining directly into a cryovial and frozen. Excess yolk was removed from the 8-month body cavity by wiping with a Kimwipe until no yolk remained. Animals were then weighed again and flash frozen in liquid nitrogen.

Water content (%) of embryos and yolk samples was determined in a separate group of embryos exposed to 75% or 100% SW for 120 h as described above. After wet mass had been recorded, all samples were placed in an oven (55°C) for drying until a constant dry mass was achieved. Percent water was calculated as wet mass (g) – dry mass (g) / wet mass (g) × 100.

In order to determine the time to recovery after exposure to 75% SW, a separate group of 4- and 8-month embryos were exposed to dilute seawater for 120 h as described above and then recovered in 100% SW. Excretion rates were measured as described above over a 3 h period immediately following replacement in 100% seawater and every 24 h thereafter for 72 h. Tissues were sampled at the end of the recovery period as described above.

Water sample analysis

Water samples were analyzed for urea content using a colorimetric assay described by Rahmatullah and Boyde (1980). The detection limits of this assay were 0.1–100 μmol l^{-1} urea.

The ammonia concentration in water samples was measured using the Indophenol Blue method described by Ivancic and Degobbis (1984). The detection limits of this assay were 0.1–300 μmol l^{-1} ammonia.

Tissue osmolyte analysis

Embryos were ground to a fine powder using a mortar and pestle under liquid nitrogen. Urea was measured using the method described by Rahmatullah and Boyde (1980). All tissue and yolk samples were prepared for urea analysis as described by Steele et al. (2001), with the following exceptions. Tissue supernatant was diluted an additional 500-fold for a total dilution of 5000× (in order to bring the concentrations within the detection limits of the assay), and instead of 8% perchloric acid, 5% trichloroacetic acid (TCA) was used for deproteinization (no difference in urea content was observed when TCA was substituted; data not shown).

TMA/TMAO was measured in both embryonic tissue and yolk using the ferrous sulphate/EDTA method described by Wekell and Barnett (1991). Samples (ground embryo or yolk) were dissolved in 10 volumes of 5% TCA (samples were vortexed extensively to ensure homogeneous mixing) and centrifuged at 16 000 ***g*** for 10 min. The supernatant was diluted an additional fivefold with 5% TCA for TMAO analysis. All spectrophotometric measurements for urea, ammonia and TMA/TMAO were made using a Perkin Elmer UV/VIS Lambda 2 spectrophotometer (Perkin Elmer Corp., Norwalk, CT, USA). The detection limits of this assay were 0.5–3 mmol l^{-1} TMA.

Additional potential osmolytes (glucose, myo-inositol, GPC, taurine, urea, serine, betaine, sarcosine, glycine, proline, creatine, β-alanine) were measured using the HPLC method of Wolff et al. (1989). Samples were prepared by homogenizing in 9 volumes of 7% perchloric acid and centrifuging for 20 min at 14 000 ***g*** in a microcentrifuge. Supernatants were neutralized with 2 mol l^{-1} KOH, centrifuged for 5 min, then passed through a C18 cartridge (Analytichem, Bowie, MD, USA) and a 0.45 μm filter (Millipore, Billerica, MA, USA).

Tissue OUC analysis

In preliminary analysis it was determined that the yolk fraction contained no detectable OUC enzyme activity (data not shown) and therefore analysis was performed on embryonic tissue alone. Samples were homogenized in approximately 30 volumes (w/v) ice-cold extract buffer (0.05 mol l^{-1} Hepes, pH 7.5, 0.05 mol l^{-1} KCl, 0.5 mol l^{-1} EDTA, 1 mmol l^{-1} DL-dithiothreitol) and centrifuged for

10 min at 14 000 ***g*** (4°C). The tissue homogenate supernatant was then passed through a cold 60 ml Sephadex (G25) column in order to remove low molecular mass solutes.

In order to account for the presence of any potential remaining endogenous substrates, controls without exogenous substrate were included in each enzyme assay. Enzyme reactions for GSase, CPSase III, OTCase and arginase were run according to Steele et al. (2001), except that GSase, OTCase and arginase activities were determined by the amount of product produced from 0 to 6 min, while the CPSase III reaction was stopped after 18 min. Trial experiments using ammonia (NH_4Cl) as the N-donating substrate showed that activity was less than 5% of total CPSase activity occurring in the presence of glutamine (data not shown). Enzyme activities were measured at 26°C.

Statistical analysis

Differences between osmolyte contents (TMAO, urea, total other osmolytes) or enzyme activities in 100% and 75% SW groups were determined using a General Linear Models means comparison procedure, and urea and ammonia excretion rates were analyzed using repeated-measures analysis of variance (ANOVA) with a General Linear Models procedure, both using the SAS system (version 8e; SAS Institute Inc., Cary, NC, USA). A Tukey test was applied if significant differences were detected between control and 75% seawater exposed animals. Statistical significance in all tests was declared at $P<0.05$. Due to the limited number of embryos available for these experiments, a very small number of animals (N=3 or 4) was used in all experiments. Data are presented as means ± S.E.M.

Results

Tissue osmolytes in 100% seawater: yolk vs. embryo

Water content of 4- and 8-month embryo and yolk samples was significantly higher after 120 h of exposure to 75% SW relative to 100% SW controls (Table 1). Tissue levels of urea, TMAO and other osmolytes were therefore corrected for tissue water content (Table 2). The sum of eleven 'other' osmolytes (–glucose) constitutes the total of osmolytes besides urea and TMAO. Glucose is not normally considered an important organic osmolyte and was only detected in the yolk (Table 2). Several individual organic osmolytes were significantly different between the embryonic tissues and the yolk in 4- and 8-month embryos (Table 2). Myo-inositiol and taurine were the only two osmolytes that were significantly higher in embryonic tissue relative to yolk at both the 4- and 8-month stage, whereas serine, proline and TMAO were significantly lower in both stages. β-alanine, betaine and sarcosine were significantly lower in 4-month embryo *versus* yolk. β-alanine, sarcosine and creatine were significantly higher in 8-month embryo *versus* yolk.

