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Compensation for Hypercapnia by a Euryhaline Elasmobranch: Effect of Salinity and Roles of Gills and Kidneys in Fresh Water

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ABSTRACT Specimens of the euryhaline elasmobranch, *Dasyatis sabina* were acclimated to seawater and fresh water, and exposed to normocapnic (air) and hypercapnic (1% CO_2 in air) environmental water. Blood pH, P_{CO_2}, and [HCO_3^-], as well as whole-animal net-acid excretion, were measured for up to 24 h of hypercapnia. In a separate experimental series, urine was collected from freshwater acclimated stingrays during 8 h of normocapnia and hypercapnia. Stingrays in both salinities at least partially compensated for the respiratory acidosis by accumulating HCO_3^- in their extracellular spaces. The degree of compensation for blood pH was 88.5% in seawater, but only 31.0% in fresh water after 24 h of hypercapnia. Whole-animal net-acid excretion was also greater in seawater than in fresh water, as was the increase in extracellular fluid [HCO_3^-]. Mean urinary net-acid excretion rates were slightly negative, and never increased above normocapnic control rates during hypercapnia. Since whole-animal net-acid excretion rates increased with blood [HCO_3^-], and urinary excretion was always negative, the gills were probably the primary organ responsible for compensation from environmental hypercapnia. The faster, and more complete, compensation for hypercapnia in seawater than in fresh water for this euryhaline elasmobranch is consistent with data for euryhaline teleosts, and probably reflects Na^+-dependent mechanisms of branchial acid excretion. *J. Exp. Zool. 297A:52–63, 2003.*

INTRODUCTION

Several studies have shown that exposing seawater and freshwater teleosts and seawater elasmobranchs to hypercapnia induces a respiratory acidosis that is compensated for by accumulating bicarbonate in body fluids (Claiborne, '98; Claiborne et al., 2002). Initially, non-bicarbonate buffering and transfers of bicarbonate from intracellular to extracellular compartments are important, but the majority of the long-term, steady-state increase in bicarbonate concentration is caused by transepithelial movement of acid-base relevant ions across the gill epithelium (Heisler, '93).

The rate and degree that plasma pH recovers in teleosts varies with species and environmental water conditions. For example, during exposure to 1% CO_2 in air, the seawater (SW) teleosts *Onchorynchus kisutch* and *Conger conger* compensated for 100% of their maximum pH decrease by 24 h (Perry, '82; Toews et al., '83), but the freshwater (FW) teleosts *Onchorynchus mykiss* and *Cyprinus carpio* only compensated for 21 and 32% of their pH decreases after 24 h and 48 h, respectively (Perry, '82; Claiborne and Heisler, '84). Therefore, it appears that environmental salinity has a positive effect on the rate of pH recovery from hypercapnia-induced respiratory acidosis in teleosts. This was best demonstrated by Iwama and Heisler ('91) who showed that *O. mykiss* exposed to 1% CO_2 compensated for 61, 82, and 88% of their pH decreases when held in 3, 100, and 300 mmol l^{-1} NaCl, respectively. Bicarbonate was accumulated faster in 100 and 300 mmol l^{-1} NaCl, despite a constant water [HCO^{3-}] in the three salinities, suggesting that acid-excretion into, and/or bicarbonate accumulation from, environmental water increases with environmental [Na^+] and [Cl^-]. This positive effect of salinity is thought to reflect cellular mechanisms of branchial H^+ and/or NH_4^+ excretion that depend on external Na^+.

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Environmental salinity also influences the relative contribution of gills and kidneys to systemic acid-base regulation. In the SW teleosts, *Myoxocephalus octodecimspinosus* and *Parophrys vetulus*, the kidneys contributed only 5% of whole-animal net-acid excretion (McDonald et al., '82; Claiborne et al., '94) during acid-base disturbances. Likewise, SW elasmobranch kidneys have been shown to contribute less than 1% of whole-animal net-acid excretion during acidosis (Heisler et al., '76; Evans et al., '79; Holeton and Heisler, '83). This low renal contribution to pH regulation reflects relatively low urine flow rates (UFRs) and efficient branchial acid transport in SW fishes. However, in FW, where osmotic gradients are reversed and UFRs are greater (Hickman and Trump, '69), the kidneys of teleosts have been shown to contribute 5–33% of whole-animal net-acid excretion during control and acidotic conditions (Cameron, '80; Cameron and Kormanik, '82; Smatresk and Cameron, '82a; Hyde and Perry, '87; Perry et al., '87).

Unlike stenohaline FW elasmobranchs of the family Potamotrygonidae, euryhaline elasmobranchs retain the ability to store high concentrations of urea in their tissues when in FW. Consequently, the osmotic gradient between environmental water and euryhaline elasmobranchs in FW is the highest reported for any group of fishes (>600 mOsm l^{-1}; Smith, '31; Thorson et al., '73; Piermarini and Evans, '98), and their UFRs are the highest reported for any group of fishes (>10 ml kg^{-1} h^{-1}; Smith, '31). This creates the potential for kidneys of euryhaline elasmobranchs to be large contributors to systemic acid-base regulation in FW.

