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Nitrogen metabolism, acid–base regulation, and molecular responses to ammonia and acid infusions in the spiny dogfish shark (*Squalus acanthias*)

C. Michele Nawata^{1,2,3} · Patrick J. Walsh^{1,4} · Chris M. Wood^{1,2,5}

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Abstract Although they are ureotelic, marine elasmobranchs express Rh glycoproteins, putative ammonia channels. To address questions raised by a recent study on high environmental ammonia (HEA) exposure, dogfish were intravascularly infused for 24 h at 3 ml kg⁻¹ h⁻¹ with isosmotic NaCl (500 mmol l⁻¹, control), NH₄HCO₃ (500 mmol l⁻¹), NH₄Cl (500 mmol l⁻¹), or HCl (as 125 mmol l⁻¹ HCl + 375 mmol l⁻¹ NaCl). While NaCl had no effect on arterial acid–base status, NH₄HCO₃ caused mild alkalosis, NH₄Cl caused strong acidosis, and HCl caused lesser acidosis, all predominantly metabolic in nature. Total plasma ammonia (T_{Amm}) and excretion rates of ammonia (J_{Amm}) and urea-N (J_{Urea-N}) were unaffected by NaCl or HCl. However, despite equal loading rates, plasma T_{Amm} increased to a greater extent with NH₄Cl, while J_{Amm} increased to a greater extent with NH₄HCO₃ due to much greater increases in blood-to-water PNH₃ gradients. As with HEA, both treatments caused large (90 %) elevations

of J_{Urea-N}, indicating that urea-N synthesis by the ornithine-urea cycle (OUC) is driven primarily by ammonia rather than HCO₃⁻. Branchial mRNA expressions of *Rhbg* and *Rhp2* were unaffected by NH₄HCO₃ or NH₄Cl, but *v-type H⁺-ATPase* was down-regulated by both treatments, and *Rhbg* and Na⁺/H⁺ exchanger *NHE2* were up-regulated by HCl. In the kidney, *Rhbg* was unresponsive to all treatments, but *Rhp2* was up-regulated by HCl, and the urea transporter *UT* was up-regulated by HCl and NH₄Cl. These responses are discussed in the context of current ideas about branchial, renal, and OUC function in this nitrogen-limited predator.

Keywords Elasmobranchs · Rh glycoproteins · Gene expression · Urea · Ammonia · HCl · NH₄Cl · NH₄HCO₃ · Gills · Kidney · *Rhp2* · Ornithine-urea cycle

Introduction

In marine elasmobranchs, the focus of research in N-metabolism has long been on urea rather than ammonia, ever since the classic work of Homer Smith (1929, 1936) showing that these animals are ureotelic, retaining high levels of urea in the body fluids so as to approximately match the osmolality of the external seawater. Nevertheless, the initial end product of amino acid oxidation is ammonia, most of which is immediately funneled into urea synthesis via reaction with HCO₃⁻ in the ornithine-urea cycle (OUC). The initial trapping of ammonia is by a high-affinity glutamine synthetase (Shankar and Anderson 1985; Ballantyne 1997), but small amounts of ammonia do escape, to be excreted at the gills and kidney (King and Goldstein 1983; Evans 1982; Evans and More 1988; Wood et al. 1995, 2005; Kajimura et al. 2006, 2008). In the last few years, there has

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✉ C. Michele Nawata
cmnawata@email.arizona.edu

- ¹ Bamfield Marine Sciences Centre, 100 Pachena Road, Bamfield, BC V0R 1B0, Canada
- ² Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada
- ³ Department of Physiology, University of Arizona, 1501 N. Campbell Ave., Tucson, AZ 85724, USA
- ⁴ Department of Biology and Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON K1N 6N5, Canada
- ⁵ Department of Zoology, University of British Columbia, #4321-6270 University Blvd., Vancouver, BC V6T 1Z4, Canada

been renewed interest in ammonia metabolism in elasmobranchs, stemming from the discovery that they express Rhesus (Rh) glycoproteins in various tissues (Nakada et al. 2010; Anderson et al. 2010, 2012); these channel proteins facilitate ammonia transport in both invertebrates (Weihrauch et al. 2004, 2012) and vertebrates (Marini et al. 2000; Bakouh et al. 2004; Ripoche et al. 2004; Mouro-Chanteloup et al. 2010), including teleost fish (Nakada et al. 2007; Nawata et al. 2007, 2010b).

Recently, we have shown that dogfish sharks respond to high environmental ammonia (HEA) by actively taking up ammonia, up-regulating urea-N production, and down-regulating the mRNA expression of *Rhbg* (a putative basolateral ammonia transporter) in the gills (Nawata et al. 2015). Increased ureagenesis is reminiscent of the responses of several ureotelic teleosts (Magadi tilapia, gulf toadfish) to HEA as a mechanism for ammonia detoxification (Wood et al. 1989, 2013a, b; Walsh et al. 1990). The decrease in *Rhbg* expression was similar to the response of a marine ammoniotelic teleost (pufferfish) to HEA, which was interpreted as a mechanism to reduce the permeability of the gills to further ammonia influx (Nawata et al. 2010a). We offered a similar explanation in the dogfish, that *Rhbg* expression was decreased to prevent the homeostatic systems being overwhelmed in the face of increased ammonia scavenging during HEA (Nawata et al. 2015). Alternatively, or additionally, it may be part of an overall ammonia retention strategy, signaled by an increase in plasma ammonia concentration. In nature, sharks probably rarely encounter HEA; more commonly, ammonia loading occurs internally, as a result of feeding. These opportunistic predators appear to be N-limited in the wild (Haywood 1973) and respond to feeding by immediate increases in urea-N production and down-regulation of overall N-losses at the gills (Wood et al. 2005, 2007a; Kajimura et al. 2006).