Tissue osmolytes in 100% seawater: 4-month vs. 8-month

There were also a number of significant differences between the 4- and 8-month embryos. Contents of several osmolytes (betaine, creatine, sarcosine and β-alanine, with the latter rising the most) increased (3- to 12-fold) from 4 to 8 months, although taurine and glutamine decreased by 2.8- and 2.1-fold, respectively (Table 2). Interestingly, an almost opposite trend was observed in the yolk where osmolyte levels (taurine, proline, β-alanine, sarcosine) were significantly lower in the 8-month relative to the 4-month old yolks, except for GPC, which increased over fivefold, and glycine, which increased to detectable levels (Table 2).

Table 1. *Percent water content of 4- and 8-month old* Raja erinacea *embryos and yolk samples after exposure to 100% or 75% seawater (SW) for 5 days*

Age (months)	Sample	100% SW	75% SW
4	Embryo	86.3±0.37 (4)	88.6±0.29 (4)*
	Yolk	51.5±0.32 (4)	54.1±0.46 (4)*
8	Embryo	81.0±0.40 (3)	83.3±0.27 (4)*
	Yolk	47.4±0.67 (3)	51.2±2.43 (3)*

Values are means ± S.E.M. (N).
*Significantly different from control (100% SW) value ($P<0.05$).

The total of all the organic osmolytes measured (minus TMAO and urea), as well as urea alone, was lower in 4-month *versus* 8-month embryonic tissue (Table 2). Tissue ratios of urea:TMAO and other osmolytes were significantly higher in 8-month embryonic tissue and yolk compared to 4-month embryos (Table 2).

Response to dilute (75%) seawater

No mortalities occurred in either 4- or 8-month embryos due to dilute seawater exposure or recovery. Urea excretion rates were significantly increased (tenfold) in 4-month embryos at the onset of exposure to 75% SW (over the first 3 h), and although excretion fell by 24 h, rates remained significantly elevated over the control (100% SW) rate (Fig. 1). Excretion rates returned to control levels by 48 h of 75% SW exposure and remained there for the rest of the 120 h exposure period. Upon return to 100% seawater, urea excretion was slightly, but significantly elevated over control rates for up to 24 h but returned to control values by 48 h (Fig. 1). Similarly, in 8-month embryos, there was a significant increase in urea excretion (fivefold) just after exposure to 75% SW, with excretion rates returning to control levels by 48 h (Fig. 2). Urea excretion rates increased significantly (11-fold) at the onset of recovery in 100% SW but returned to control levels 24 h into recovery (Fig. 2). Ammonia excretion was not detected in 4-month embryos; however, in 8-month embryos ammonia excretion constituted approximately 20–66% of total nitrogen excretion [% ammonia excretion = (ammonia N excretion rate / ammonia N excretion rate + urea N excretion rate) × 100] over the course of the experimental period (Fig. 3). There was no change in ammonia excretion in response to dilute seawater exposure; however, ammonia excretion significantly increased (1.4-fold) at the onset of 100% SW recovery (Fig. 3).

Tissue TMAO content was significantly lower in 4-month

Table 2. *Content of various osmolytes in the embryo and yolk of 4- and 8-month old* Raja erinacea *embryos exposed to 100% or 75% seawater for 5 days*

	4 months				8 months			
[Osmolyte]	100% SW		75% SW		100% SW		75% SW	
(mmol kg^{-1} tissue water)	Embryo	Yolk	Embryo	Yolk	Embryo	Yolk	Embryo	Yolk
Other (see text)								
Myo-Inositol	5.95±0.48	1.33±0.06*	4.84±0.59	1.18±0.20	4.5±0.52	1.18±0.34*	3.26±0.23	0.76±0.07
Taurine	31.9±1.24	14.6±1.78*	28.2±2.52	12.3±1.40	12.3±0.39†	5.52±0.64*,†	9.72±0.77	3.00±0.26
GPC	1.10±0.14	1.08±0.21	0.47±0.06	0.53±0.15	1.79±0.20	6.59±0.92†	0.65±0.09‡	3.13±0.20‡
Serine	0.98±0.09	8.23±0.91*	0.85±0.15	11.3±2.25	2.48±0.34	6.48±2.17*	0.90±0.17	3.78±1.48
Betaine	1.96±0.04	7.34±0.86*	1.26±0.18	11.0±1.26	7.32±0.62†	6.56±1.24	6.52±0.90	4.79±0.77
Glycine	1.22±0.13	BLD	BLD‡	BLD	2.28±0.28	4.29±0.60	BLD‡	2.23±0.16‡
Proline	BLD	11.0±0.56	BLD	10.8±1.60	BLD	2.71±0.52†	BLD	2.00±1.00
Creatine	1.66±0.17	3.46±0.40	2.04±0.54	7.94±1.46	8.84±0.83†	4.08±0.68*	7.44±0.43	2.09±0.53
β-alanine	2.23±0.32	8.79±0.47*	1.68±0.24	10.8±1.60	27.3±0.96†	6.36±0.78*,†	16.9±1.52‡	4.11±0.31
Glutamine	7.57±0.43	BLD	7.30±0.53	BLD	3.86±0.58†	BLD	2.58±0.26	BLD
Sarcosine	0.29±0.06	1.85±0.52*	0.10±0.03	1.82±0.26	3.81±0.38†	0.47±0.06*,†	2.21±0.42	0.22±0.03
Total other	54.9±2.33	57.7±3.80	46.7±4.01	67.7±4.93	74.4±1.66†	33.2±6.84	50.2±2.15‡	26.1±3.71
Glucose	BLD	1.52±0.28	BLD	1.12±0.12	BLD	3.08±2.23	BLD	1.42±0.67
Urea	362±13.5	497±36.7	315±18.5	409±38.8	458±14.0†	515±14.1	364±21.1‡	434±23.1
TMAO	104±4.75	155±6.09*	76.3±5.64‡	127±8.02	94.3±2.02	132±15.9*	74.7±3.94	113±11.7
Urea:TMAO+total other	2.27±0.06	2.32±0.10	2.59±0.30	2.10±0.13	2.72±0.13†	2.96±0.37†	2.97±0.10	3.18±0.48

Values are means ± S.E.M.; N=4 except for 8-month yolk in 100% SW (N=3).
BLD, below level of detection.
*Significant difference within a developmental stage between different tissue types (P<0.05).
†Significant difference between stages for the same tissue type (P<0.05).
‡Significant difference within stage and tissue type between 100% and 75% SW conditions (P<0.05).