To our knowledge, acid-base regulation of an elasmobranch in FW has never been described. This is partly because they are rare, with less than five percent of extant elasmobranch species found in FW. Of these, 29 are stenohaline FW stingrays of the family Potamotrygonidae, and 14 are euryhaline species that include the bull shark (*Carcharhinus leucas*), Ganges River shark (*Glyphis gangeticus*), sawfishes (*Pristis sp.*), and whiptail stingrays (*Dasyatis sp.* and *Hiamantura sp.*) (Compagno and Cook, '95). The only North American elasmobranch species that is regularly found in FW is the Atlantic stingray (*Dasyatis sabina*). This euryhaline species ranges from Chesapeake Bay to Central America where it is common near the coast (Bigelow and Schroeder, '53). It enters FW rivers seasonally, and has breeding populations in the St. John's River of Florida over 300 km up-river from the Atlantic ocean (Johnson and Snelson, '96). Therefore, it can serve as a physiological model for other euryhaline elasmobranch species. In this study, we measured blood acid-base parameters and rates of whole-animal, net-acid excretion in SW and FW acclimated Atlantic stingrays from the St. John's River during 24 h of normocapnia and hypercapnia, to establish baseline values for a euryhaline stingray. In addition, the degree of blood pH compensation, and rates of acid excretion during hypercapnia were compared between the two salinities to determine if salinity has a positive effect on compensation for acidosis in elasmobranchs. We hypothesized that like teleosts, *D. sabina* would compensate for hypercapnia faster in SW than in FW, because it is also thought to use a Na^+ dependent mechanism for acid excretion (Piermarini and Evans, 2001). In a separate series, net-acid excretion rates were measured in the urine of normocapnic and hypercapnic FW acclimated stingrays to determine the renal contribution to compensation for acidosis. We hypothesized that kidneys would be important contributors to compensation for hypercapnia in FW *D. sabina* because of high UFRs. This is the first study of acid-base regulation of an elasmobranch in FW, and the first to determine the effect of salinity on acid-base regulation in an elasmobranch.

MATERIALS AND METHODS

Experimental animals and acclimations

Thirty-three Atlantic stingrays (*Dasyatis sabina*) were captured from Lake George of the St. John's River, Florida using trotlines baited with shrimp. Stingrays, of both sexes, were captured between May 2000 and December 2001 (Range 229 to 748 g, 463 ± 23 g, mean $\pm$ S.E.) and transported to the University of Florida where they were held in 380 l Rubbermaid tanks (up to four stingrays in each tank). After two or three days of exposure to buffered Gainesville tap water (approximate concentrations in mmol l^{-1}: Na^+ 3.50, Ca^{2+} 1.16, K^+ 0.03, Cl^- 0.40), stingrays were divided into two groups; one group remained in FW, and the other was transferred to a separate 380 l Rubbermaid tank where they were gradually exposed to buffered SW (approximate concentrations in mmol l^{-1}: Na^+ 517.36, Ca^{2+} 8.66, K^+ 11.54, Cl^- 485.60), from the Atlantic ocean (one day in 25% SW, two days in 50% SW, and one day in 75% SW) (as in Piermarini and Evans, 2000). Commercial carbonate salt buffers (Seachem Laboratories Inc.,

TABLE 1. Control acid-base status of seawater and freshwater acclimated Atlantic stingrays (D. sabina)[1]

Normocapnia	Seawater	Fresh water
Environmental Water		
pH	8.10 ± 0.07 (5)	8.17 ± 0.07 (5)
P_{CO_2} (mmHg)	0.72 ± 0.18 (5)	0.95 ± 0.16 (5)
$[HCO_3^-]$ (mmol l^{-1})	3.84 ± 0.24 (5)	3.85 ± 0.39 (5)
Blood/Plasma		
pH	7.66 ± 0.02 (10)	***7.81 ± 0.01 (13)
P_{CO_2} (mmHg)	3.64 ± 0.25 (10)	3.58 ± 0.14 (13)
$[HCO_3^-]$ (mmol l^{-1})	5.12 ± 0.25 (10)	***7.03 ± 0.27 (13)
Whole-animal Fluxes		
Net-acid (μmol kg^{-1} h^{-1})	36.7 ± 27.6 (10)	*−56.5 ± 20.0 (12)
T_{amm} (μmol kg^{-1} h^{-1})	58.4 ± 11.5 (10)	57.2 ± 6.5 (12)
TA (μmol kg^{-1} h^{-1})	−21.7 ± 29.1 (10)	*−113.7 ± 19.7 (12)
Urine		
pH	ND	7.1 ± 0.1 (10)
Urine Flow Rate (ml kg^{-1} h^{-1})	ND	14.7 ± 0.5 (10)
Net-acid (μmol kg^{-1} h^{-1})	ND	−14.0 ± 6.8 (10)
T_{amm} (μmol kg^{-1} h^{-1})	ND	10.6 ± 2.3 (10)
TA-HCO_3^- (μmol kg^{-1} h^{-1})	ND	−24.6 ± 6.1 (10)

Values are means ± S.E. (N).
ND=not determined.
*$P < 0.050$ and ***$P < 0.001$ for unpaired t-test between seawater and fresh water.

Covington, GA) were added to the acclimation and experimental waters to make the pH and $[HCO_3^-]$ stable and approximately equal in the two salinities (Table 1). Stingrays remained in either FW or SW for 1 to 4 weeks while being fed raw shrimp three times a week, until two days before surgery. Plasma Na^+, K^+, and Cl^- concentrations in stingrays exposed to SW for this amount of time approximate those of stingrays caught in SW (Piermarini, personal communication).

Surgery for blood collection series

One day before surgery, each stingray was transferred to a darkened flux chamber (described below) supplied with flowing aerated water of the appropriate salinity. The stingrays were anaesthetized by an initial immersion in 150 mg l^{-1} MS–222 (Sigma) diluted in FW (buffered with 1 g l^{-1} $NaHCO_3$) or SW. After their pectoral fins stopped moving, but before their gills stopped ventilating, the anesthetic was diluted to 75 mg l^{-1} by addition of either FW or SW for the remainder of surgery. Using a high MS–222 concentration initially, followed by dilution, minimizes struggling, and therefore acidosis in fish (Heisler, '84). The fish were transferred to an angled operation table that allowed their gills to remain immersed in water, but not their posterior-dorsal surface. They were each fitted with one chronic indwelling polyethylene catheter (PE50) in a cutaneous vein that drains the tail. This was done by finding and puncturing the vessel with a heparinized 25–gauge needle and syringe, then inserting a short length (15 cm) of PE50 using a conical-tipped guide wire. A longer length (40–50 cm) of PE50 was then connected to this short cannula with a 2–3 cm piece of 1/32'' inside diameter silicone tubing (Cole Parmer Instrument Company, Vernon Hills, IL), and the entire length of cannula was kept clear with heparinized elasmobranch Ringer's (Forster et al., '72). For FW stingrays, the Ringer's was modified by reducing NaCl concentration by 200 mmol l^{-1}, urea concentration by 200 mmol l^{-1}, and trimethylamine oxide by 41 mmol l^{-1} as described by Piermarini and Evans (2000). After surgery, stingrays were transferred back into a flux chamber and were allowed to recover for at least 24 h before any measurements were recorded.