Therefore, in the present study, we hypothesized that internal ammonia loading, by infusion of ammonium salts into the bloodstream, would increase urea-N production and down-regulate the expression of *Rhbg* in the gills of the dogfish shark, *Squalus acanthias*. Blood plasma [HCO_3^-] increases during HEA exposure (Nawata et al. 2015) and also after feeding (the “alkaline tide”; Wood et al. 2005, 2007b). Therefore, in order to evaluate whether an acid–base signal plays any role in these responses (cf. Wood et al. 2008), we infused ammonia in two forms—as NH_4HCO_3 , which raises plasma [HCO_3^-], and as NH_4Cl , which lowers plasma [HCO_3^-]. By comparing excretion rates and plasma levels of ammonia and urea-N in the two treatments, as well as blood acid–base status, we were also able to evaluate whether provision of just one substrate (ammonia) or both substrates (ammonia and HCO_3^-) is critical to the ureogenic response (cf. Atkinson and Bourke 1984; Atkinson 1992). Nawata et al. (2015) reported that

the mRNA expression levels of several other transporters (Na^+ , K^+ -ATPase, *v*-type H^+ -ATPase) were also altered in the gills during HEA exposure, so these were additionally measured in the present infusion experiments, together with other acid–base-related proteins (*NHE2*, *carbonic anhydrase IV*).

A final objective focused on the role and regulation of a primitive Rhesus glycoprotein *Rhp2* ($p = \text{“primitive”}$) originally discovered by database mining (Huang and Peng 2005). The presence of *Rhp2* was first reported in the banded hound shark, *Triakis scyllium*, by Nakada et al. (2010) who found it only in the kidney. Using a more sensitive technique, we found that *Rhp2* was expressed in many tissues of *Squalus acanthias*, including both gills and kidney, but surprisingly it did not respond to HEA exposure (Nawata et al. 2015). In the hound shark, Nakada et al. (2010) reported that renal expression of *Rhp2* increased with elevations in salinity (and vice versa), and speculated that it served to reabsorb ammonia from the urine to fuel urea synthesis. However, an alternative possibility is that *Rhp2* serves to secrete ammonia into the urine. Elevated renal ammonia production and excretion have been shown to contribute to increased renal acid excretion during metabolic acidosis in the dogfish shark (King and Goldstein 1983). Furthermore, increases in salinity generally cause metabolic acidosis in teleost and elasmobranch fish due to a reduction in the ‘strong ion difference’ (Stewart 1981) as plasma Cl^- rises more than plasma Na^+ (e.g. Walker et al. 1989; Maxime et al. 1990; Wilson and Taylor 1992; Cooper and Morris 2004). In the present study, we therefore examined whether *Rhp2* expression in kidney and gills would respond to a signal of acidosis alone (induced by HCl infusion) and/or to an ammonia signal either coupled (by NH_4Cl infusion) or not coupled (by NH_4HCO_3 infusion) to acidosis.

Materials and methods

Experimental animals

All experimental procedures were approved by the Animal Care Committees of Bamfield Marine Sciences Centre (BMSC) and McMaster University, and conformed to the guidelines of the Canadian Council on Animal Care. Male Pacific spiny dogfish (*Squalus acanthias suckleyi* L., 0.9–2.4 kg) were collected by trawl netting in Barkley Sound, British Columbia, Canada in May and June 2009 and 2010, under a license from the Dept. of Fisheries and Oceans, Canada. Note that taxonomy is currently controversial, and we have retained the more established nomenclature. Ebert et al. (2010) have recently proposed that these animals endemic to the north-east Pacific may be a separate

species (*Squalus suckleyi*) rather than a subspecies of *Squalus acanthias*.

At BMSC, these small sharks were held until the experimental period (July and August) in a large 151,000-L circular tank served with running seawater at the experimental salinity (30 ± 2 ‰), pH (7.90 ± 0.15), and temperature (11 – 12 °C). The animals were fed every 3–4 days with a 3 % ration (relative to body mass) of dead hake (*Merluccius productus*) during the holding period. At 1–2 weeks prior to experimentation, the dogfish were transferred to smaller 1500-L tanks in batches of 6–10. The animals were then fasted to standardize their metabolic status.

At 24 h prior to surgery, the fish were placed in covered polyurethane-coated wooden fish boxes (105 cm length \times 16.5 cm width \times 25 cm depth), as described by Wood et al. (1995). The boxes were served with a flow-through of 1 l min^{-1} and were placed in an external seawater bath to maintain temperature during periods when the boxes were closed for flux measurements. After this initial adjustment period, all animals were fitted with indwelling caudal artery catheters for subsequent infusion and blood sampling and then returned to the boxes for 24-h recovery. The catheter was filled with 500 mM NaCl containing 50 i.u. ml^{-1} lithium heparin (Sigma–Aldrich, St. Louis, MO, USA).