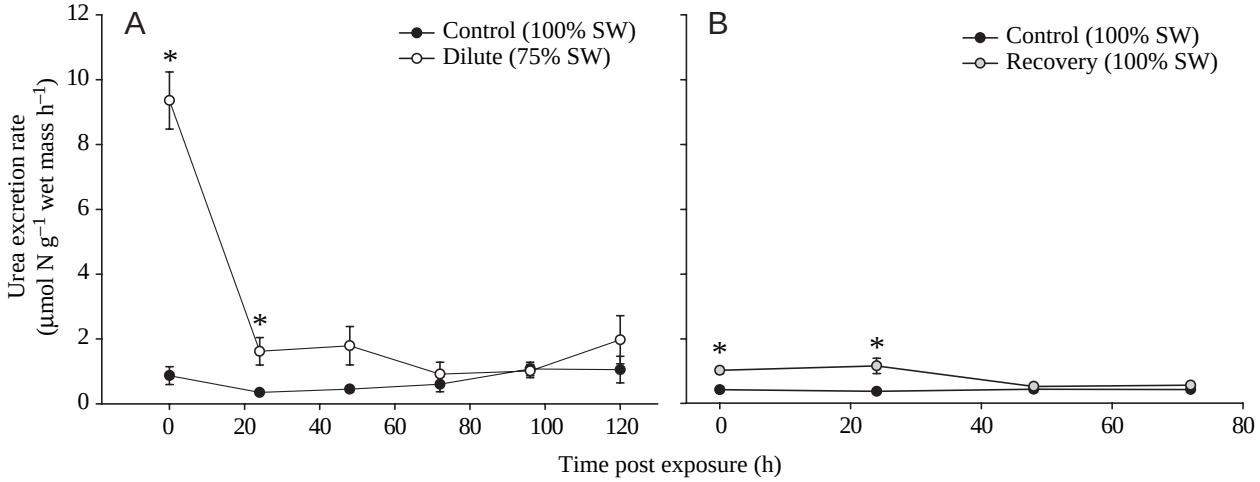

Fig. 1. Urea excretion rates (mean ± S.E.M.) in 4-month old *Raja erinacea* embryos over a 120 h period of exposure to 75% seawater (A) and a subsequent 78 h reintroduction to 100% seawater (B), N=4. *Significantly different from control value at that time point (P<0.05).

embryos exposed to 75% SW for 120 h, with full recovery by 72 h in 100% seawater (Table 2, Fig. 4). Similarly, glycine was significantly decreased in the embryonic tissue of 4-month embryos exposed to 75% SW (Table 2). However, levels of other osmolytes in the embryo, and all osmolyte levels in the yolk, did not change between 100% and 75% SW (Table 2, Fig. 4). Contents of tissue urea and several other osmolytes were significantly lower in 8-month embryos after 120 h of 75% SW exposure, with full recovery by 72 h (Table 2, Fig. 5). GPC and glycine decreased significantly in 8-month

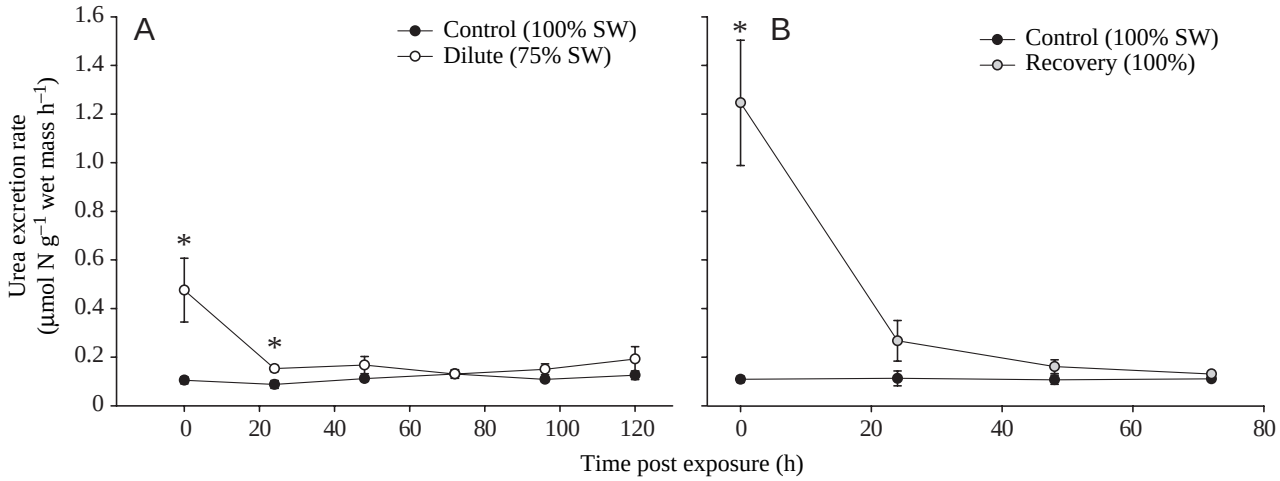

Fig. 2. Urea excretion rates (mean ± S.E.M.) in 8-month old *Raja erinacea* embryos over a 120 h period of exposure to 75% seawater (A) and a subsequent 78 h reintroduction to 100% seawater (B), *N*=4. *Significantly different from control value at that time point (*P*<0.05).