Surgery for urine collection series

Attempts to cannulate female stingrays failed, so all the urinary collections were done with male stingrays. Ten male FW stingrays were anaesthetized as described above and put ventral-side up on the operation table. One 50 cm indwelling polyethylene catheter (PE–50) was inserted into each urinary opening (2 in males) on the distal portion of each urinary papilla. Two purse-string sutures were then used to seal the papillae around the cannulas. Two more sutures in the skin of each clasper were used to secure each cannula to the stingray. After surgery, the stingrays were transferred back into flux chambers and were allowed to recover for at least 24 h. During this recovery, the cannulas were left loose to drain into the water.

Flux chamber

Flux chambers were made from polypropylene boxes that could be adjusted to hold 6.5 or 10.5 l of water, depending on the size of the fish. Water from the chambers was pumped (4–5 l/min) countercurrent to gas mixtures though a 500 ml exchange column, and then recycled back into the chamber. Chambers were partially submerged in a trough that was supplied with water that circulated from a 100 l aquarium to maintain a relatively constant temperature (23–25°C). Air or 1% CO_2 in air from a Cameron Instrument gas

mixer was pumped into the exchange columns through air diffusers at 700 to 1000 cc/min.

Protocols

After recovery, blood cannulated stingrays were exposed to a pretreatment period of 4 to 14 h, when the exchange column received air from the gas mixer. Following this pretreatment period, the gas mixture was left pumping air (control) for 24 h, or switched to 1% CO_2 in air (hypercapnia) for 24 h. Samples of blood (100–150 μl) were taken on the following schedule: at least 3 pretreatment samples and 2 h, 4 h, 8 h, and 24 h after initiation of the treatment period. This blood sampling schedule did not cause a change in any of the blood acid-base measurements (Fig. 1). Water samples were taken on a slightly different schedule: beginning and end of pretreatment period and 4, 8, 9, and 24 h after initiation of the treatment. One hour before the pretreatment period, and right at 8 h, the water in the flux chambers was flushed with new water of the appropriate salinity and gas composition to limit accumulation of waste products. Therefore, the 9 h water sample began the final flux period.

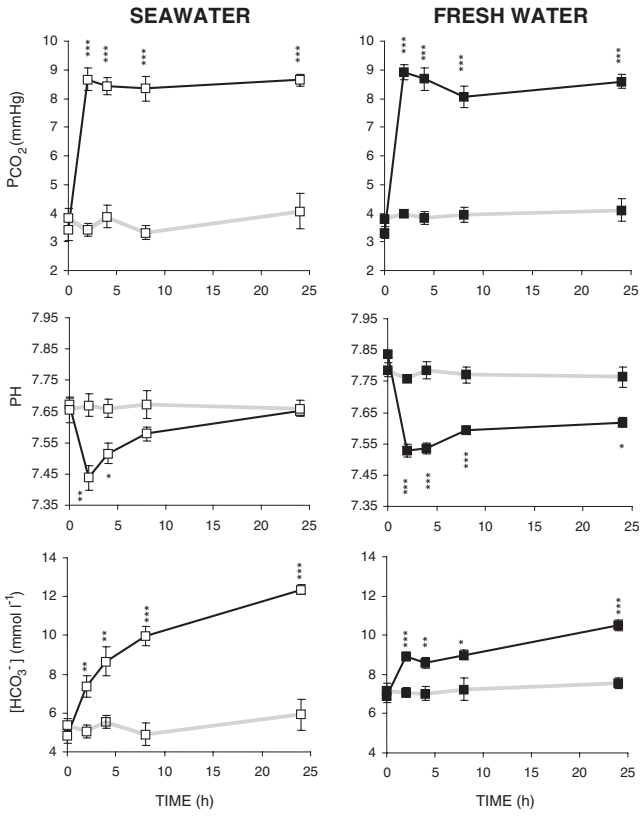

Fig. 1. Plasma P_{CO_2}, pH, and $[HCO_3^-]$ in seawater and fresh water acclimated *D. sabina* during normocapnia (broken lines) or before and during 1% hypercapnia (solid lines). (mean ± S.E.; seawater normocapnic controls: N=5 for 0, 2, & 4 h, N=4 for 8 h, N=3 for 24 h; seawater hypercapnia: N=5; freshwater normocapnic controls: N=7 for 0, 2, 4, & 8 h, N=6 for 24 h; freshwater hypercapnia: N=6 for 0, 2, 4, & 8 h, N=4 for 24 h); *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$ for unpaired t-test between control and experimental means.

After recovery, urinary cannulated stingrays were exposed to a pretreatment period as above. However, urine collections were limited to 4 h. Urine was collected by passing the catheters over the rim of the flux chambers, and into a common glass collection vial that was covered with parafilm. The ends of the catheters were 7 cm below the surface of the water. Following the pretreatment period, the collection vials were switched for urinalysis, and the gas mixture was either left as air or switched to hypercapnia, as above. Urine collection vials were switched again after 4 and 8 h of the treatment period. The depressiform shape of stingrays allowed them to completely rotate when in the flux chambers. This often tangled the urinary catheters, and therefore periodic untangling was required to keep catheters working. This made overnight urine collections impossible, so urine was collected for only the pretreatment period and the first eight hours of the treatment period. However, this is when most of the acid-base compensation occurred, and therefore represents the time when the most important changes in urinary function would be occurring.