Cannulation followed methods described by De Boeck et al. (2001). The fish were anesthetized in MS-222 (0.15 g l^{-1} ; Syndel Laboratories, Qualicum, BC, Canada), weighed, and artificially ventilated on an operating table. After surgical exposure of the vertebral column in the region of the caudal peduncle between the epaxial and hypaxial muscle masses, a small hole was made in the cartilaginous haemal canal using a 21-gauge needle. The catheter (Clay-Adams PE50, Becton–Dickinson, Franklin Lakes, NJ, USA) was then rapidly inserted through the hole into the artery and advanced approximately 10 cm anteriorly. A PE160 sleeve, heat flared at both ends, was cemented over the PE50 tubing at its exit from the incision and firmly anchored with several silk sutures so as to secure the catheter in place. The wound was then dusted with powdered oxytetracycline (Sigma–Aldrich) to avoid infection and tightly closed with silk ligatures. The catheter was filled with 500 mM NaCl containing 50 i.u. ml^{-1} lithium heparin (Sigma–Aldrich). After surgery, the dogfish were revived in anesthetic-free water, returned to their boxes, and allowed to recover for 24 h prior to the start of infusion experiments.

Infusion treatments

Dogfish were subjected to infusion treatments designed to change plasma ammonia and blood acid–base status ($n = 6$ – 12 per treatment). These and other plasma parameters were measured prior to, and during 24 h of infusion,

together with flux rates of ammonia and urea-N to the external water. Blood samples (1 ml) were drawn prior to infusion (0 h control) and at 2, 6, 12, and 24 h and processed as described below. Water samples (10 ml) for flux measurements were taken at the start and end of a 12-h pre-infusion control period and then over two successive 12-h infusion periods; samples were immediately frozen for later measurements of ammonia and urea-N concentrations. For each flux period, water flow to the box was stopped and the water level set to a mark representing 40 l. Between flux periods, the water was renewed by filling and partially emptying the box 3 times (avoiding air exposure of the fish) and then resetting the level to the 40-l mark.

Infusions were carried out via the caudal artery catheter using either a peristaltic pump (Gilson Minipuls 3, Middleton, WI, USA) or a cassette pump (Manostat 72-500-000, Woodstock, NY, USA) by methods similar to those of Wood et al. (1995). The nominal infusion rate was $3 \text{ ml kg}^{-1} \text{ h}^{-1}$, monitored gravimetrically by periodically weighing the reservoir; average rates per fish varied from 2.5 to $3.5 \text{ ml kg}^{-1} \text{ h}^{-1}$. The infusion treatments were (i) 500 mmol l^{-1} NaCl (control), (ii) 125 mmol l^{-1} HCl + 375 mmol l^{-1} NaCl, (iii) 500 mmol l^{-1} NH_4HCO_3 , and (iv) 500 mmol l^{-1} NH_4Cl , all approximately isosmotic to the blood plasma. These concentrations were chosen based on both preliminary experiments and on previous experience in infusing dogfish with acidic and basic loads (Wood et al. 1995, 2007c, 2008). At the end of the infusion period, most of the fish were sampled for molecular endpoints as described below.

Blood sampling

At each sampling time, arterial blood samples (400 and 600 μl) were drawn into two ice-cold gas-tight glass syringes (Hamilton, Reno, NV, USA). The smaller blood sample was immediately analyzed for arterial pH (pHa) and oxygen tension (PaO_2). The larger blood sample was immediately centrifuged in a sealed tube (1 min at 5000 g) to separate the plasma, which was then divided into three separate aliquots. One was analyzed immediately for true plasma total CO_2 concentration; the other two were flash frozen in liquid N_2 and then stored at -80 °C for subsequent analyses of (i) plasma total ammonia (T_{Amm}) and urea-N ($T_{\text{Urea-N}}$) concentrations, and (ii) plasma electrolytes. Analytical techniques are described below. Red blood cells separated by centrifugation were re-suspended in saline, combined with blood recovered from the electrodes, and re-infused together with sufficient saline to restore blood volume.

Terminal sampling for molecular endpoints

After the end of the 24-h infusion protocol, an additional blood sample was drawn. The red blood cells were

Table 1 Primers used for qPCR

Primer	Forward/reverse (5' → 3')	Accession #
Rhag	ctttgttgattggcttc/aatgaaatcaggacggttc	KJ960196
Rhbg	gctcatctgcctctgtt/gggatcacctgaagtagaactcg	KJ960197
Rhp2	ggagctgctgaagatcaagg/agaccacagcaacaagctc	KJ960198
CA	caatgcctcacaacacaagg/ggagaagaaatgcgctctg	DQ092628
HATP	ggaatctcggcaatagatgg/tggcaaaattctctgactg	EU004205
NHE	ttctggaacggcttcgact/ccagagggtgatctcaaagg	DQ324545
NKA	tactgctcgaggtgtgtcg/gcttcaagccagctgtatcc	AJ781093
UT	aagaaagcagcaattgtggaag/gcaccacgtaagatccagtc	AF257331

immediately separated by centrifugation (1 min at 5000 *g*) and then frozen in liquid N₂, taking care to avoid contamination with white blood cells. The fish was then anesthetized (0.15 g l⁻¹ MS-222) and artificially ventilated on an operating table. In order to clear blood from the tissues, the ventral aorta was surgically exposed, cannulated with PE160 tubing, and perfused with an ice-cold 500 mmol l⁻¹ NaCl containing 10 i.u. ml⁻¹ of lithium heparin. A Model 1100 cardiac pump (Harvard Apparatus, Holliston, MA, USA) was used, set to a working mode of 1/3 systole, 2/3 diastole, a heart rate of 30 beats min⁻¹, and a stroke volume scaled to the body weight such the output was approximately 20 ml kg⁻¹ min⁻¹. Once tissues were cleared of blood (2–4 min), samples of gill and kidney were removed and stored in RNAlater (Sigma–Aldrich).

