Effect of low environmental salinity on plasma composition and renal function of the Atlantic stingray, a euryhaline elasmobranch

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IN GENERAL, MARINE ELASMOBRANCH fishes (sharks, skates, and rays) maintain body fluids hyperosmotic to the ambient environment as part of their osmo- and volume-regulatory strategies. In the ocean, for example, plasma osmolality is 2–10% higher than the osmolality of the surrounding seawater (for review, see Ref. 28). These fish have a high fractional renal water reabsorption, free water clearance; glomerular filtration rate; tubular urea reabsorption.

Atlantic stingrays were maintained in harbor water [control salinity sustained 50% reduction in the osmolality of the external medium. The Atlantic stingray is a marine elasmobranch that habits a remarkable range of environmental salinities. We hypothesized that the ability of these stingrays to regulate fluid volume in low-salinity environments is due primarily to a renal glomerular and tubular functional reserve. We tested this hypothesis by measuring renal excretory function after a rapid and sustained 50% reduction in the osmolality of the external medium. Atlantic stingrays were maintained in harbor water [control salinity (CS) ~850 mosmol/kgH2O] for 1 wk. Rays were then either transferred to dilute harbor water [low salinity (LS) ~440 mosmol/kgH2O] or maintained in CS for a further 24 h. Renal excretory function was markedly higher in the rays subjected to low salinity. Glomerular filtration rate was threefold higher and urine flow rate ninefold higher in the LS group. The clearance of solute-free water was greater, and solute-free water comprised a significantly larger proportion of the urine output for the stingrays transferred to dilute harbor water. We conclude that 1) the kidneys of Atlantic stingrays have a remarkable glomerular and tubular functional reserve, and 2) the marked increase in renal function attenuates the increase in fluid volume when these fish move into low-salinity habitats.

Most species of marine elasmobranchs are confined to marine habitats (9) and only encounter relatively small variations in environmental salinity. However, some species are found in estuaries, as well as marine environments, and therefore can be considered marginally euryhaline (9). Furthermore, a small number of species are euryhaline; i.e., they are able to reside in marine, estuarine, riverine, and even freshwater habitats for extended periods of time. Although, these euryhaline elasmobranchs appear to migrate between habitats on a seasonal basis (48, 54), at least one species, the Atlantic stingray, Dasyatis sabina, can reproduce and complete its life cycle in freshwater (36). Because the gills of elasmobranchs are freely permeable to water (6), in the ocean, the osmotic gradient between seawater and the extracellular fluid leads to a small osmotically driven influx of solute-free water. The kidneys maintain volume homeostasis by excreting the excess water (for review, see Refs. 16 and 27). These fish have a high fractional renal water excretion (3, 17, 21, 47), and urine is usually hypotonic to both plasma and seawater (3, 47, 57; for review, see Ref. 28).

The very high extracellular fluid osmolality and the ability of water to move freely across the gills present a challenge to volume homeostasis for elasmobranchs that swim between environments of differing salinities or are resident in low-salinity habitats. In response to this challenge, two strategies are employed: “osmoconformity”; i.e., plasma osmolality is varied in the same direction as the change in external salinity by altering the plasma concentrations of urea and/or Na+ and Cl− (13, 21, 22, 45, 47, 53, 54, 57); and, renal excretory function is altered inversely with changes in environmental salinity (21, 40, 47, 57; for review, see Ref. 10). However, the changes in internal osmolality are not directly proportional to changes in the external salinity. Marginally euryhaline elasmobranchs are generally “partial osmoconformers,” whereas euryhaline elasmobranchs are the least osmoconforming (for review, see Ref. 10). As a result of this lack of proportionality between plasma and external osmolality, there is an increase in the osmotic driving force for water uptake on exposure to low external salinity. In freshwater environments of 40 mosmol/kgH2O, plasma osmolalities of euryhaline elasmobranchs are 550–700 mosmol/kgH2O (only 30–40% lower than that in 100% seawater). Therefore, the osmotic gradient between plasma and the ambient environment is markedly increased from near isosmotic in 100% seawater to 500–600 mosmol/kgH2O in freshwater (13, 35, 43, 44, 54).

When marginally euryhaline elasmobranchs are exposed to a dilute environment, branchial water uptake is counterbalanced...
by increased glomerular filtration rate, urinary flow rate, free water clearance ($C_{H2O}$), and urea and electrolyte excretion (21, 22, 40, 47, 57). Interestingly, because euryhaline elasmobranchs can penetrate into very low-salinity environments, they experience larger external:internal osmotic gradients than marginally euryhaline elasmobranchs but have smaller increases in body weight and smaller decreases in hematocrit on dilution, and/or a more rapid return of hematocrit to predilution values (7, 10, 13, 17, 21, 22). These latter findings indicate that the volume-regulatory mechanisms of euryhaline elasmobranchs can effectively counterbalance water influx. Although the renal excretory response induced by exposure to low salinity has not been fully characterized for any euryhaline elasmobranch, Atlantic stingrays in fresh water excrete very dilute urine, with urine osmolality one-tenth of plasma and $C_{H2O}$ ~90% of urine flow (35).

We hypothesized that the ability of euryhaline elasmobranchs to regulate fluid volume following large changes in external salinity is primarily the result of a remarkable renal glomerular and tubular functional reserve that can be utilized to rapidly counteract a large increase in osmotically driven water uptake. This functional reserve would underpin the ability of these elasmobranchs to efficiently regulate fluid volume when they encounter very low-salinity habitats. We tested our hypothesis by utilizing acute clearance techniques to evaluate the effect on plasma urea and electrolyte concentrations, plasma and urine osmolality, hematocrit, renal excretory function, and $C_{H2O}$ of Atlantic stingrays 24 h following a rapid and sustained 50% reduction in the osmolality of the external medium.