Measurements

All blood samples were immediately analyzed for pH and total CO_2. Blood pH was measured with a Mettler InLab 423 micro pH electrode that was customized to fit tightly into a 500 μl tube to minimize gas equilibration with the room air. The electrode was calibrated every few hours with buffers made from desiccated phosphate salts. Total CO_2 was measured in 20 μl of plasma by differential conductivity using a Cameron Instruments Capni-Con II. The Capni-Con was calibrated with standards made from desiccated $NaHCO_3$.

Water samples (50 ml) were stored at 4°C and analyzed for titratable base and total ammonia (T_{amm}) within 3 days of collection. Titratable base was measured in duplicate 10 ml samples, by adding 0.1 N HCl with a micrometer syringe burette (model SB2, Micro Metric Instrument Co.) to a pH of 4.2. Each sample was bubbled with

nitrogen to enhance CO_2 liberation as the solution was acidified (Claiborne and Evans, '92). T_{amm} was measured in triplicate with a micro plate modification of the salicylic acid, hypochlorite assay (Verdouw et al., '78), using standards made from desiccated NH_4Cl in either FW or SW.

Urine samples were analyzed immediately for pH, volume, and titratable acidity (TA) – [HCO_3^-], and a portion was saved for T_{amm} analysis. Urine pH was measured as described for blood. Volume was measured gravimetrically. TA-HCO_3^- was measured by first titrating 500 μl of urine with 0.02 mol l^{-1} HCl to a pH below 5.0. It was then bubbled for 15 min with N_2 gas to remove CO_2. Finally, it was titrated to the mean blood pH of the corresponding time interval with 0.02 mol l^{-1} NaOH. T_{amm} was assayed as above for water, but had to first be diluted 1/10 to fit within the linear range of the salicylic acid, hypochlorite assay.

Calculations and statistics

Plasma P_{CO_2} was calculated from pH and total CO_2 using αCO_2 and *pk'* values calculated from equations given by Heisler ('84) and plasma osmolarity and [Na^+] given by Piermarini and Evans ('98). Plasma [HCO_3^-] was then calculated as total CO_2–($P_{CO2} \times \alpha CO_2$).

Mass specific whole-animal net-acid excretion rates (μmol kg^{-1} h^{-1}) were calculated as the sum of T_{amm} excretion rate and titratable acidity excretion rate for each flux period. T_{amm} excretion rate was calculated with the following equation:

$$T_{amm} = \frac{([T_{amm}]_f - [T_{amm}]_i) \times Volume}{Mass \times Time},$$

where $[T_{amm}]_f$ and $[T_{amm}]_i$ were the concentrations of total ammonia at the end and beginning of a flux period, respectively. Titratable acidity excretion rate was calculated from titratable base in the same manner, except the sign was changed to reflect acid excretion instead of base excretion (Heisler, '84).

Percent pH compensation was calculated with the following equation:

$$\%pH_{24} = \frac{pH_{24} - pH_2}{pH_{pre} - pH_2} \times 100,$$

where pH_x is the pH at time x. Preliminary experiments indicated that the lowest pH occurred at about 2 hours of hypercapnia.

Mass specific urinary net-acid excretion rates (μmol kg^{-1} h^{-1}) were calculated as the sum of T_{amm} excretion rate and TA-HCO_3 excretion rate for each urine collection as described previously (Hills, '73; Wood et al., '99). T_{amm} excretion rate was calculated with the following equation:

$$T_{amm} = \frac{[T_{amm}] \times Volume}{Mass \times Time},$$

and TA-HCO_3^{-1} was calculated as:

$$\text{TA-}HCO_3^- = \frac{VolumeHCl - VolumeNaOH \times VolumeUrine}{Mass \times Time}.$$

Unpaired Student's t-tests were used to compare all corresponding time interval means between hypercapnic and control stingrays. Unpaired student's t-tests were also used to compare control blood variables between SW and FW stingrays, and percent pH compensation and cumulative acid excretion between SW and FW hypercapnic stingrays. Alpha was set at 0.05.

RESULTS

Control acid-base status

Control (normocapnic) environmental water pH, P_{CO_2}, and [HCO_3^-] were similar in SW and FW (Table 1). The similarity in water P_{CO_2} was reflected by almost identical plasma P_{CO_2} in normocapnic SW and FW acclimated stingrays. However, despite the similarities in plasma P_{CO_2} and water acid-base conditions, FW stingrays had a mean blood pH 0.15 units higher, and a mean [HCO_3^-] 1.91 mmol l^{-1} higher than SW stingrays (Table 1).

Control whole-body fluxes from stingrays were also different between the two salinities (Table 1). This was due to a net titratable base excretion rate (more negative net titratable acid excretion) in FW that was 92 μmol kg^{-1} h^{-1} greater than in SW. Conversely, net T_{amm} excretion rates were almost identical in the two salinities. This left calculated net-acid excretion rates of 36.7 μmol kg^{-1} h^{-1} and −56.5 μmol kg^{-1} h^{-1} in SW and FW, respectively.

Control urine flow rates were large (14.7 ml kg^{-1} h^{-1}), but T_{amm}, and net-acid excretion rates were only 24.8 and 18.5 % of the corresponding whole-animal excretion rates, respectively (Table 1). TA-HCO_3^- excretion rates were always negative, with a mean of –24.6 indicating that there was a net bicarbonate loss from the kidneys. Urine pH was always slightly acidic to the blood, with a mean of 7.1 (Table 1).