Blood and water analyses and calculations

T_{Amm} in plasma was measured immediately after thawing using an enzymatic method (L-glutamate dehydrogenase; Mondzac et al. 1965) with a commercial kit (Product No. 85446, Ammonia Reagent, Raichem Life Sciences, Mumbai, India). T_{Amm} in water was measured by the colorimetric salicylate–hypochlorite method of Verdouw et al. (1978); the two methods were cross-validated in sea water. Plasma and water ammonia partial pressures (PNH₃) and ammonium ion concentrations ([NH₄⁺]) were calculated from the respective T_{Amm} and pH measurements, using the Henderson–Hasselbalch relationship, as detailed by Wright and Wood (1985). In the absence of data for elasmobranch plasma, constants (α_{NH3}, pK_{amm}) were taken from the study of Cameron and Heisler (1983) on trout, using an ionic strength equivalent to 500 mmol l⁻¹ NaCl and their adjustments for plasma. The PNH₃ gradient across the gills (ΔPNH₃) was calculated as the difference between simultaneous measurements of water PNH₃ and arterial blood plasma PaNH₃.

Arterial blood oxygen tensions (PaO₂), pHa, and water pH were measured using Radiometer electrodes (Copenhagen, Denmark; E5036 for PO₂, GK2401C for pH) thermostatted to the experimental temperature. Outputs of the

electrodes were displayed on Radiometer pHM 71 and pHM 72 acid–base analyzers. A Corning 965 analyzer (Midland, MI, USA) was used to measure true plasma total CO₂ concentrations. Arterial blood carbon dioxide tensions (PaCO₂) and bicarbonate concentrations ([HCO₃⁻]_a) were calculated using rearrangements of the Henderson–Hasselbalch equation and coefficients for the solubility of carbon dioxide (α_{CO2}) and the apparent pK (pK_{app}) in dogfish plasma from Boutilier et al. (1984).

Urea-N concentrations (T_{Urea-N}) in both plasma and water were determined by the colorimetric diacetyl monoxime method of Rahmatullah and Boyde (1980). Plasma sodium, potassium, calcium, and magnesium concentrations were measured by flame atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Australia), and chloride was measured with a Radiometer CMT-10 chloridometer.

Flux rates of total ammonia (J_{Amm}) and urea-N (J_{Urea-N}) were calculated from changes of concentration in the flux boxes, factored by fish mass, volume, and time (see Nawata et al. 2007 for equation). All concentrations and rates of urea have been expressed in units of urea-N (i.e., two N per urea molecule) to allow comparison with ammonia (one N per ammonia molecule).

Molecular analyses

First-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) using 1 μg of DNaseI-treated total RNA (gill and kidney) that was extracted with Trizol (Invitrogen).

mRNA levels of *carbonic anhydrase IV (CA-IV)*, *Rhbg*, *Rhp2*, *v-type H⁺-ATPase (B subunit) (H⁺-ATPase)*, *Na⁺/H⁺ exchanger type 2 (NHE2)*, and *Na⁺,K⁺-ATPase (α subunit) (NKA)* in the gill and *Rhbg*, *Rhp2*, and urea transporter (*UT*) in the kidney were then analyzed by qPCR using the primer sets in Table 1. cDNA cloning of *Squalus acanthias Rhbg* and *Rhp2* has been described earlier (Nawata et al. 2015). Reactions were performed on a Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) following the protocol described previously (Nawata et al. 2007). Values were extrapolated from standard curves generated from control template. We tested three housekeeping genes (beta-actin, ribosomal protein L8 and 18S rRNA) and found that all were unstable in the experimental samples. Therefore, we chose to normalize to total RNA, another acceptable method of normalization (Bustin 2000, 2002).

Statistics

Data have been expressed as means ± 1 SEM (*n*), where *n* = number of fish. Variables that failed the normality test

Fig. 1 Levels of pH, PCO_2 (Torr), HCO_3^- (mmol l^{-1}) and PO_2 (Torr) in the arterial blood of *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c** NH_4HCO_3 , or **d** NH_4Cl for 24 h. “INF” represents the period of infusion. Asterisks indicate a significant difference ($P < 0.05$) from the corresponding pre-infusion control levels. Data are mean \pm SEM, $n = 5\text{--}12$