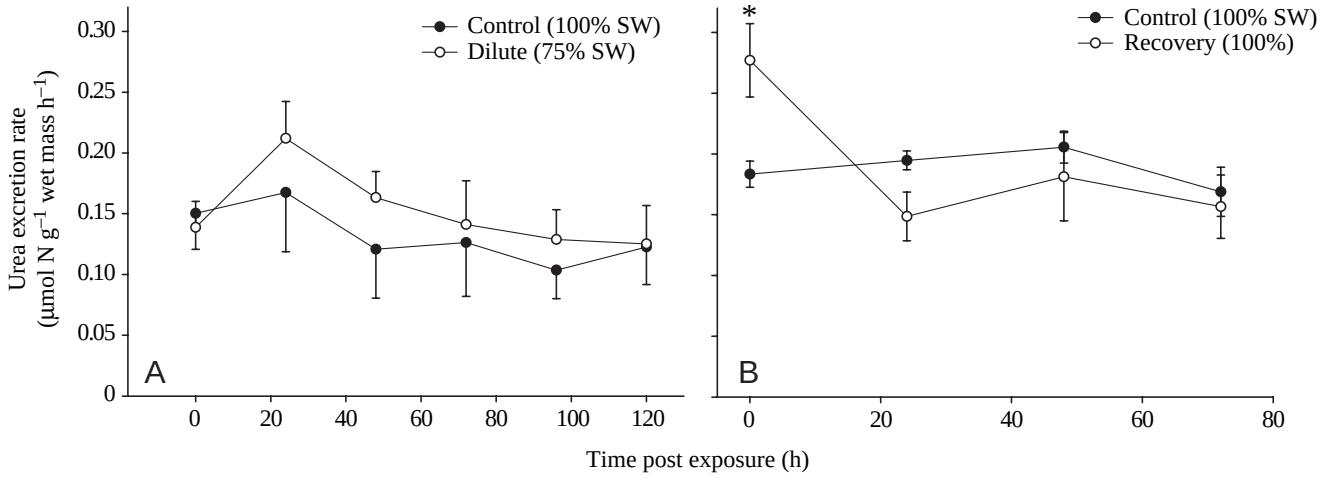

Fig. 3. Ammonia excretion rates (mean ± S.E.M.) in 8-month old *Raja erinacea* embryos over a 120 h period of exposure to 75% seawater (A) and a subsequent 78 h reintroduction to 100% seawater (B), *N*=4.

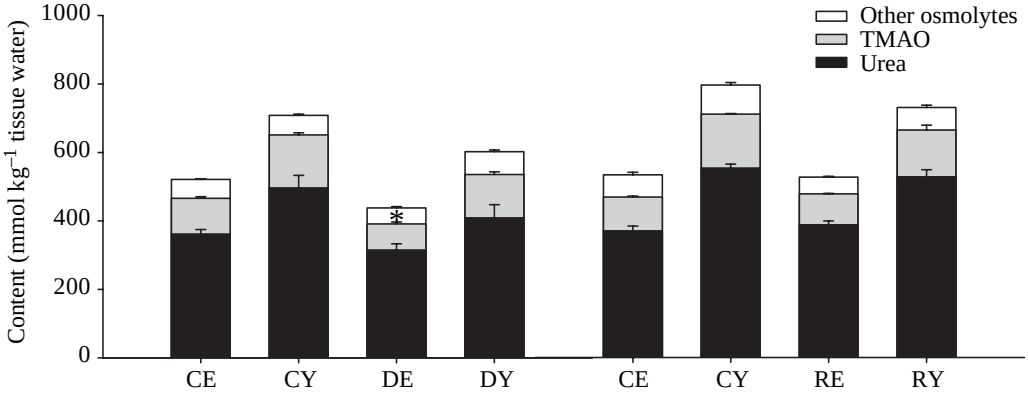

Fig. 4. Stacked plot of urea, TMAO, and total other osmolytes content (mean ± S.E.M.) in 4-month old embryos (E) and yolk (Y) samples after both 120 h of 75% seawater exposure (D) and 78 h of recovery in 100% (R) seawater, *N*=4. C, control. *Significantly different from control value (*P*<0.05).

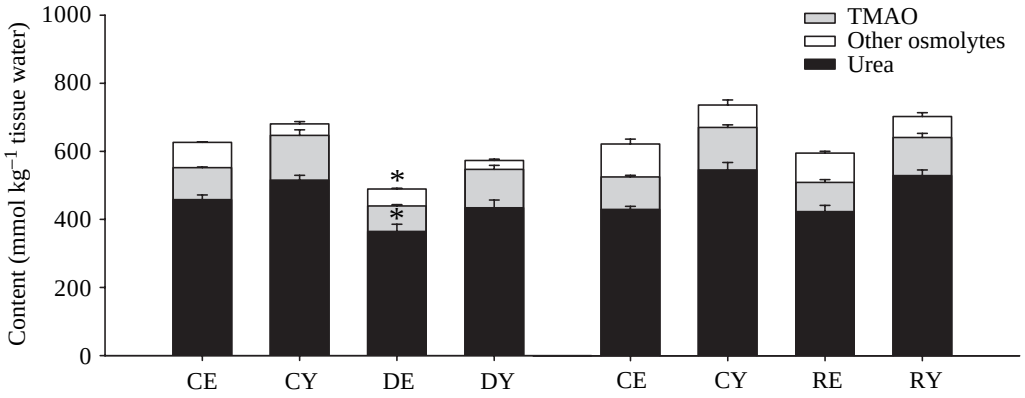

Fig. 5. Stacked plot of urea, TMAO, and total other osmolytes content (mean ± S.E.M.) in 8-month old embryos (E) and yolk (Y) samples after both 120 h of 75% seawater exposure (D) and 78 h of recovery in 100% (R) seawater, *N*=4. C, control. *Significantly different from control value ($P<0.05$).

embryonic tissue and yolk after 75% SW exposure, and β-alanine was significantly decreased in the embryonic tissue alone (Table 2). Many other individual osmolytes in both tissues of both stages showed lower averages after 75% SW exposure (Table 2), but were not statistically different.

CPSase III and other OUC enzyme activities (GSase, OTCase and arginase) were detected in both 4- and 8-month embryos (Table 3). There were no significant differences between enzyme activities and developmental stage. Additional enzyme assays were performed on skeletal muscle tissue isolated from 8-month embryos (Gsase 1.26±0.42; CPSase III 0.008±0.001; OTCase 10.02±1.97; arginase 1.22±0.17 μmol min^{-1} g^{-1}, *N*=6). Whole embryo CPSase III and other OUC enzyme activities were unchanged by exposure to 75% SW exposure, either on a per g wet mass or a per mg protein basis (Table 3).

Discussion

Our study provides the first evidence that embryos of the little skate *R. erinacea* have a full range of osmoregulatory strategies in place well before hatching. Although the literature is sparse, previously published data indicated that skate and dogfish embryos maintain elevated urea levels independent of the mother (Read, 1968a,b; Evans and Kormanik, 1985). Our findings confirm these earlier reports and extend previous observations in three ways.