The application of acute clearance techniques to the determination of renal function in anesthetized teleost (bony fish) and elasmobranchs is controversial. Handling, anesthesia, and surgical procedures can alter blood chemistry, fluid volume, and renal function (for review see Refs. 11 and 56). Therefore, to test our hypothesis in Atlantic stingrays, we developed an intact, anesthetized, and cannulated preparation that was designed to overcome possible limitations in the use of acute clearance techniques for the determination of elasmobranch renal function during anesthesia.

**METHODS**

The experiments described in this manuscript were conducted with approval of the Medical University of South Carolina Animal Care and Use Committee. The experiments described in this manuscript were conducted in accordance with the procedures and practices in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Animals**

Atlantic stingrays ($D.\ sabina$, Lesueur, 1824) were caught in Charleston Harbor and neighboring estuaries by the South Carolina Department of Natural Resources (SCDNR; Fort Johnson, Charleston, SC) and Michael Janesch using trammel nets, during scheduled surveys of local fisheries. The stingrays were held in a filtered, closed circulating, 15,000-liter holding tank. Fresh harbor water [28°C, (parts per thousand); ~850 mosmol/kgH$_2$O] was pumped at high tide from Charleston Harbor to a settling tank from where it was then pumped to the holding tank. The salinity of the water in the holding tank was maintained at ~850 mosmol/kgH$_2$O. Water temperature and pH were maintained between 20 and 24°C and 8.0 and 8.3, respectively. The animals were allowed at least 1 wk to adapt to the holding conditions. During this period, they were fed a daily diet of shrimp.

Female and male rays (0.25–0.79 kg) were divided into two salinity groups: control salinity (CS; $n = 4$) or low salinity (LS; $n = 4$). Twenty-four hours before blood collection, rays were weighed and then transferred to one of two small holding tanks containing ~500 liters of either harbor water (CS, 853 mosmol/kgH$_2$O) or dilute harbor water (LS, 462 mosmol/kgH$_2$O). The harbor water was diluted until the target osmolality was reached by adding tap water (conditioned by standing for at least 14 days) to the tank. The pH of the tanks was monitored and, if necessary, adjusted to pH 8.0–8.3 (Marine Buffer, SeaChem, Stone Mountain, GA). The rays were fed shrimp at the start of the 24-h treatment period.

Following the 24-h treatment period, the rays were anesthetized by placement in aerated, pH-buffered medium (either CS or LS as appropriate, pH maintained between 8.0 and 8.3 with Marine Buffer) containing 0.01% MS-222 (aminobenzoic acid ethyl ester, Sigma, St. Louis, MO) and again weighed. The pericardial cavity was then exposed by a ventral incision, and blood was collected into a heparinized syringe by direct cardiac puncture.

**Protocol 1: Effect of Environmental Dilution on Body Weight and Hematocrit**

Experiment 1: Identification of Peripheral Vascular Sites. The protocol for Local Anaesthesia. The stingrays were held in a filtered, closed circulating, 15,000-liter holding tank maintained at ~850 mosmol/kgH$_2$O. Water temperature and pH was 850 mosmol/kgH$_2$O. Water temperature and pH were maintained between 20 and 24°C and 8.0 and 8.3, respectively. The animals were allowed at least 1 wk to adapt to the holding conditions. During this period, they were fed a daily diet of shrimp.
pressure was monitored and arterial plasma was sampled via an indwelling polyethylene cannula (pulled to approximately the external diameter of PE-10) in the right pelvic fin (femoral) artery. Mean arterial pressure (MAP) was measured using either a Stratham pressure transducer (model P23Dc) and recorded on a Grass model 7 polygraph or a Digi-Med TXD-310 transducer connected to a Digi-Med LPA-200 low blood pressure analyzer. The animal was placed head and ventral side down on a slant board (≈30°) so that the rostrum, mouth, spiracles, and eyes were completely under the water. The caudal end of the animal was kept moist by covering it with cotton soaked in either CS or LS as appropriate. At the end of the surgical preparation, a bolus dose (0.9 ml) of 10% polyfructosan (Inutest, Laevozan-Gesellschaft, Linz, Austria) in elasmobranch Ringer solution (in mM: 280 NaCl, 6 KCl, 5 CaCl₂, 3 MgCl₂, 0.5 NaSO₄, 1 NaHPO₄, 8 NaHCO₃, 350 urea, and 5 glucose; see Ref. 17) was given over 10 min into the lateral cutaneous vein followed by a continuous infusion of the polyfructosan-elasmobranch Ringer solution at 0.45 ml/h. Animals were allowed to equilibrate for 1 h before urine collection. Urinary cannulas exited under the slant board, and urine was collected in centrifuge tubes above the water line at the same level as the kidneys. Urine was collected in four 30-min samples during two 60-min clearance periods, and urine volume was determined gravimetrically. Plasma samples (0.5 ml) were collected at the beginning of each 60-min clearance period and then at the end of the experiment. Aliquots of the bathing medium were taken at the end of each experiment for the determination of osmolality and electrolyte concentrations.