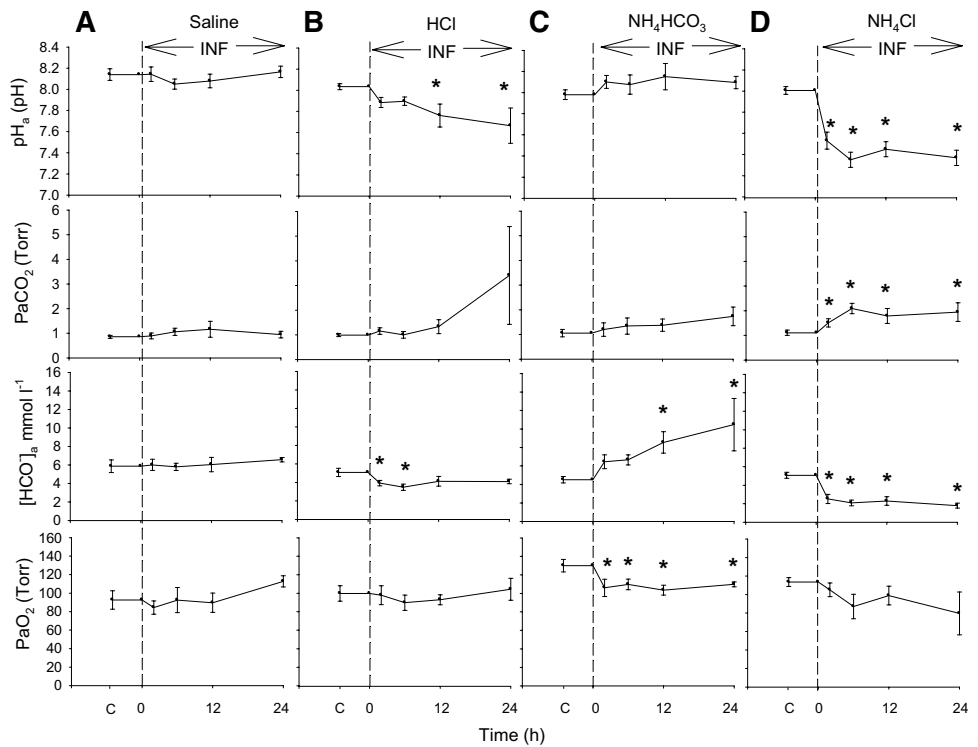
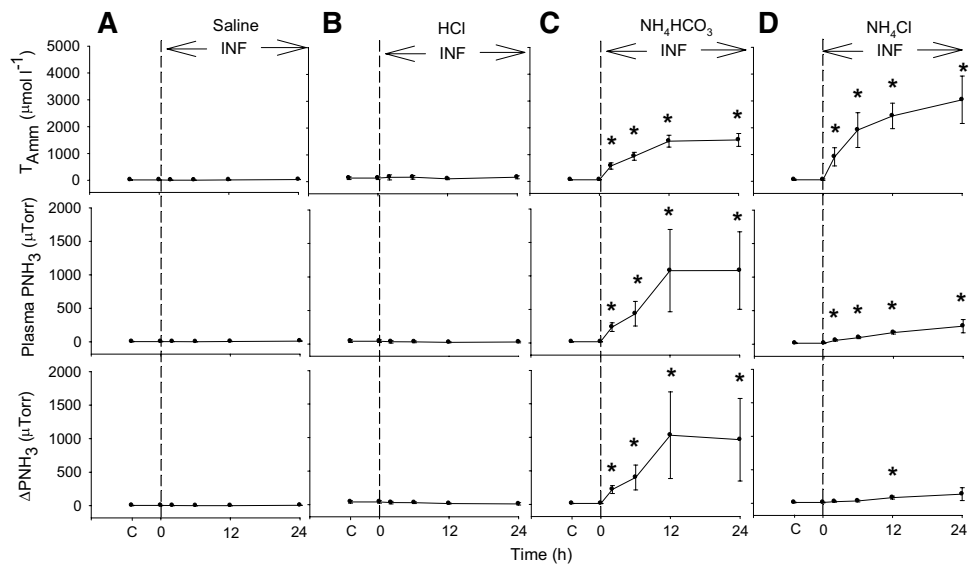


Fig. 2 Plasma total ammonia (T_{Amm} ; $\mu\text{mol l}^{-1}$), PNH_3 (μTorr), and ΔPNH_3 (μTorr) in *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c** NH_4HCO_3 , or **d** NH_4Cl for 24 h. “INF” represents the period of infusion. Asterisks indicate a significant difference ($P < 0.05$) from the corresponding pre-infusion control levels. Data are mean \pm SEM, $n = 6\text{--}12$



were log transformed. A repeated measures ANOVA followed by a Holm-Sidak or Dunnett’s multiple comparison test was used to detect specific differences relative to the pre-exposure control value and differences between treatments (Figs. 2, 3, and 4) were analyzed by multivariate ANOVA (MANOVA). qPCR data were analyzed by one-way ANOVA followed by LSD post hoc test. All tests were two-tailed and a significance level of 0.05 was used throughout. Statistical tests were run using SYSTAT (version 13.1; Systat Software, San Jose, CA, USA).