First, by measuring the contents of the destabilizing osmolyte urea and an array of compatible and counteracting osmolytes, we have established the urea:TMAO + other osmolyte ratio to be 2.3–2.7:1, similar to published values for adult elasmobranchs (2–2.4:1; reviewed by Yancey and Somero, 1980). As in adult elasmobranchs, these ratios may be closer to 2:1 in the intracellular compartment, because osmolyte analyses were conducted on whole-tissue extracts. Urea is generally equilibrated between intra- and extracellular fluids (Forster and Goldstein, 1976), thus the whole-tissue urea content per kg water (Table 2) should be fairly close to actual *in vivo* concentrations (mmol l^{-1}). However, the other organic osmolytes are typically more concentrated in the intracellular

Table 3. *Glutamine synthetase (GSase), carbamoyl phosphate synthetase III (CPSase III), ornithine transcarbamoylase (OTCase) and arginase activities in 4- and 8-month old* Raja erinacea *embryos exposed to 100% or 75% SW for 5 days*

	4-month embryo		8-month embryo	
	100% SW	75% SW	100% SW	75% SW
GSase	10.72±1.08	9.40±1.35	6.92±0.53	5.50±0.30
	0.70±0.13	**0.53±0.13**	**0.34±0.05**	**0.33±0.04**
CPSase III	0.010±0.002	0.012±0.008	0.018±0.003	0.016±0.004
	$6\times10^{-4}\pm1\times10^{-4}$	**$7\times10^{-4}\pm1\times10^{-4}$**	**$8\times10^{-4}\pm2\times10^{-4}$**	**$10\times10^{-4}\pm7\times10^{-5}$**
OTCase	11.03±1.58	9.36±2.46	14.29±1.10	13.11±0.52
	0.72±0.15	**0.53±0.21**	**0.69±0.05**	**0.80±0.07**
Arginase	7.90±0.77	8.69±2.07	9.25±0.37	7.88±0.44
	0.52±0.10	**0.49±0.19**	**0.47±0.07**	**0.48±0.07**

Values are means ± S.E.M. (*N*=4).
Enzyme activities are μmol min^{-1} g^{-1} wet mass and μmol min^{-1} mg^{-1} protein (in bold).

compared to extracellular compartment (Forster and Goldstein, 1976). Hence, the whole-tissue organic osmolyte content (Table 2) is probably less than actual *in vivo* intracellular concentrations (Yancey and Somero, 1980). Nevertheless, the ratios of urea:TMAO+other osmolytes were determined using whole-tissue extracts in both adults (Yancey and Somero, 1980) and embryos (present study) and are therefore comparable.

Second, the presence of significant levels of the rate-limiting OUC enzyme CPSase III, along with OTCase, arginase and GSase, strongly suggests that the OUC is functional very early in development. In particular, the activity of CPSase III is more than sufficient to account for urea excretion rates in embryos (Table 3, Figs 1, 2; for example, see Appendix).

Third, no mortalities occurred in embryos exposed to and recovered from a 5-day exposure to dilute seawater (75%), an osmotic shock that would drive water in and osmolytes out of embryonic tissues. Furthermore, by studying embryos of known age we were able to differentiate between the osmoregulatory abilities of very young embryos (4 months, 0.35 g embryonic tissue only) from more developed, but still encapsulated embryos (8 months, 4.13 g embryonic tissue only). Although there were some marked differences between 4- and 8-month embryos (see below), overall we can conclude that little skate embryos synthesize and retain urea, as well as a suite of other osmolytes, in order to regulate osmotic balance with external seawater.

Developmental differences were observed in the osmolyte ratios and the response to environmental dilution. The urea:TMAO + other osmolyte ratio was significantly higher in 8-month than in 4-month embryos (Table 1). This difference is due, for the most part, to the significantly higher (+27%) urea levels in the 8-month old embryos, but only modestly higher levels of other osmolytes and similar TMAO contents (Table 2). There were also differences in individual osmolytes other than urea and TMAO. Taurine was the principle 'other' osmolyte found in the embryonic tissue of 4-month embryos, but was significantly lower in the 8-month stage. The predominant 'other' osmolyte at 8 months was β-alanine (Table 2). Sarcosine was also significantly higher in 8-month than in 4-month embryonic tissue (Table 2). As well as having protein stabilizing properties (Yancey and Somero, 1979, 1980), these compounds are arguably some of the most important free amino acids involved in cell volume regulation in fish (King and Goldstein, 1983). Taurine and β-alanine in particular are distributed in comparatively large quantities in a variety of tissues, such as the elasmobranch brain, red blood cells, myocardium and muscle, while sarcosine is a major component of the amino acid pool in skate muscle (for a review, see King and Goldstein, 1983). Their importance as osmolytes probably stems from the fact that, in addition to being compatible or stabilizing towards proteins, they are relatively metabolically inert compared to other amino acids and can therefore be accumulated without interfering with metabolic pathways within the cell (King and Goldstein, 1983). It would be logical to consider, therefore, that early life stages may be actively regulating the metabolism and retention of these amino acids as development progresses to establish the high physiological levels required in adulthood.

The relative balance between TMAO and other compatible and stabilizing osmolytes varies between species and possibly between developmental stages. Some shark tissues are known to have relatively high TMAO levels, whereas the little skate appears to retain lower TMAO levels but higher levels of osmolytes such as sarcosine, β-alanine, taurine, etc. (King and Goldstein, 1983). Our data indicate that TMAO levels are relatively high (two- to threefold higher) at both embryonic stages (Table 2) compared to TMAO levels in adult muscle cells and plasma (64 mmol l^{-1} and 39 mmol l^{-1}, respectively; Forster and Goldstein, 1976) and adult liver and muscle tissue (~40-50 mmol kg^{-1} tissue water; S. Steele, P. Yancey, P. Wright, manuscript submitted for publication). The total of other osmolytes in embryos are comparable to adult liver tissue, but are only about one half of the total in adult muscle tissues (~180 mmol kg^{-1} tissue water, S. Steele, P. Yancey, P. Wright, manuscript submitted for publication). The embryonic total osmolytes are also low compared to published values for adult wing muscle taurine, sarcosine, proline, glycine and β-alanine reported by Boyd et al. (1977), which add up to approximately 92 mmol kg^{-1}. When considering the collection of compatible and counteracting osmolytes in the little skate, therefore, the present data indicate that TMAO may play a larger role than other non-urea osmolytes in embryos than in adults.