Analytic Procedures

Urine, plasma, and bathing medium Na⁺ and K⁺ concentrations were determined using flame photometry (model 943, Instrumentation Laboratory, Lexington, MA). Chloride was determined using a chloride titrator (model CMT-10, Radiometer, Copenhagen, The Netherlands). Total osmolality was determined using a vapor pressure osmometer (model 5110B, Wescor, Logan, UT). Urea nitrogen concentration was determined colorimetrically using a diacetylmonoxime reagent (Sigma). Polyfructosan concentration was determined colorimetrically using a method modified after Fuhr et al. (20). Glomerular filtration rates (GFR), urine flow rate, urinary excretion rates, and fractional solute and water excretions were calculated using standard formulas. Osmolar clearance was calculated as C_{osm} = \left(\frac{U_{osm} \times P_{osm}}{U_{urea} \times P_{urea}}\right)_{\text{urea}}, where \( U_{osm} \) and \( P_{osm} \) are urine and plasma osmolality, respectively, and \( U_{urea} \) is the urine flow rate. C_{H₂O} was calculated as C_{H₂O} = \frac{U_{H₂O} - C_{osm}}{U_{urea}}.

For the renal function experiment, the plasma values pertaining to each hour were averaged and these averaged values were used in the above calculations. The contribution of Na⁺, Cl⁻, and urea to plasma and urine osmolality was calculated using osmolar coefficients of 0.9 for Na⁺ and Cl⁻ and 0.96 for urea (46).

Statistical Analysis

Data are presented as means ± SE. Data from protocol 1 were analyzed by Student’s t-test. Data from the renal function study were analyzed using two-factor ANOVA for repeated measures, with treatment (CS vs. LS) and time (hour 1 vs. hour 2) as the factors. Post hoc comparisons were tested using either paired or unpaired t-tests with the Bonferroni modification. The Kruskal-Wallis test was used to compare data that were not normally distributed. Significance was accepted at \( P < 0.05 \).

RESULTS

Protocol 1: Effect of Environmental Dilution on Body Weight, Plasma Osmolality, and Hematocrit

Stingrays exposed to low salinity for 24 h had a lower plasma osmolality than rays maintained in harbor water (731 ± 10 vs. 886 ± 5 mosmol/kgH₂O for dilution and control groups, respectively; \( P < 0.001 \), Table 1). The osmotic gradient between the medium (harbor water or dilute harbor water) and plasma was markedly steeper in the rays exposed to the dilute harbor water compared with the rays in the control medium (Table 1). The rays exposed to low salinity had a very modest increase in body weight compared with the control group, whereas hematocrit did not differ between the groups (Table 1). Atlantic stingrays appear to regulate intravascular volume and attenuate increases in extravascular volume even in the face of a marked increase in the osmotic gradient for water intake across the gills during the first 24 h of dilution.

Protocol 2: Effect of Environmental Dilution on Plasma Composition and Renal Function

Stability of measurements. For both salinity groups, values for the measured parameters did not differ between the first and second hours of study (Table 2; see Table 4 and Fig. 3), indicating that the animals were stable for the duration of the experiment. Therefore, for both groups, data from the first and second hours were averaged, and these averaged values were used to evaluate the effects of dilution (Table 2; see Table 4 and Fig. 3).

Blood pressure and plasma composition. MAP was not significantly different for animals held at the control salinity or those subjected to environmental dilution (Table 2). Similarly, mean hematocrits were not different between the two groups (Table 2).

Plasma osmolality was lower for stingrays held at LS compared with that for rays in CS (741 ± 13 and 891 ± 4 mosmol/kgH₂O for dilution and control groups, respectively; \( P < 0.001 \), Table 2). Plasma sodium and chloride concentrations were also significantly lower in the dilution group (Table 2). In contrast, there was no statistically significant difference in plasma urea concentration between the groups (Table 2).

Osmolality and electrolyte concentrations for the two media were 856 ± 12 and 431 ± 4 mosmol/kgH₂O; Na⁺, 407 ± 16 and 194 ± 5 mM; and Cl⁻, 482 ± 8 and 241 ± 4 mM for CS and LS, respectively. The calculated osmolar and electrolyte gradients between plasma and seawater for the two groups of rays are given in Table 3. The difference in plasma osmolality between the rays in the LS and CS groups was less than the difference in the osmolality of the harbor water and the dilute harbor water. Therefore, the water-to-plasma osmotic gradient was steeper in the dilution group and thus would be expected to result in a greater influx of water across the gills compared with the control group. In contrast to the osmotic gradient, the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Salinity</th>
<th>Low Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium osmolality, mosmol/kgH₂O</td>
<td>853</td>
<td>462</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH₂O</td>
<td>886±5</td>
<td>731±10†</td>
</tr>
<tr>
<td>Osmotic gradient, mosmol/kgH₂O</td>
<td>33±10</td>
<td>269±19†</td>
</tr>
<tr>
<td>Change in body weight, %</td>
<td>0.8±0.5</td>
<td>4.5±1.3*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>21±3</td>
<td>20±1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for each group. *P < 0.05, †P < 0.001 vs. control group.
seawater-to-plasma gradient for chloride was abolished and the gradient for sodium was partially reversed in the dilution group (Table 3).

The calculated contributions of sodium, chloride, and urea to plasma osmolality are presented in Fig. 1. In both groups of stingrays, Na$^+$, Cl$^-$, and urea were the major contributors to plasma osmolality, accounting for 94–96% of the osmotic pressure. In the present study, the lower plasma osmolality obtained for rays in dilute medium was primarily due to lower concentrations of Na$^+$ and Cl$^-$. Together these osmolytes contributed 511 ± 8 mosmol/kgH$_2$O to plasma osmolality of the rays in the control medium but only 405 ± 4 mosmol/kgH$_2$O (P < 0.001) in the dilute medium. In contrast to this finding, there was no significant difference between the two groups in the contribution of urea to plasma osmolality. Interestingly, the contribution of the three osmolytes to plasma osmotic pressure, expressed as a percentage of the total osmolality, did not differ significantly between the two groups of stingrays. Na$^+$ and Cl$^-$ contributed 57 ± 1 and 35 ± 1% to plasma osmotic pressure of rays in CS and LS, respectively, whereas urea contributed 37 ± 2 and 41 ± 2% (P > 0.05).