Results

Blood gases and acid–base status

NaCl infusion had no effect on pH_a , $[\text{HCO}_3^-]_a$, PaCO_2 , or PaO_2 over the 24-h period (Fig. 1a). However, HCl infusion caused significant decreases in pH_a at 2 and 6 h, and significant decreases in plasma $[\text{HCO}_3^-]_a$ at 12 and 24 h (Fig. 1b). PaO_2 and PaCO_2 did not change, though the latter became highly variable by 24 h. The acid–base responses

Table 2 Mean plasma concentrations of urea-N, Cl^- , Na^+ , Ca^{++} and Mg^{++} (mmol l^{-1}) in *Squalus acanthias* during 0, 2, 6, 12, and 24 h of infusion of saline (NaCl), HCl, NH_4HCO_3 , or NH_4Cl

	0 h	2 h	6 h	12 h	24 h
Saline					
Urea-N	687.32 ± 14.04	677.18 ± 2.48	668.08 ± 25.34	652.50 ± 38.56	650.90 ± 31.76
Cl^-	285.25 ± 7.07	294.00 ± 10.44	309.00 ± 11.44*	307.33 ± 14.53*	310.29 ± 12.06*
Na^+	289.46 ± 5.42	288.87 ± 6.52	303.96 ± 13.34	311.25 ± 13.86	310.08 ± 11.23
Ca^{++}	4.74 ± 0.15	4.43 ± 0.12	4.32 ± 0.24	4.38 ± 0.15	4.18 ± 0.16
Mg^{++}	2.42 ± 0.40	2.04 ± 0.33	2.06 ± 0.36	2.08 ± 0.45	2.29 ± 0.30
HCl					
Urea-N	681.08 ± 20.16	680.36 ± 19.26	669.12 ± 23.88	657.76 ± 15.58	657.96 ± 10.12*
Cl^-	292.67 ± 6.14	302.11 ± 9.38	314.22 ± 8.27*	310.75 ± 5.05*	310.17 ± 5.50*
Na^+	313.59 ± 9.95	302.83 ± 10.89	322.37 ± 10.06	313.27 ± 7.77	308.3 ± 7.66
Ca^{++}	4.72 ± 0.28	4.76 ± 0.43	4.49 ± 0.29	4.40 ± 0.40	4.54 ± 0.41
Mg^{++}	2.65 ± 0.66	2.69 ± 0.81	2.45 ± 0.67	1.98 ± 0.24	1.73 ± 0.24
NH_4HCO_3					
Urea-N	698.62 ± 13.22	706.80 ± 15.34	706.86 ± 26.8	694.50 ± 22.40	671.54 ± 15.48
Cl^-	287.00 ± 7.72	282.63 ± 10.19	288.71 ± 10.02	288.75 ± 8.53	276.17 ± 7.42
Na^+	295.94 ± 8.28	302.54 ± 14.24	304.76 ± 9.94	305.76 ± 9.73	290.96 ± 8.04
Ca^{++}	4.93 ± 0.23	4.60 ± 0.22*	4.70 ± 0.18*	4.58 ± 0.14*	4.43 ± 0.15*
Mg^{++}	1.77 ± 0.22	1.86 ± 0.25	2.06 ± 0.22	2.27 ± 0.31*	2.21 ± 0.17*
NH_4Cl					
Urea-N	764.22 ± 15.60	766.36 ± 17.98	750.50 ± 17.80	735.74 ± 16.16*	729.38 ± 35.72*
Cl^-	264.08 ± 3.03	279.67 ± 3.58*	288.83 ± 3.66*	286.50 ± 4.64*	294.00 ± 7.03*
Na^+	290.25 ± 5.05	287.83 ± 4.33	296.48 ± 4.90	293.47 ± 4.31	292.99 ± 6.63
Ca^{++}	4.31 ± 0.29	4.04 ± 0.11	4.19 ± 0.11	4.20 ± 0.13	4.26 ± 0.12
Mg^{++}	1.73 ± 0.23	1.51 ± 0.15	1.64 ± 0.15	1.58 ± 0.17	1.79 ± 0.30

Data are mean ± SEM, $n = 6-12$. Asterisks indicate significant difference from the corresponding value at 0 h

Fig. 3 Net rates of ammonia flux (J_{Amm}) in *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c** NH_4HCO_3 , or **d** NH_4Cl for 24 h. “INF” represents the period of infusion. Asterisks indicate a significant difference from the pre-infusion control levels. Dagger indicates that the total efflux rate over 24 h in the NH_4HCO_3 group is significantly greater than that in the NH_4Cl group ($P < 0.05$). Data are mean ± SEM, $n = 5-12$