Hypercapnic acid-base status

During hypercapnia, environmental water pH, P_{CO_2}, and [HCO_3^-] were 7.20 ± 0.03, 8.24 ± 0.22

mmHg, and 4.36 ± 0.18 mmol l^{-1} in SW and 7.35 ± 0.02, 7.87 ± 0.27 mmHg, and 4.02 ± 0.20 mmol l^{-1} in FW, respectively. After two hours of this hypercapnia, plasma P_{CO_2} increased about 2.25 fold relative to controls in both salinities, and remained elevated for the rest of the experiment (Fig. 1), indicating that stingrays were exposed to similar levels of hypercapnic stress in SW and FW. After two hours of this hypercapnia, blood pH decreased by 0.23 and 0.31 units, to the lowest values of 7.44 and 7.53 in SW and FW, respectively. In SW, stingrays compensated for almost all of this respiratory acidosis by 24 h (0.01 pH units below controls), but in FW, hypercapnic stingrays were still 0.24 units below normocapnic controls (Fig. 1). In both salinities, most of the compensation occurred in the first eight hours of hypercapnia due to increased plasma $[HCO_3^-]$. Stingrays increased their plasma $[HCO_3^-]$ by 7.51 and 3.64 mmol l^{-1} after 24 h, in SW and FW, respectively. Blood pH, P_{CO_2}, and $[HCO_3^-]$ remained relatively constant in the SW and FW control series of stingrays (Fig. 1).

Hypercapnic whole-animal fluxes

Whole-animal net-acid excretion rates increased from 49 and —63 μmol kg^{-1} h^{-1}, to the greatest values of 265 and 164 μmol kg^{-1} h^{-1} in the first four hours of hypercapnia, and remained statistically above controls up to eight and 24 h in SW and FW, respectively (Fig. 2). Although there were qualitatively similar increases in net-acid excretion rates in the two salinities, changes in the components used to calculate net fluxes were opposite. For example, the increases in net-acid excretion in SW were solely due to increased titratable acidity excretion (190 and 124 μmol kg^{-1} h^{-1}, 0 to 4 and 4 to 8 hours, respectively); T_{amm} excretion rates were never statistically different from controls (Fig. 2). Alternatively, the increases in net-acid excretion in FW were solely due to increased T_{amm} excretion rates; titratable acidity excretion rates were never statistically different from controls (Fig. 2). Net-acid excretion, T_{amm}, and titratable acidity excretion rates remained relatively constant in the SW and FW control series of stingrays (Fig. 2).

SW vs. FW compensation

Direct comparisons between SW and FW stingrays were made to determine if salinity had effects on the rate and degree of compensation for hypercapnia. The mean blood pH compensation

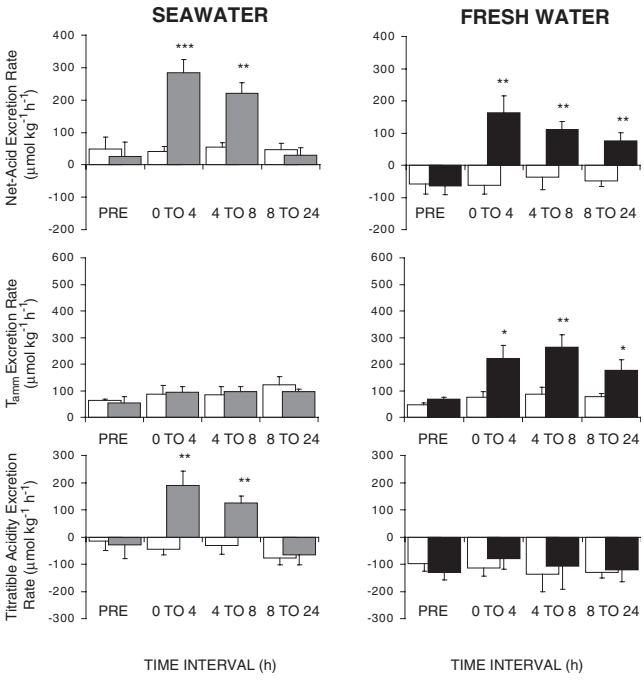

Fig. 2. Whole-animal net-acid, T_{amm}, and titratable acidity excretion rates in seawater and fresh water acclimated *D. sabina* during normocapnia (white bars) or before and during 1% hypercapnia (hatched and solid bars). (mean + or − S.E.; seawater normocapnic controls and hypercapnia: N=6; freshwater normocapnic controls: N=6 for 0, 2, 4, & 8 h, N=5 for 24 h; freshwater hypercapnia: N=6); $^*P < 0.050$, $^{**}P < 0.010$, $^{***}P < 0.001$ for unpaired t-test between control and experimental means.

after 24 h of hypercapnia was 88.5% in SW, but only 31.0% in FW (Fig. 3A), clearly showing that stingrays compensated faster, and more completely, in the higher salinity. In both salinities, most of this compensation was in the first eight hours of hypercapnia, so cumulative net-acid fluxes were compared for this time interval. As hypothesized, SW stingrays had a greater cumulative net-acid excretion than FW stingrays (Fig. 3B).

FW urine fluxes

Urinary net-acid, T_{amm}, and TA-HCO_3^-, excretion rates were never different from normocapnic control rates in FW acclimated *D. sabina* (Fig. 4). Specifically, individual urinary net-acid excretion rates were never greater than 25 μmol kg^{-1} h^{-1}, and there was always a slight mean base-excretion (negative net-acid excretion). In addition, there was a slight cumulative net-base excretion (negative net-acid excretion) during the first eight hours of hypercapnia (the interval of the most compensation) (Fig. 5).