Renal excretory function. Although urine osmolality was significantly higher in the control group (437 ± 70 vs. 158 ± 18 mosmol/kgH$_2$O for control and dilute harbor water, respectively; P < 0.001), both groups excreted urine that was hyposmolar to plasma and the external harbor water. Calculated contributions of Na$^+$, Cl$^-$, and urea to urine osmolality are presented in Fig. 2. Similar to plasma, the contribution of Na$^+$ and Cl$^-$ to urine osmotic pressure was markedly lower in the dilution group. Together, Na$^+$ and Cl$^-$ contributed 249 ± 46 mosmol/kgH$_2$O in controls and 59 ± 12 mosmol/kgH$_2$O (P < 0.01) in the dilution group (56 ± 2 vs. 35 ± 3%, P < 0.01 for control and dilution groups, respectively). Urea, in contrast, contributed 48 ± 11 mosmol/kgH$_2$O in the control medium and 73 ± 8 mosmol/kgH$_2$O (P > 0.05) in the dilute medium. Although urea contributed relatively little to urine osmotic pressure in controls (11 ± 1%), it was the major osmolyte in the urine of the dilution group (46 ± 2%) (P < 0.001). Unidentified osmolytes contributed markedly (32%) to the urine osmolality of the rays held in CS but to a lesser extent (19%) for the rays in LS (Fig. 2).

GFR and urine flow rate data are presented in Fig. 3. The GFR of stingrays in the dilution group was approximately threefold greater than that in controls (12.4 ± 0.4 vs. 3.8 ± 0.8 ml·h$^{-1}$·kg body wt$^{-1}$, respectively; P < 0.001). Furthermore, a concomitant ninefold elevation in urine flow rate was observed in the dilution group (8.1 ± 0.3 vs. 0.9 ± 0.2 ml·h$^{-1}$·kg body wt$^{-1}$ for LS and CS respectively; P < 0.001).

Mean sodium and chloride excretion rates were ~3-fold higher, and the urinary urea excretion rate was ~19-fold higher for the dilution group compared with the control group (Table 4). The clearance of urea was 1.94 ± 0.18 ml·h$^{-1}$·kg body wt$^{-1}$ (P < 0.001) for the rays exposed to low salinity and the control group, respectively.

### Table 3. Osmotic and electrolyte concentration gradients between plasma and the external medium for rays maintained in harbor water or exposed to dilute harbor water for 24 h

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Osmolality, mosmol/kgH$_2$O</th>
<th>[Na$^+$], mmol/l</th>
<th>[Cl$^-$], mmol/l</th>
<th>[K$^+$], mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35 ± 15</td>
<td>−128 ± 11</td>
<td>−192 ± 7</td>
<td>−3.9 ± 0.2</td>
</tr>
<tr>
<td>Low</td>
<td>310 ± 13*</td>
<td>22 ± 4*</td>
<td>−6.3 ± 3*</td>
<td>−1.2 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. *P < 0.001 vs. control saline.
Fig. 2. Calculated contribution of osmolytes to urine osmolality of D. sabina exposed to either CS (n = 6) or LS (n = 6).

Absolute reabsorption of Na⁺, Cl⁻, and urea was increased in animals transferred to LS (Fig. 4). Absolute reabsorption of Na⁺ and Cl⁻ increased ∼2.5-fold (Na⁺, 976 ± 260 vs. 2,376 ± 101; Cl⁻, 1,015 ± 217 vs. 2,672 ± 188 μmol·h⁻¹·kg body wt⁻¹, CS vs. LS, respectively; P < 0.001), and urea reabsorption increased ∼3-fold (1,282 ± 282 vs. 3,439 ± 322 μmol·h⁻¹·kg body wt⁻¹, P < 0.001). Absolute water reabsorption, however, was not different between the two groups (2.9 ± 0.6 vs. 4.3 ± 0.5 ml·h⁻¹·kg body wt⁻¹, CS vs. LS, respectively).

Fractional excretion values were calculated for Na⁺, Cl⁻, urea, and water (Fig. 5). Fractional excretion of both Na⁺ and Cl⁻ was not different between animals held in LS and CS. This finding is consistent with glomerulotubular balance over a wide range of salinity for these electrolytes. In contrast, fractional urea excretion was markedly higher in the dilution group (0.16 ± 0.03 vs. 0.03 ± 0.01, P < 0.001). Fractional water excretion was elevated threefold in the dilution group (Fig. 5).

Cᵦₒ₋₅ (Fig. 6A) and fractional free water clearance (Cᵦₒ₋₅/GFR, Fig. 6B) values were calculated to determine the effect of dilution on tubular water reabsorption. Both Cᵦₒ₋₅ and Cᵦₒ₋₅/GFR were higher for the stingrays transferred to the dilute harbor water (Cᵦₒ₋₅, 6.3 ± 0.2 vs. 0.5 ± 0.2 ml·h⁻¹·kg body wt⁻¹, P < 0.001; Cᵦₒ₋₅/GFR, 0.50 ± 0.01 vs. 0.10 ± 0.03 ml·h⁻¹·kg body wt⁻¹, P < 0.001). Total solute-free water comprised a significantly greater proportion of the urine output of stingrays in the dilute medium compared with those in harbor water (Table 5). Osmolar clearance adjusted for GFR (Cₒₛ₉/GFR) did not differ between the two groups of rays (Table 5).