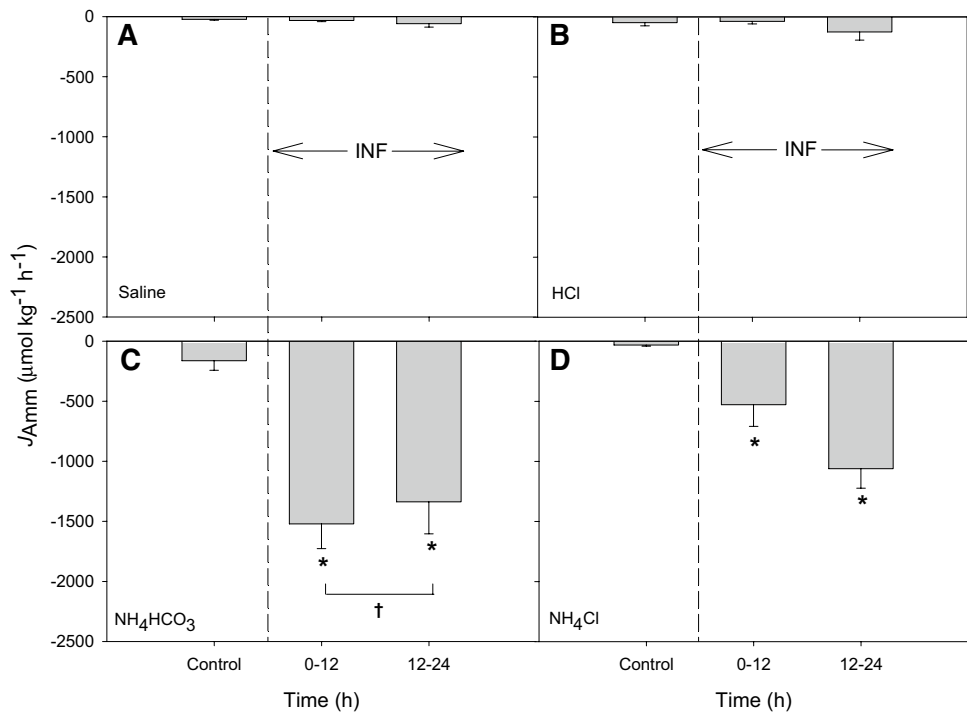
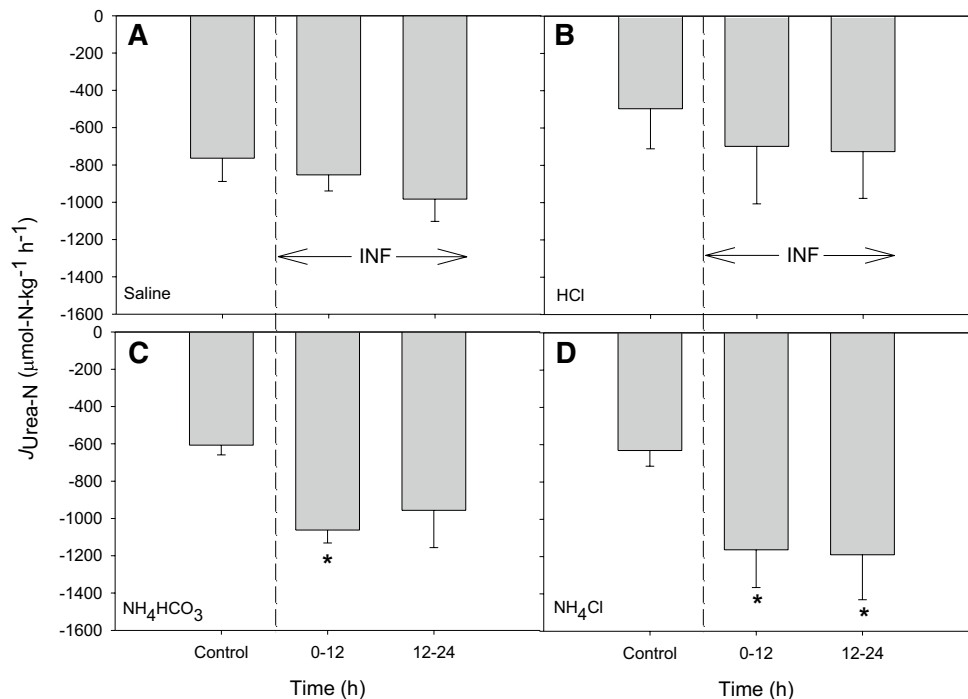


Fig. 4 Net rates of urea-N flux ($J_{\text{Urea-N}}$) in *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c** NH_4HCO_3 , or **d** NH_4Cl for 24 h. “INF” represents the period of infusion. Asterisks indicate a significant difference ($P < 0.05$) from the pre-infusion control levels. Data are mean \pm SEM, $n = 4$ –9



to NH_4HCO_3 versus NH_4Cl infusions were very different. NH_4HCO_3 infusion caused significant increases in plasma $[\text{HCO}_3^-]_a$ by 12 h and an almost doubling by 24 h; small increases in pHa and PaCO_2 were not significant, but PaO_2 was significantly depressed by about 20 % at all time points (Fig. 1c). In contrast, NH_4Cl infusion caused significant 50–70 % losses of plasma $[\text{HCO}_3^-]_a$ at all time points, accompanied by significant decreases in pHa of 0.5–0.6 units, and increases in PaCO_2 which approximately doubled from 6 h onwards (Fig. 1d). Decreases in PaO_2 were comparable to those with NH_4HCO_3 infusion but were not significant due to variability.

Plasma ammonia, urea-N, and ions

Infusion with NaCl or HCl had no effect on any of the plasma ammonia parameters (Fig. 2a, b). However, there were marked, rather different responses to NH_4HCO_3 versus NH_4Cl infusions. The ammonia loading rates with these two treatments were the same, but the resulting increases in plasma T_{Amm} , which were significant in both by 2 h, were approximately twice as large in the NH_4Cl treatment by 24 h (Fig. 2c, d). Despite this, the accompanying significant increases in PaNH_3 , and therefore in the ΔPNH_3 gradient across the gills, were substantially higher in the sharks infused with NH_4HCO_3 . As a result of the Henderson–Hasselbalch relationship, these PNH_3 differences reflected the large differences in pHa response between the two treatments (Fig. 1c, d).