Yolk levels of urea, TMAO and other osmolytes are in most cases significantly higher compared to embryonic tissues when calculated as per tissue water (Table 2). If one calculates contents based on the wet mass of the tissue, however, yolk osmolyte contents are typically lower than comparable embryonic tissue levels (data not shown), due to the much lower water content (Table 2). The yolk of teleost species ranges from 54% water in freshwater salmonids (e.g. Rombough, 1988) to 93% water in marine pelagic teleosts (e.g. Ying and Craik, 1993). As discussed above, *in vivo* tissue concentrations may be the same (urea) or higher (other organic osmolytes) than whole-tissue contents, but the situation may be more complex in yolk because of its composition. The distribution of urea and other osmolytes in the yolk water, yolk lipid and extracellular fluid is unknown, but is necessary in order to understand the actual *in vivo* yolk concentrations.

TMAO content was significantly lower in 4-month embryos (–27%) but not in 8-month embryos exposed to 75% seawater. In contrast, the total of other osmolytes was significantly lower in the 8-month embryos (–33%) but not in the 4-month embryos. Glycine was downregulated to undetectable levels in both 4- and 8-month embryonic tissue, although it only constitutes 2–3% of the total value for other osmolytes and is not strongly implicated in tissue osmotic regulation in adult elasmobranchs. Boyd et al. (1977) reported a significant decrease in erythrocyte glycine content as well as brain, muscle and erythrocyte β-alanine levels in *R. erinacea* exposed to 50%

SW. Our findings agree with the adult data, in that there was a large decrease in β-alanine in 8-month embryonic tissue with exposure to 75% SW. Three of the solutes measured here – myo-inositol, GPC and creatine – have not been typically analyzed in elasmobranch osmotic studies, with a couple of exceptions (see Yancey, 2001a). They are included here because they have been shown to act as osmolytes in other animals and tissues, especially mammals (Yancey, 2001b). Creatine did not exhibit any significant changes between 100% and 75% SW; it most likely has a different primary function, e.g. in energy storage with creatine phosphate. GPC acts like a regulated osmolyte at the 8-month stage as it is significantly lower in these embryos exposed to 75% seawater (Table 2). Whether myo-inositol can be considered to function as an osmolyte in this animal will require further study.

Urea content in the embryonic tissue decreased significantly in the tissues of 8-month (–20%), but not 4-month, embryos exposed to 75% SW (Table 2). As predicted, urea excretion rates were initially elevated in both the 4- and 8-month embryos following the onset of 75% SW exposure. In fact, the magnitude of this response was much higher in the 4-month embryos (Fig. 1) relative to the 8-month embryos (Fig. 2). Indeed, the difference in whole embryo urea content (tissue plus yolk) between control and 75% seawater exposed embryos is approximately 68 mmol kg^{-1} wet mass, less than that lost by excretion over the entire 5 day period (145 mmol kg^{-1} wet mass). In 8-month embryos, however, the decrease in urea content of the whole embryo (90 mmol kg^{-1} wet mass) is much larger than the amount of urea lost by excretion (7 mmol kg^{-1} wet mass). Therefore, urea synthetic rates are probably altered under dilute seawater exposure during the 8-month stage, perhaps by changes in substrate flux through the pathway or the availability of cofactors and/or modulators.

Higher urea levels in the older (8-month) embryos do not correlate with higher OUC activities. As the embryo grows between 4 and 8 months (approximately a 12-fold increase in embryonic tissue mass), there is probably a corresponding increase (~12-fold) in the amount of OUC enzymes to maintain the per g wet mass (or per mg protein) activity. The urea tissue content will also be influenced by the rate of urea loss to the environment. One factor that plays a role in the rate of urea loss is the extent to which embryos can exchange materials with the external seawater environment, that is, the relative permeability of the egg case and the embryonic membranes. In our study the egg case was removed, but it is not known to be a barrier to urea and other small molecular mass solutes (for a review, see Kormanik, 1995). The egg cases of oviparous elasmobranchs develop an opening to the environment when the albumin within the case disappears, approximately 1/3 of the way into embryonic development (Koob, 1999). At 4 months post-oviposition, therefore, the embryos used in the present study have only recently been exposed directly to seawater. In the face of such a large tissue:water urea gradient and a comparatively low rate of urea loss, it is very likely that the embryonic membranes are relatively impermeable to urea, as reported for adult elasmobranchs (see Introduction). Kormanik (1995) points out that the large size of the skate embryos compared to teleost embryos would also be advantageous in reducing urea loss, since a low surface area:volume ratio would facilitate urea retention. Indeed, older embryos (8-month) had significantly lower urea excretion rates (eightfold). Whether the lower rates of urea loss are simply due to a lower surface area:volume ratio in the older, larger embryos, or also to the development of urea retention mechanisms by the gill and kidneys (see Introduction), is not known.

CPSase III activity in both 4- and 8-month embryos was, not surprisingly, much lower than activities in adult liver. Lechenault et al. (1993) reported that the liver constituted only 4.8–6.8% of the total body wet mass of *Scyliorhinus canicula* (an oviparous dogfish) neonates. If one assumes that all of the CPSase III activity is localized in the liver of *R. erinacea* embryos, then we have severely underestimated the hepatic CPSase III activity in embryos. This may not be the case, because in 8-month muscle tissue the CPSase III activity was 0.008±0.001 μmol min^{-1} g^{-1} or 44% of the total whole embryo CPSase III activity (Table 3). Thus, a significant component of CPSase III activity resides in the skeletal muscle tissue, and possibly other tissues as well. Skeletal muscle CPSase III has been reported in teleost fish (Julsrud et al., 1998; Kong et al., 1998; Lindley et al., 1999; Todgham et al., 2001; Steele et al., 2001), but to our knowledge never in elasmobranchs. In addition, Gsase, OTCase and arginase activities in skeletal muscle tissue were 18%, 70% and 13% compared to whole embryo enzyme activities, respectively (Table 3). It appears, therefore, that skeletal muscle may play a role in urea synthesis, at lease during early developmental stages.