The finding that solutes other than urea, Na⁺, Cl⁻, and K⁺ contribute only a small amount to the osmolality of plasma but contribute substantially (19 - 32%) to urine osmolality indicates that some of these solutes are unreabsorbed and others are secreted into the tubular fluid. Although the identity of the secreted osmolytes was not determined, they were most likely the divalent ions, Mg²⁺, PO₄³⁻, and SO₄²⁻ (52, 57). The secretion of osmolytes in tubular fluid would be accompanied by basolateral-to-apical transtubular water movement and would obligate water excretion above that necessary to excrete the unreabsorbed solute component of the filtered load. Because it is not possible to determine the relatively small amount of water that remains unreabsorbed as a result of the unreabsorbed osmolytes, or that is added to the tubular fluid as a result of tubular secretion of osmolytes, our calculated values of fractional water excretion and Cᵦₒ₋₅ can be considered to be minimum values. Because these unidentified osmolytes contributed a greater proportion to the osmolar load of the stingrays in harbor water compared with those exposed to dilute harbor water, we calculated the contribution of rate of formation of solute-free water to urine flow following adjustment for the contribution of the all the non-urea, non-Na⁺/Cl⁻/K⁺ osmolytes to osmolar clearance (assuming that all of the osmolytes that are secreted would result in the calculation of a maximal value for this parameter). The adjusted Cᵦₒ₋₅/Uᵦ values are presented in Table 5. The rate of formation of solute-
free water still comprised a significantly greater proportion of the urine output of stingrays in the dilute medium compared with those in harbor water.

Although plasma and urine osmolalities were significantly higher in the control group, the plasma-to-urine osmotic gradient did not differ between the two groups of rays (Table 5).

**DISCUSSION**

Atlantic stingrays equilibrated to harbor water were subjected to a rapid and sustained 50% reduction in external salinity. Although the reduction in the osmolality and composition of the external medium represents a substantial decrease in the external salinity, the salinity of the dilute harbor water is well within the normal range of salinities in which these stingrays are found. Atlantic stingrays are commonly caught in oligohaline (very low salinity, 5.0‰) to freshwater environments along the coastline of the southern United States [24, 36, 43; W. Roumillat (SCDNR), personal communication]. Locally, these rays are migratory, spending cooler months in fresh and estuarine habitats. They appear to move rapidly between oligohaline environments and the more estuarine habitats of Charleston Harbor, surrounding tidal creeks and salt marshes.

Atlantic stingrays exposed to low salinity partially osmoconform. This partial osmoconformation resulted in a marked increase in the transbranchial (medium:plasma) osmotic gradient. An elevated osmotic gradient would be expected to result in a large influx of water across the gills because they are freely permeable to water (6), which, in turn, would result in a marked increase in body weight and hemodilution (markers of extracellular, and intravascular volume, expansion, respectively). However, the stingrays had only a 4.5% increase in body weight after 24 h of exposure to low salinity. Furthermore, mean hematocrit was not different between the rays subjected to low salinity and those maintained in harbor water. From these findings, we conclude that tissue-water volume was only minimally elevated and intravascular volume did not appear to be altered in the rays subjected to low salinity. Therefore, Atlantic stingrays caught in Charleston Harbor and neighboring estuaries and equilibrated in harbor water in our facility appear to effectively maintain fluid balance after a marked, rapid, and sustained environmental dilution.

We hypothesized that the primary mechanism that underpins the ability of euryhaline elasmobranchs to effectively maintain fluid balance when present in, or moving into, very low-salinity habitats is a remarkable renal (glomerular and tubular) functional reserve that is invoked to rapidly excrete water taken up as a result of the large increase in branchial water influx. To test this hypothesis, we developed a preparation that allowed the application of acute clearance techniques to the measurement of renal function. The absolute values of renal functional parameters of the rays in harbor water were similar to those reported for conscious Atlantic stingrays (8), and marginally euryhaline elasmobranchs (21, 40, 47).

**Table 4. Renal excretory function for stingrays maintained in harbor water or exposed to dilute harbor water for 24 h**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Salinity</th>
<th>Low Salinity</th>
<th>Mean of Hours 1 and 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hour 1</td>
<td>Hour 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$U_{Na}$, μmol•h$^{-1}$•kg$^{-1}$</td>
<td>114±15</td>
<td>94±10</td>
<td>104±12</td>
</tr>
<tr>
<td>$U_{Cl}$, μmol•h$^{-1}$•kg$^{-1}$</td>
<td>84±15</td>
<td>81±13</td>
<td>83±14</td>
</tr>
<tr>
<td>$U_{urea}$, μmol•h$^{-1}$•kg$^{-1}$</td>
<td>38±4</td>
<td>28±4</td>
<td>33±4</td>
</tr>
<tr>
<td>$U_{H_2O}$, μmol•h$^{-1}$•kg$^{-1}$</td>
<td>85±64</td>
<td>460±70</td>
<td>176±89</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 6$ for each group. Data are presented for the 1-h clearance periods and for the overall group (average of hours 1 and 2). $U_{Na}$, renal sodium excretion; $U_{Cl}$, renal chloride excretion; $U_{urea}$, renal urea excretion; $F_{H_2O}$, fractional excretion of water. *$P < 0.05$, †$P < 0.001$ vs. overall mean of control group.
The mean GFR of the Atlantic stingrays in harbor water (3.8 ± 0.8 ml·h⁻¹·kg body wt⁻¹) was approximately midrange between the values reported for conscious marginally euryhaline spiny dogfish, in 100% seawater and after exposure to 75% seawater for 24 h (47). The Atlantic stingrays subjected to a rapid reduction in salinity for 24 h had a GFR almost threefold higher than that of the rays maintained at the control salinity. The mean GFR of the rays in dilute harbor water (12.4 ± 0.4 ml·h⁻¹·kg body wt⁻¹) was close to the highest value reported for elasmobranchs (for review, see Ref. 27). This finding is consistent with the very high renal excretory function reported for conscious or anesthetized Atlantic stingrays in freshwater (8, 35). The mean urine flow rate obtained from conscious Atlantic stingrays in freshwater was ~15 ml·h⁻¹·kg⁻¹; therefore, the GFR of these rays would be expected to be even higher than those subjected to acute dilution in the present study.