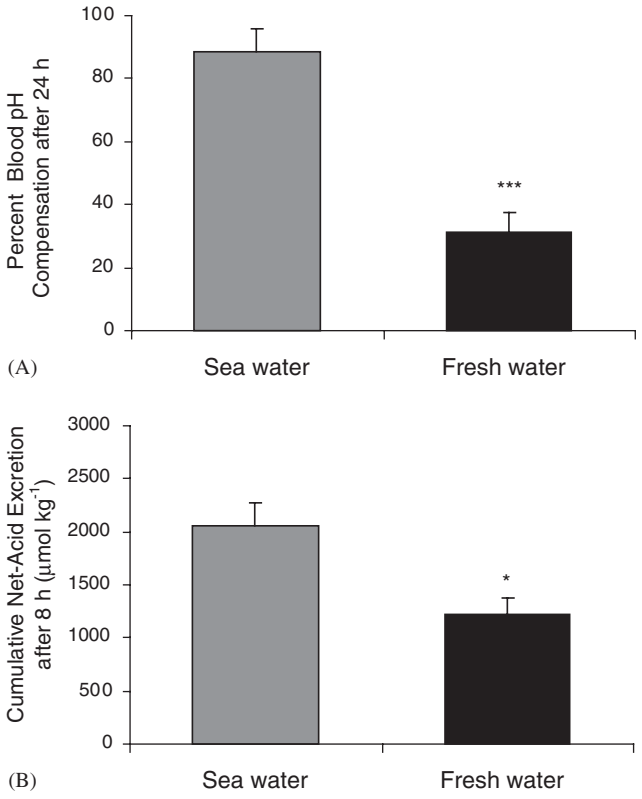

Fig. 3. Comparison of compensation for 1% hypercapnia between seawater and fresh water acclimated *D. sabina*. A, Percent plasma pH compensation after 24 h of 1% hypercapnia (mean+S.E.; seawater: N=5 and fresh water: N=4); ***$P < 0.001$ for unpaired t-test. B, Cumulative net-acid excretion after eight hours of 1% hypercapnia. (mean+S.E.; N=5 for seawater and N=6 for fresh water); *$P < 0.05$ for unpaired t-test.

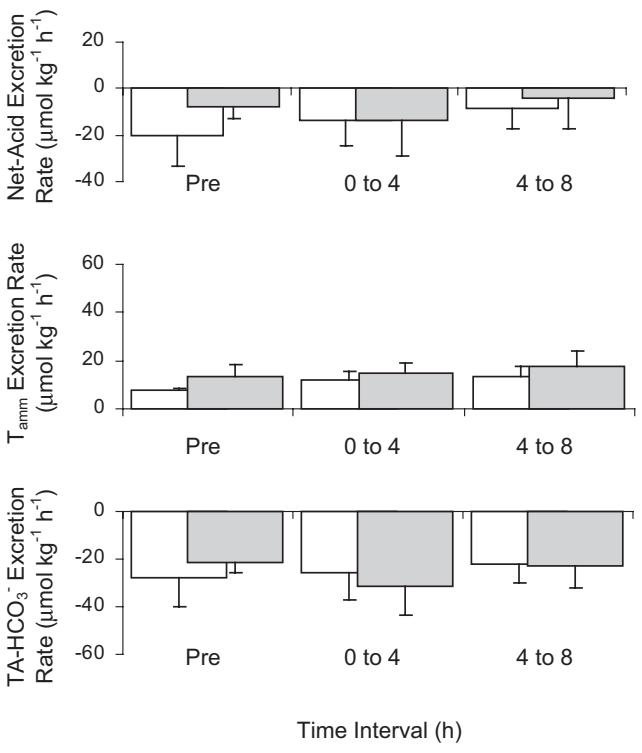

Fig. 4. Urinary net-acid, T_{amm}, and titratable acidity—HCO_3^- excretion rates in freshwater acclimated *D. sabina* during normocapnia (white bars) or before and during 1% hypercapnia (lightly shaded bars). (mean+or − S.E.; N=5). Hypercapnic means were never significantly different from normocapnic controls as determined with unpaired t-tests.

DISCUSSION

Control blood acid-base status

Blood pH of a euryhaline elasmobranch in FW has been reported for bull sharks from Lake Nicaragua (Thorson et al., '73). However, blood was sampled by caudal vessel and cardiac puncture from specimens that had been recently caught with hook and line gear. Therefore, the reported mean blood pH of 6.83 for sharks caught in Lake Nicaragua likely represents severe metabolic and respiratory acidosis from struggling. To our knowledge, our pH of 7.81, P_{CO_2} of 3.58 mmHg, and [HCO_3^-] of 7.03 for normocapnic control *D. sabina* are the first to be reported for blood from a cannulated, steady-state elasmobranch in FW.

Our measured pH of 7.66, and calculated P_{CO_2} of 3.64 mmHg and [HCO_3^-] of 5.12 mmol l^{-1} for SW stingrays appear to be slightly respiratory acidotic relative to values from studies that have sampled arterial blood from other cannulated marine elasmobranchs (Piiper et al., '72; Heisler et al., '76; Holeton and Heisler, '83; Cameron, '86; Claiborne and Evans, '92). This discrepancy is

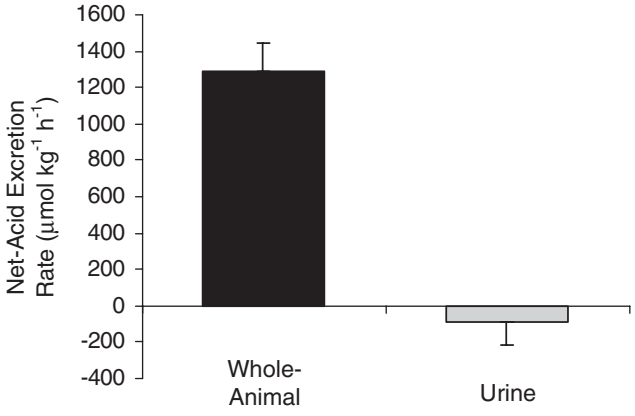

Fig. 5. Comparison of cumulative whole-animal and urinary net-acid excretion during the first eight hours of 1% hypercapnia in fresh water acclimated *D. sabina*. (mean +or − S.E.; whole-animal: N=6 and urine: N=5).