Activities of OTCase and arginase presented in the current study are much higher (4- to 70-fold) than those reported by Read (1968b) in *R. binoculata* (OTCase ~0.2 μmol min^{-1} g^{-1} wet mass; arginase ~1.7 μmol min^{-1} g^{-1} wet mass; skate mass ~0.1 g). This is especially surprising since Read performed his assays at a higher temperature (38°C) compared to the present study (26°C). The discrepancy may be due to species or methodological differences.

We predicted that if embryos were capable of acclimating to 75% SW, they would decrease the rate of urea synthesis by lowering the levels of OUC enzyme activities and/or increase the rate of urea excretion, in order to achieve a lower tissue content of urea. There were several distinct differences in the response of 4- and 8-month old embryos to 75% SW, but at both stages there were no significant changes in OUC enzyme activities (Table 3). However, flux through the OUC may be modulated by the intracellular levels of substrates, cofactors, modifiers and/or other cellular conditions. Goldstein and Forster (1971) reported an increase in renal urea clearance, but no change in total body urea clearance in adult little skates exposed to 50% SW for 5 days. They suggested that urea synthesis may be diminished in dilute seawater (although this has not been tested in adults), which

would explain the significantly lower tissue urea levels under these conditions (e.g. Price and Creaser, 1967; Goldstein and Forster, 1971). Indeed, we have discovered a downregulation of hepatic arginase in adult skates acclimated to 75% SW (S. Steele, P. Yancey, P. Wright, manuscript submitted for publication).

After 5 days in dilute seawater, embryos were recovered in 100% SW for 3 days. An initial decrease in urea excretion rates might enhance the retention of urea to bring levels close to pre-dilute seawater exposure. The results are opposite to what we expected in that urea excretion rates increased immediately and dramatically (11-fold) in the 8-month embryos (Fig. 2) and marginally, but significantly, in the 4-month embryos (Fig. 1). The explanation for this counter-intuitive response is unknown. Such an increase could be related to a transient increase in water efflux, as tissue water content would presumably recover towards control levels. This would in turn increase excretion rates if, for example, urea retention mechanisms were not equipped to retain urea under such conditions in early life stages. Indeed, it is also interesting that recovery in 100% SW (8-month embryos) was accompanied by a significant increase in ammonia excretion rates (Fig. 3). In adult little skates, ammonia excretion was unchanged by exposure to 50% SW or recovery in 100% SW (Goldstein and Forster, 1971). These developmental differences may relate to a higher proportion of nitrogenous wastes excreted as ammonia in embryos (up to 66%) *versus* adults (19%; Goldstein and Forster, 1971).

Ammonia excretion was detected in 8-month, but not 4-month, embryos. It is not clear why the rate of ammonia excretion was below the level of detection very early in development. Ammonia excretion constitutes approximately 19% of total nitrogen excretion in the adult little skate (Goldstein and Forster, 1971), which is lower than values obtained for 8-month embryos in the present study (up to 66%). Clearly, there are a number of ontogenic changes in nitrogen metabolism that alter the proportion of nitrogen excretory products throughout development.

In summary, ureosmotic mechanisms appear to be active very early in development in the little skate, as indicated by the presence of significant levels of OUC enzymes and the ability of these embryos to survive and recover from dilute seawater exposure. The ratios of urea to compatible and counteracting osmolytes (e.g. TMAO, other methylamines and amino acids) in skate embryos are similar to those found in adult elasmobranchs. Upon exposure to dilute seawater, 8-month old embryos downregulate tissue contents of these osmolytes as observed in adult elasmobranchs; however, they are unique to their adult counterparts in that urea excretion is increased in this milieu. Finally, although independent ureosmotic regulation is in place as early as 4 months, significant developmental changes in nitrogen metabolism and excretion, as well as osmoregulation, occur over the time period we examined between 4 and 8 months post-conception.

Appendix

Example: 4-month urea excretion *versus* urea production at Time 0, control (N=4)

1. Mass of embryos=0.28±0.07
2. Excretion rate (μmol N g^{-1} h^{-1})=0.60±0.09,
   Excretion rate (nmol urea $individual^{-1}$ min^{-1})=1.40±0.41.
3. CPSase III activity (nmol g^{-1} min^{-1})=10.13±2.43,
   CPSase III activity (nmol $individual^{-1}$ min^{-1})=2.66±0.64.

Urea produced *versus* excreted = CPSase III activity/Excretion rate (nmol/individual min^{-1})=2.48±1.09.

Therefore, at this stage, the capacity of CPSase III to produce urea is 2.5 times that which is excreted.

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References

Anderson, P. M. (1995). Urea cycle in fish: molecular and mitochondrial studies. In *Fish Physiology*, vol. 14. *Ionoregulation: Cellular and Molecular Approaches* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 57-83. New York: Academic Press.

Bedford, J. J., Harper, J. L., Leader, J. P., Yancey, P. H. and Smith, R. A. J. (1998). Betaine is the principle counteracting osmolyte in tissues of the elephant fish *Callorhincus millii* (Elasmobranchii, Holocephali). *Comp. Biochem. Physiol.* **119B**, 521-526.

Boyd, T. A., Cha, C., Forster, R. P. and Goldstein, L. (1977). Free amino acids in tissues of the skate *Raja erinacea* and the stingray *Dasyatis sabina*: effects of environmental dilution. *J. Exp. Zool.* **199**, 435-442.

Browning, J. (1978). Urea levels in plasma and erythrocytes of the southern fiddler skate, *Tryogogorhina fasciata guanerius*. *J. Exp. Zool.* **230**, 325-330.

Cooper, A. R. and Morris, S. (1998). Osmotic, ionic and haematological response of the Port Jackson shark *Heterodontus portusjacksoni* and the common stingaree *Trygonoptera testacea* upon exposure to diluted seawater. *Mar. Biol.* **132**, 29-42.

Evans, D. H. and Kormanik, G. A. (1985). Urea efflux from the *Squalus acanthias* pup: the effect of stress. *J. Exp. Biol.* **119**, 375-379.

Forster, R. P. and Goldstein., L. (1976). Intracellular osmoregulatory role of amino acids and urea in marine elasmobranchs. *Am. J. Physiol.* **230**, 925-931.

Goldstein, L. and Forster, R. P. (1971). Osmoregulation and urea metabolism in the little skate *Raja erinacea*. *Am. J. Physiol.* **220**, 742-746.