Despite the markedly higher absolute value of the GFR of the rays in dilute medium compared with those in harbor water, the degree of augmentation of GFR following exposure to dilute medium is similar to that reported for other elasmobranchs following exposure to low salinity; e.g., the GFR of little skates adapted to 50% seawater was fourfold higher than that of skates in 100% seawater, and the GFR of spiny dogfish exposed to 75% seawater for 24 h was 2.5-fold higher than the GFR in those in 100% seawater (21, 47). The ability to increase GFR three- to fourfold indicates that both marginally euryhaline and euryhaline elasmobranchs possess a remarkable and similar functional reserve for glomerular filtration. The mechanisms underlying glomerular functional reserve in elasmobranchs have yet to be elucidated. Both increased single-nephron function (single-nephron GFR) (3, 55) and recruitment of previously nonfiltering glomeruli have been proposed as mechanisms that contribute to this glomerular functional reserve (37). However, the fraction of nonfiltering nephrons is small even for elasmobranchs in seawater (3, 55), so that recruitment of these nephrons is unlikely to account for the 3- to 4-fold increase in GFR. Therefore, the increase in whole kidney GFR induced by exposure to low salinity appears to be due primarily to a marked increase in single-nephron GFR. The factors regulating filtration across the glomeruli of elasmobranch kidneys have yet to be determined. The glomerular functional reserve may involve recruitment of filtration surface area within the glomeruli and/or altered filtration hemodynamics, resulting in increased filtration pressure.

The urine flow rate of stingrays exposed to dilute harbor water was ninefold higher than that of stingrays maintained in harbor water, indicating that the renal response to the increased branchial water uptake also involved a marked diuresis. This diuresis was due, in large part, to the generation of a substantial amount of solute-free water. Adjusted for GFR, the amount of free water excreted by the rays in low salinity was fivefold higher than that of the rays maintained in harbor water, and solute-free water comprised a greater proportion of the urine flow rate than that observed for stingrays maintained in harbor water. Unadjusted for the osmotic load of secreted osmolytes, solute-free water contributed 51 and 79% to the urine flow rate for Atlantic stingrays equilibrated to harbor water and the rays exposed to low salinity, respectively. The contribution of solute-free water to the urine flow rate for the rays in harbor water was ninefold higher than that of stingrays maintained in harbor water, and the GFR of spiny dogfish exposed to 75% seawater for 24 h was 2.5-fold higher than the GFR in those in 100% seawater (21, 47). The ability to increase GFR three- to fourfold indicates that both marginally euryhaline and euryhaline elasmobranchs possess a remarkable and similar functional reserve for glomerular filtration. The mechanisms underlying glomerular functional reserve in elasmobranchs have yet to be elucidated. Both increased single-nephron function (single-nephron GFR) (3, 55) and recruitment of previously nonfiltering glomeruli have been proposed as mechanisms that contribute to this glomerular functional reserve (37). However, the fraction of nonfiltering nephrons is small even for elasmobranchs in seawater (3, 55), so that recruitment of these nephrons is unlikely to account for the 3- to 4-fold increase in GFR. Therefore, the increase in whole kidney GFR induced by exposure to low salinity appears to be due primarily to a marked increase in single-nephron GFR. The factors regulating filtration across the glomeruli of elasmobranch kidneys have yet to be determined. The glomerular functional reserve may involve recruitment of filtration surface area within the glomeruli and/or altered filtration hemodynamics, resulting in increased filtration pressure.

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Table 5. Parameters of renal function for stingrays maintained in harbor water or exposed to dilute harbor water for 24 h

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Salinity</th>
<th>Low Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/P_inulin</td>
<td>4.71±0.33</td>
<td>1.55±0.07‡</td>
</tr>
<tr>
<td>C_H2O/Ur, %</td>
<td>51±8</td>
<td>79±2‡</td>
</tr>
<tr>
<td>Adjusted C_H2O/Ur, %</td>
<td>60±8</td>
<td>82±2*</td>
</tr>
<tr>
<td>P_unosm – U_unosm, mosmol/kgH2O</td>
<td>454±72</td>
<td>582±14</td>
</tr>
<tr>
<td>C_unosm/GFR, %</td>
<td>10.5±1.7</td>
<td>14.3±2.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 for each group. C_H2O, solute-free water clearance; U_r, urine flow rate; U_unosm, urine osmolality; C_unosm, osmolar clearance. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. control salinity.
euryhaline, lip shark (Hemiscyllium plagiostomum) exposed to 50% seawater (57), and similar to the 50% reported for the marginally euryhaline, S. acanthias moved from 100 to 75% seawater for 24 h (47). The contribution of solute-free water to urine flow rate for the rays in the dilute harbor water approached the 90% reported for Atlantic stingrays in freshwater (35). These findings indicate that Atlantic stingrays maintained in harbor water have a remarkable capacity to generate dilute urine and this capacity for urinary dilution is further enhanced following exposure to low salinity.