probably not due to differences between venous blood used in this study and arterial blood used in the others, because the vein used in this study drains the tail, which is not used for propulsion in stingrays. In addition, differences in water temperature between our study and previous studies may explain more of this disparity. The elasmobranchs measured in previous studies have all been temperate species held in water 14 to 19°C, but *D. sabina* is a sub-tropical species that was held at 23–25°C. Using a steady-state $\Delta pH/\Delta^{\circ}C$ of —0.012 taken from Heisler et al. ('80), the theoretical steady-state pH of blood from *D. sabina* at 16°C is 7.74 to 7.77, close to the range of pHs reported for temperate marine elasmobranchs (7.78–7.87) (Piiper et al., '72; Heisler et al., '76; Holeton and Heisler, '83; Cameron, '86; Claiborne and Evans, '92). In addition, the aortic blood pH of FW spotted gar (*Lepisosteus oculatus*) at high temperature (26°C), was about 7.68, close to the pH we measured for *D. sabina* in SW (Smatresk and Cameron, '82a).

Control SW acclimated stingrays had a metabolic acidosis of 0.15 pH units and 1.91 mmol l^{-1} HCO_3^- relative to control FW stingrays. This effect of salinity on acid-base status has not been reported previously for an elasmobranch, but appears to be a common response in teleosts transferred from FW to a higher salinity (Perry and Heming, '81; Smatresk and Cameron, '82b; Wilkes and McMahon, '86; Nonnotte and Truchot, '90; Maxime et al., '91; Madsen et al., '96). Conversely, *Myoxocephalus octodecimspinosus* (sculpin) and *Salmo salar* (Atlantic salmon) developed a metabolic alkalosis when transferred from SW to lower salinities (Maxime et al., '90; Claiborne et al., '94). This relationship between external salinity and extracellular fluid $[HCO_3^-]$ was first reported for invertebrates (reviewed by Truchot, '87), and when considered with studies on teleosts and this study on an elasmobranch, now appears to be ubiquitous for euryhaline animals. The reasons for acid-base changes with environmental salinity are complex and unresolved, but have been correlated to changes in strong ion difference (SID) that are not due to lacticacidosis (Smatresk and Cameron, '82b; Wilkes and McMahon, '86; Maxime et al., '90). Explanations for these changes in SID include differential net fluxes of Na^+ and Cl^- between the environment, ECF, and intracellular fluid that cause changes in net fluxes of acidic equivalents.

Other explanations involve changes in metabolism of organic metabolites used for osmoregulation (Truchot, '87; Gaumet et al., '94). This may partially explain the higher ECF $[HCO_3^-]$ and net-base excretion observed for control FW stingrays in this study. For example, Goldstein and Forster ('71a, '71b) showed that urea synthesis of little skates (*Raja erinacea*) in 50% SW was about 100 μmoles kg^{-1} h^{-1} lower than in 100% SW, and that urea synthesis in the stenohaline FW stingray (*Potamotrygon*) was negligible. Because bicarbonate is a substrate for urea synthesis (Anderson, '95), lower urea synthesis rates for *D. sabina* in FW than in SW would spare bicarbonate, and therefore explain the relative metabolic alkalosis when in FW.

The mean UFR of control normocapnic FW stingrays was 14.7 ml kg^{-1} h^{-1}. This is over ten-fold greater than UFRs reported for SW elasmobranchs (Cross et al., '69; Holeton and Heisler, '83; Swenson and Maren, '86), and even higher than UFRs reported for FW teleosts (Hickman and Trump, '69). This copious urine is likely because of the ~600 mOsm l^{-1} osmotic gradient that *D. sabina* maintains when in FW (Piermarini and Evans, '98).

Hypercapnic blood acid-base status

The decrease in pH during hypercapnic (1% CO_2) exposure, and the subsequent compensation by increasing ECF $[HCO_3^-]$ was qualitatively similar between FW and SW, and characteristic of fishes exposed to hypercapnia (Graham et al., '90; Heisler, '93; Claiborne, '98). As hypothesized, stingrays in SW compensated toward control pH faster, and more completely, than stingrays in FW, suggesting a positive effect of salinity on compensation for acidosis. This has also been shown directly for the teleost, *O. mykiss* (Iwama and Heisler, '91) exposed to hypercapnia and following exhaustive exercise (Tang et al., '89). In addition, separate studies have shown that FW teleost species compensate for hypercapnia slower than do SW teleosts (Perry, '82; Toews et al., '83; Claiborne and Heisler, '84; Perry et al., '86). Therefore, increased salinity appears to promote compensation for acidosis in elasmobranchs and teleosts, and may therefore represent a universal effect of ambient ions that is not species specific.

It should be noted that the blood pHs of stingrays after 24 hours of hypercapnia were similar in the two salinities (7.65 in SW and 7.62 in FW), suggesting that there may be a common target pH that both groups reached. Unfortunately, blood sampling beyond 24 h was not usually successful

because of clotting, so it is unclear if blood pH continues to increase beyond this range in FW. However, in preliminary experiments, FW stingrays continued to have elevated net-acid excretion rates from 24 to 48 hours (47.76 ± 13.06 μmoles kg^{-1} h^{-1}, N=5) of hypercapnia, even though their blood pHs had compensated to the range of control SW stingrays. Alternatively, SW animals were almost always back to control net-acid excretion rates by 24 hours. These results (although sparse) suggest that compensation was continued beyond the blood pH range of 6.60–6.65 in FW, but not in SW, contradicting the possibility of a common target pH for the two salinities.