Goldstein, L., Oppelt, W. W. and Maren, T. H. (1968). Osmotic regulation and urea metabolism in the lemon shark *Negaprion brevirostris*. *Am. J. Physiol.* **215**, 1493-1497.

Holmes, W. M. and Donaldson, E. M. (1969). The body compartments and the distribution of electrolytes. In *Fish Physiology* (ed. W. S. Hoar and D. J. Randall), pp. 1-89. New York: Academic Press.

Ivancic, L. and Deboggis, D. (1984). An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. *Water Res.* **18**, 1143-1147.

Julsrud, E. A., Walsh, P. J. and Anderson, P. M. (1998). *N*-acetyl-*L*-glutamate and the urea cycle in gulf toadfish (*Opsanus beta*) and other fish. *Arch. Biochem. Biophys.* **350**, 55-60.

King, P. and Goldstein, L. (1983). Organic osmolytes and cell volume regulation in fish. *Mol. Physiol.* **4**, 53-66.

Kong, H., Edberg, D. D., Korte, J. J., Salo, W. L., Wright, P. A. and Anderson, P. M. (1998). Nitrogen excretion and expression of carbamoyl-phosphate synthetase III activity and mRNA in extrahepatic tissues of largemouth bass (*Micropterus salmoides*). *Arch. Biochem. Biophys.* **350**, 157-168.

Koob, T. J. (1999). Elasmobranch reproduction. In *Encyclopedia of Reproduction*, vol. 1 (ed. E. Knobil and J. D. Neill), pp. 1009-1018. San Diego: Academic Press.

Kormanik, G. A. (1995). Maternal-fetal transfer of nitrogen in chondrichthyans. In *Nitrogen Metabolism and Excretion* (ed. P. J. Walsh and P. A. Wright), pp. 243-258. Boca Raton: CRC Press.

Lechenault, H., Wrisez, F. and Mellinger, J. (1993). Yolk utilization in *Scyliorhinus canicula*, an oviparous dogfish. *Env. Biol. Fishes* **38**, 241-252.

Leonard, J. B. K., Summers, A. P. and Koob, T. J. (1999). Metabolic rate of embryonic little skate, *Raja erinacea* (Chondrichthyes: Batoidea): The cost of active pumping. *J. Exp. Zool.* **283**, 13-18.

Lindley, T. E., Scheiderer, C. L., Walsh, P. J., Wood, C. M., Bergman, H. L., Bergman, A. L., Laurent, P., Wilson, P. and Anderson, P. M. (1999). Muscle as the primary site of urea cycle enzyme activity in an alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami*. *J. Biol. Chem.* **274**, 29858-29861.

Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* **48**, 583-593.

Payan, P., Goldstein, L. and Forster, R. P. (1973). Gills and kidneys in ureosmotic regulation in euryhaline skates. *Am. J. Physiol.* **224**, 367-372.

Price, K. S., Jr. and Creaser, E. P., Jr. (1967). Fluctuations in two osmoregulatory components, urea and sodium chloride, of the clearnose skate, *Raja eglanteria* Bosc 1802 – I. Upon laboratory modification of external salinities. *Comp. Biochem. Physiol.* **23**, 65-76.

Rahmatullah, M. and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta* **107**, 3-9.

Read, L. J. (1968a). Urea and trimethylamine oxide levels in elasmobranch embryos. *Biol. Bull.* **135**, 537-547.

Read, L. J. (1968b). Ornithine-urea cycle enzymes in early embryos of the dogfish *Squalus suckleyi* and the skate *Raja binoculata*. *Comp. Biochem. Physiol.* **24**, 669-674.

Robertson, J. D. (1975). Osmotic constituents of the blood plasma and parietal muscle of *Squalus acanthias* L. *Biol. Bull.* **148**, 303-319.

Rombough, P. J. (1988). Growth, aerobic metabolism, and dissolved oxygen requirements of embryos and alevins of steelhead, *Salmo gairdneri*. *Can. J. Zool.* **66**, 651-660.

Smith, H. W. (1936). The retention and physiological role of urea in the elasmobranchii. *Biol. Rev.* **11**, 49-82.

Spicer, J. I. and Gaston, K. J. (ed.) (1999). *Physiological Diversity and Its Ecological Implications*. Malden: Blackwell Science.

Steele, S. L., Chadwick, T. D. and Wright, P. A. (2001). Ammonia detoxification and localization of urea cycle enzyme activity in embryos of the rainbow trout (*Oncorhynchus mykiss*) in relation to early tolerance to high environmental ammonia levels. *J. Exp. Biol.* **204**, 2145-2154.

Todgham, A. E., Anderson, P. M. and Wright, P. A. (2001). Effects of exercise on nitrogen excretion, carbamoyl phosphate synthetase III activity and related urea cycle enzymes in muscle and liver tissues of juvenile rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **129A**, 527-539.

Wekell, J. C. and Barnett, H. (1991). New method for analysis of trimethylamine oxide using ferrous sulphate and EDTA. *J. Food Sci.* **56**, 132-138.

Wolff, S., Yancey, P. H., Stanton, T. S. and Balaban, R. (1989). A simple HPLC method for quantitating the major organic solutes of the renal medulla. *Amer. J. Physiol.* **256**, F954- 956.

Wourms, J. P. (1977). Reproduction and development in chondrichthyan fishes. *Amer. Zool.* **17**, 379-410.

Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH and sodium uptake blockade. *J. Exp. Biol.* **114**, 329-353.

Yancey, P. H. (2001a). Nitrogen compounds as osmolytes. In *Fish Physiology*, vol 20. *Nitrogen Excretion* (ed. P. Wright and P. Anderson), pp. 309-341. San Diego: Academic Press.

Yancey, P. H. (2001b). Water stress, osmolytes and proteins. *Amer. Zool.* **41**, 699-709.

Yancey, P. H. and Somero, G. N. (1979). Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes. *Biochem. J.* **182**, 317-323.

Yancey, P. H. and Somero, G. N. (1980). Methylamine osmoregulatory compounds in elasmobranch fishes reverse urea inhibition of enzymes. *J. Exp. Zool.* **212**, 205-213.

Ying, M. and Craik, J. C. A. (1993). Biochemical changes during development of eggs and yolk-sac larvae of herring and plaice. *Oceanol. Limnol. Sin./Haiyang Yu Huzhao* **24**, 157-165.