Atlantic stingrays maintained in harbor water reabsorbed most of the filtered load of the primary solutes. The fractional reabsorption of Na\(^+\), Cl\(^-\), and urea was 88, 91, and 97%, respectively. Furthermore, osmolar clearance adjusted for GFR was 10.5%. This renal conservation of Na\(^+\) and/or Cl\(^-\) was similar to, or higher, than that observed for other elasmobranchs in 100% seawater (1, 17, 21, 25, 47). In addition, the fractional reabsorption of urea for the Atlantic stingray was at the high end of the range that has been obtained for other elasmobranchs in 100% seawater (21, 25, 47). In contrast, fractional water reabsorption was 78%. Therefore, a portion of the solute was reabsorbed without concomitant water reabsorption. This finding indicates that urinary dilution by the elasmobranch nephron occurs, to a large extent, by mechanisms similar to those present in amphibian and mammalian nephrons, i.e., passive or active NaCl reabsorption in nephron segments [such as intermediate IV, as designated by Lacy and Reale (38)] that are effectively impermeable to water (18, 19).

The finding that phloretin-sensitive urea reabsorption occurs across a water-impermeable segment of the spiny dogfish shark nephron [proximal V, as designated by Lacy and Reale (38)] (18) indicates that, in contrast to the mammalian nephron (where urea reabsorption is near isosmotic), urea reabsorption in the elasmobranch nephron may result in the generation of dilute tubular fluid.

Interestingly, adjusted for GFR, osmolar clearance (C_{osmol/GFR}) was 5-fold less, and free water clearance (C_{H2O/GFR}) 20-fold higher, for Atlantic stingrays maintained in harbor water than that obtained for European lesser-spotted dogfish (Scyliorhinus canicula) acclimatized to 85% seawater stepwise over 6 days (56). These findings indicate that although stenohaline dogfish can be acclimated to an estuarine salinity, their ability to reabsorb solute independently of water (and thus generate free water) is markedly less than that of the euryhaline stingray at a similar salinity.

Although absolute urinary Na\(^+\) and Cl\(^-\) excretions from the stingrays in low salinity were significantly higher than those of stingrays in harbor water, fractional excretion of these electrolytes was not different between the two groups, indicating glomerulotubular balance. Because the filtered load of Na\(^+\) and Cl\(^-\) was higher in the low-salinity group, glomerulotubular balance resulted in a marked increase in reabsorption of these two electrolytes. To our knowledge, this is the first study to conclude that glomerulotubular balance occurs in elasmobranchs. However, the finding that environmental dilution did not significantly alter the fractional excretion of Cl\(^-\) by the kidneys of little skates (21) indicates that glomerulotubular balance for Cl\(^-\) and/or Na\(^+\) could also occur in other elasmobranchs.

Stingrays in low salinity had markedly higher urinary urea excretion than rays in harbor water, due to both the increase in the filtered load and a approximately fivefold increase in fractional excretion. Despite the higher urinary urea excretion, the combination of the large increase in the filtered load and a high fractional reabsorption (84%) resulted in a markedly greater amount of urea reabsorbed by the kidney. This increase in absolute urea reabsorption following exposure to low salinity has been observed for other elasmobranchs (data recalculated from Refs. 21, 40, 47).

Unlike solute reabsorption, absolute water reabsorption in low salinity was not significantly different from that in harbor water. Therefore, the increased filtered load of Na\(^+\), Cl\(^-\), and urea following exposure to low salinity is balanced by increased absolute solute reabsorption without a concomitant increase in absolute water reabsorption. We conclude that the generation of very dilute urine in response to exposure to low salinity is due to a further augmentation of solute reabsorption at tubular site(s) that have very low water permeability. Our findings and conclusion are supported by tubular fluid-to-plasma osmolar ratios (TF/P\textsubscript{osmol}) along the nephrons of little skates exposed to either 100 or 75% seawater (52). Mean TF/P\textsubscript{osmol} obtained by micropuncture from sites in the initial and distal collecting ducts of little skates held in 100% seawater did not differ from unity. However, TF/P\textsubscript{osmol} at the end of the collecting duct was lower than unity and not different from the value obtained for the urine-to-plasma osmolar ratio (indicating that urinary dilution resulted from solute reabsorption in the terminal section of the collecting duct). In contrast, when little skates were exposed to 75% seawater, fluid along the length of the collecting duct was markedly more dilute than that obtained from skates in 100% seawater (52).

For the stingrays in harbor water, the osmotic gradient between plasma and urine was 454 ± 72 (range 224–681) mosmol/kg H2O. This osmotic gradient is similar to that reported for Atlantic stingrays and other euryhaline elasmobranchs (Carcharhinus leucas and Pristis microdon) in freshwater (35, 44; calculated from data in Ref. 50) and for the marginally euryhaline S. acanthias subjected to 24-h exposure to 75% seawater (calculated from data in Ref. 47). As expected, the osmotic gradient between plasma and dilute harbor water was markedly larger for the Atlantic stingrays exposed to low salinity compared with that measured in harbor water. To counteract the greater branchial water uptake induced by this larger gradient, these stingrays excreted urine that was hyposmolar to both plasma and the external medium as well as the urine of the stingrays maintained in harbor water. However, the plasma-to-urine osmotic gradient did not differ significantly from the value observed for the rays in harbor water. Taken together, these findings indicate that the kidneys of euryhaline and some marginally euryhaline elasmobranchs can dilute urine sufficiently to generate maximal transtubular osmotic gradients of 500–680 mosmol/kg H2O. In the present study, three of the six stingrays in the harbor water group were observed to have transtubular osmotic gradients within this maximal range, indicating that, even in estuarine salinities, Atlantic stingrays can reach the limit of urinary diluting capacity; i.e., they can excrete urine that is maximally dilute relative to plasma.