Hypercapnic fluxes

The more rapid blood pH compensation for hypercapnia in SW than in FW was probably because of a 69% greater cumulative net-acid excretion in the first eight hours of hypercapnic exposure (interval when the most compensation occurred, Fig. 3). Although our results cannot rule-out an effect of other ions, this positive effect of salinity is thought to reflect cellular mechanisms of branchial H^+ and/or NH_4^+ excretion that depend on external [Na^+]. For example, the FW teleosts *O. mykiss* and *Oreochromis mossambicus* (tilapia) are thought to use an apical vacuolar H^+-ATPase to create a negative inside membrane potential that allows Na^+ to enter the cell from the water through an apical Na^+ channel (Lin and Randall, '95; Sullivan et al., '95; Sullivan et al., '96; Wilson et al., 2000). In this model, the proton pump works against the electrical potential that it creates, and therefore addition of Na^+ to the water would be expected to increase H^+ pumping by making Na^+ entry more favorable. However, Piermarini and Evans (2001) have shown that vacuolar H^+ -ATPase occurs on the basolateral side of branchial epithelial cells in *D. sabina*, a location that is not consistent with the teleost model. Based on an immunolocalization study on elasmobranchs (Edwards et al., 2002), Piermarini and Evans (2001) predict that an apical Na^+ /H^+ -exchanger is present in Na^+/K^+ -ATPase-rich cells of *D. sabina* gills. If this model is correct, it would help explain the slower net-acid excretion in FW than in SW that we observed in this study, because Na^+/H^+ -exchangers use a Na^+ gradient to excrete H^+. However, further studies are needed to identify the apical acid transporters in *D. sabina* gills.

Another possible explanation for the different rates of acid excretion is that more transport proteins and/or transport cells are available to excrete acid in SW than in FW. However, this is not likely because Na^+/K^+ -ATPase and vacuolar H^+ -ATPase expression was previously shown to be greater in FW than in SW (Piermarini and Evans, 2000, 2001).

The finding that T_{amm} excretion increased in FW, and not in SW, in response to hypercapnia suggests different mechanisms of acid excretion in the two salinities. During hypercapnia, FW *D. sabina* excreted T_{amm} at rates up to 263 μmol kg^{-1} h^{-1}. Alternatively, urine T_{amm} excretion rates never exceeded 20 μmol kg^{-1} h^{-1}, meaning that the majority of the increased T_{amm} excretion during hypercapnia occurred via extra-renal routes. As in other fishes (Wood, '93; Walsh, '98), we presume that the gills were the primary site of this extra-renal T_{amm} excretion. During hypercapnia, FW *D. sabina* increased T_{amm} excretion without decreasing TA excretion (Fig. 2), suggesting that NH_4^+, and/or NH_3 and H^+ were excreted. There is evidence for both mechanisms of ammonia excretion in fishes (reviewed by Wood, '93), and both can be explained with the apical Na^+/H^+ -exchanger, basolateral Na^+/K^+-ATPase model proposed by Piermarini and Evans (2001). In proximal tubules of mammalian nephrons, NH_4^+ regularly substitutes for H^+ and K^+ in these transporters (Weinstein, '94; Paillard, '97), and is thought to do the same in some fish gills (Claiborne et al., '82; Evans et al., '89). In addition, there is a four-fold greater Na^+/K^+-ATPase activity and number of Na^+/K^+-ATPase-rich cells in gills from FW acclimated *D. sabina* than in gills from SW acclimated *D. sabina* (Piermarini and Evans, 2000). This was hypothesized to drive Na^+ absorption from hypoionic water, but would also increase the ability for cells to absorb NH_4^+ from the blood across the basolateral membrane. Ammonia excretion across the apical membrane could then either be by Na^+/NH_4^+ exchange or Na^+/H^+ exchange followed by NH_3 via diffusion trapping. In both cases, ammonia would be acting as a buffer, minimizing the apical H^+ gradient formed by acid excretion. Ammonia functions in this way in mammalian proximal tubules (Koeppen and Stanton, '97), and may be important in FW *D. sabina*, where the water-to-intracellular fluid Na^+ gradient is reduced. In SW, the water-to-intracellular fluid Na^+ gradient is expected to be much larger, and therefore the apical pH gradient is not as large an obstacle to acid excretion.

FW urine

Because of higher UFRs in FW, we expected a renal contribution to compensation for hypercapnia greater than the typical 1% that is reported for SW elasmobranchs (Heisler et al., '76; Evans et al., '79; Holeton and Heisler, '83). However, there was actually a slight net-base loss in control urine, and net-acid, T_{amm}, and TA-HCO_3^- excretion rates never deviated from control values during hypercapnia. Therefore, despite high UFRs, *D. sabina* kidneys appear unable to respond to acute hypercapnia, and gills were probably responsible for all of the whole-animal net-acid excretion (Fig. 5). This apparent lack of renal systemic acid-base regulation despite large UFRs in FW *D. sabina*, contradicts data for FW teleosts (Cameron, '80; Cameron and Kormanik, '82; Smatresk and Cameron, '82a; Hyde and Perry, '87; Perry et al., '87), and this may be because of *D. sabina's* short evolutionary history in FW compared to teleosts. For example, the St. John's River is less than one hundred thousand years old (Cook, '39). Alternatively, teleosts have occurred in FW for probably over one hundred million years, giving them more time to evolve renal mechanisms of acid-base regulation that can take advantage of higher UFRs.

Summary

We found that *Dasyatis sabina* had a higher plasma [HCO_3^-] and blood pH in FW than in SW during normacapnia. When exposed to hypercapnia in either salinity, *D. sabina* developed a respiratory acidosis that was compensated by increasing extracellular [HCO_3^-] via increased whole-animal net-acid excretion. This compensation was faster, and more complete in SW than in FW, as has been shown for teleosts. However, unlike FW teleost kidneys, FW *D. sabina* kidneys made no contribution to whole-animal net-acid excretion, suggesting that their gills were the primary site of net-acid excretion. Studies on the cellular and molecular mechanisms of acid excretion from the gills of *D. sabina* are needed to determine how *D. sabina* increases extracellular [HCO_3^-] and why salinity effects the rate of compensation for acidosis.

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