Transtubular (plasma-to-urine) osmotic gradients of 500–680 mosmol/kg H2O represent a remarkable capacity for urinary dilution. In comparison, freshwater teleosts and mammals can only dilute urine sufficiently to generate transtubular os-
motic gradients of ~250 mosmol/kgH2O (for review, see Refs. 12, 27). The mechanism(s) underlying the ability of the elasmobranch kidney to generate such large and unique transtubular osmotic gradients remains to be elucidated. The presence of two distinct diluting segments (19, 52) suggests that the mechanism(s) underlying these large transtubular gradients is complex and that the generation of these transtubular osmotic gradients must involve the generation and maintenance of low water permeability along the distal and terminal segments of the nephron.

The increase in absolute urea reabsorption following exposure to low salinity indicates that the kidneys of marginally euryhaline and euryhaline elasmobranchs act to conserve urea when these fish migrate into or are resident in low-salinity habitats. The renal conservation of urea would allow marine elasmobranchs to remain ureosmotic even in freshwater habitats. However, the higher fractional excretion of urea following exposure of Atlantic stingrays to low salinity may also serve a physiological function. We suggest that when Atlantic stingrays are exposed to dilute harbor water the increase in urea excretion is related to the inability of these elasmobranchs to exceed the maximal plasma-to-urine osmotic gradient of 500–680 mosmol/kgH2O. The lower osmotic contribution of Na+, Cl−, and other osmolytes (mostly secreted divalent ions) results in an osmotic shortfall in urine of the stingrays in the dilute harbor water. However, as a consequence of a lower fractional reabsorption, urea becomes the major urinary osmotic gradients of

The mechanisms involved in, and the tubular site(s) of, elasmobranch renal urea reabsorption have yet to be identified. Several mechanisms have been proposed by which urea can be reabsorbed from the tubular fluid of elasmobranchs. These mechanisms involve passive reabsorption of urea down local-ished concentration gradients via facilitated urea transporters, and/or active reabsorption via Na+/urea cotransporters (2, 26, 47). The recent cloning of cDNAs encoding phloretin-sensitive, facilitated urea transporters from the kidneys of a number of elasmobranchs (30, 33, 34, 49) suggests a molecular mechanism for the passive movement of urea across the tubular epithelia. We have identified five transcripts that encode two urea transporter isoforms (strUT-1 and strUT-2) from the kidneys of Atlantic stingrays (33, 34). The expression of one of the transcripts encoding strUT-2 (strUT-2c) was lower in rays subjected to 24-h exposure to dilute harbor water compared with rays in harbor water (34). Therefore, a decrease in strUT-2 expression may play a role in the lower fractional urea reab- sorption induced by 24-h exposure to low salinity.

In summary, we developed an in vivo preparation for the determination of acute renal excretory function of batoid elasmobranchs. We utilized this preparation to test the hypothesis that the ability of euryhaline elasmobranchs to effectively maintain fluid balance during exposure to very low salinities is due to an extraordinary renal (glomerular and tubular) func-tional reserve. We demonstrated that euryhaline Atlantic stingrays exposed to low salinity have markedly elevated renal excretory function compared with rays maintained in high salinity. Although the absolute value of the GFR of the rays in dilute medium was markedly higher than that obtained from rays in harbor water, the degree of augmentation of GFR following exposure to dilute medium was similar to that reported for marginally euryhaline elasmobranchs. We con-clude that marginally euryhaline and euryhaline elasmobranchs have a remarkable and apparently similar functional reserve for glomerular filtration. Furthermore, Atlantic stingrays appear to have an even greater capacity for urinary dilution than marginally euryhaline elasmobranchs when present in the same salinity, and the capacity of these euryhaline elasmobranchs for urinary dilution is further enhanced following exposure to low salinity. This functional reserve for urinary dilution may underpin the unique ability of these fish to inhabit very low-salinity and even freshwater environments.

APPENDIX

Anesthesia

Although acute clearance techniques during anesthesia have been employed by investigators studying the renal function of various fish (see Ref. 4 for review), they are not widely employed due to the possibility of changes in blood biochemistry and renal function induced by handling, anesthesia, and surgical procedures (5, 15; see Ref. 11 for review).

The level of anesthesia used in previous studies was sufficient to halt respiration. Although aerated water is passed across the gills, the degree of gas exchange is likely to be lower than if respiration was maintained (15, 32). Therefore, the changes in plasma biochemistry and renal function most likely are a result of hypoxia and not anesthesia per se (15, 29, 31). To avoid problems associated with hypoxia, we used MS-222 throughout the experiment at a concentra-tion that inhibited swimming movements but still allowed sustained movement of the gill muscles for adequate ventilation. At this level of anesthesia, no “righting” response was observed (when placed ventral side down on the bottom of the tank, the animals remained in that position) and no changes in respiratory rate were observed during surgery. This level of anesthesia corresponded to stage III of the surgical level of anesthesia (14). In preliminary experiments, we noted that addition of MS-222 markedly altered the pH of the medium. Thus we performed all of the experiments in buffered media to maintain pH between 8.0 and 8.3.

Although blood pressure has not been determined in unanesthetized D. sabina, anesthesia does not appear to have altered systemic hemodynamics. The mean femoral arterial blood pressure obtained for the anesthetized stingrays was similar to mean caudal artery pressure reported for unanesthetized spiny dogfish sharks (41, 42). However, the vasculature of elasmobranchs has been shown to be compliant (39), so that we cannot rule out the possibility that the head-down tilt of the rays during the experiment resulted in a small undermeasurement of blood pressure.

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