Plasma $T_{\text{Urea-N}}$ tended to decrease in all infusion treatments, probably due to dilution, but the effects were only

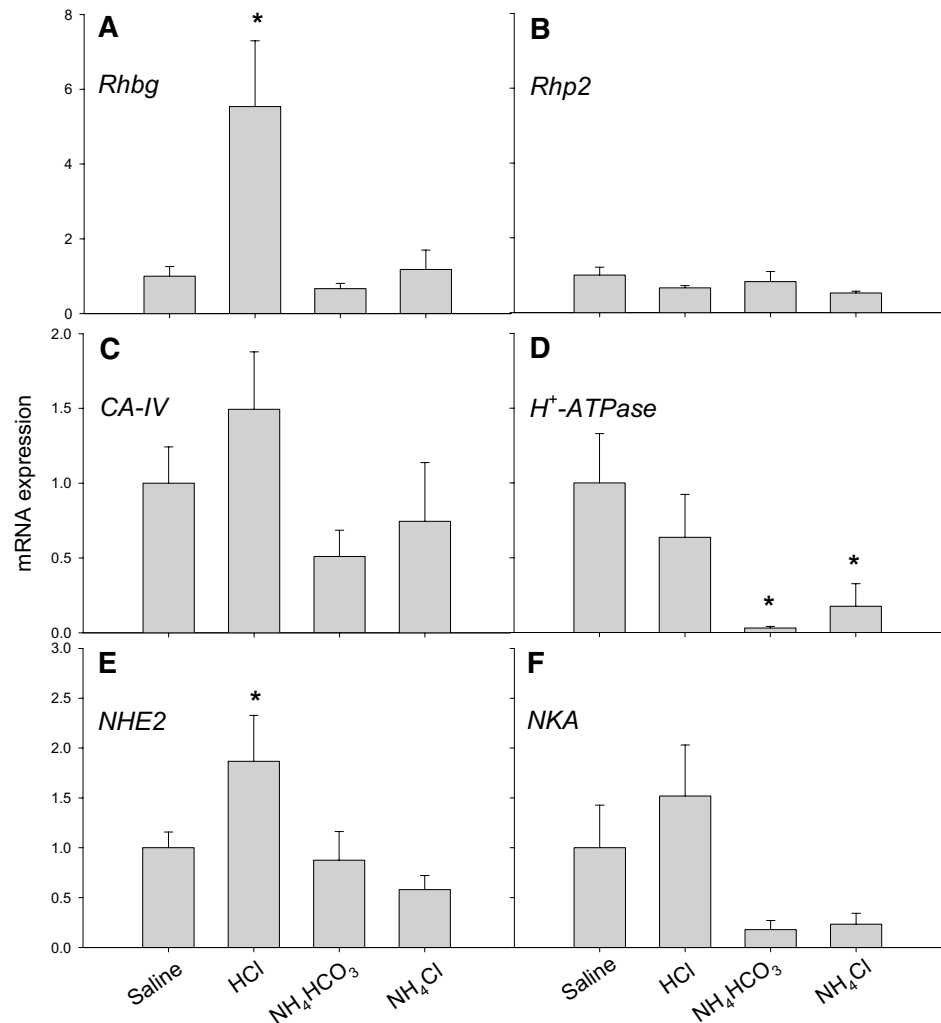
significant at 12 or 24 h in the HCl and NH_4Cl infusions (Table 2). Plasma Cl^- increased from 6 h onwards only in the NaCl and HCl treatments, reflecting the Cl^- loading (Table 2). NH_4HCO_3 infusion caused significant decreases in plasma Ca^{2+} from 2 h onwards and significant increases in plasma Mg^{2+} at 12 and 24 h. None of the other treatments significantly altered these divalent ion levels (Table 2).

Ammonia and urea-N flux rates

Prior to infusion, all groups excreted ammonia at low rates ($<200 \mu\text{mol kg}^{-1} \text{h}^{-1}$; Fig. 3) and urea-N at much higher rates (500 – $800 \mu\text{mol kg}^{-1} \text{h}^{-1}$; Fig. 4). These rates were unaffected by 24 h of infusion with either NaCl or HCl. However, NH_4HCO_3 versus NH_4Cl infusions caused qualitatively similar but quantitatively different responses in these parameters. J_{Amm} increased greatly in the two treatments at both 0–12 h and 12–24 h, but the elevation was 2-fold greater in the NH_4HCO_3 -infused fish despite the identical ammonia loading rates (Fig. 3c, d). Nevertheless, similar elevations in $J_{\text{Urea-N}}$ occurred in the two groups during both time periods (Fig. 4c, d), despite the differences in ammonia retention (Fig. 3c, d) and plasma ammonia parameters (Fig. 2c, d).

There was reasonable agreement between the amount of ammonia-N infused and the extra ammonia-N and urea-N excreted. Overall, in the NH_4HCO_3 infused fish, the elevation in total N-excretion relative to baseline control rates over 24 h equalled 113 % of the amount of

Fig. 5 mRNA expression levels in the gills of *Squalus acanthias* after 24 h of infusion with saline (NaCl), HCl, NH_4HCO_3 , or NH_4Cl . **a** *Rhbg*, **b** *Rhp2*, **c** *CA-IV*, **d** H^+ -ATPase, **e** *NHE2* and **f** *NKA*. All saline means were set to 1 in order to facilitate comparisons with the other treatments. Asterisks indicate a significant difference ($P < 0.05$) from the pre-infusion control levels. Data are mean \pm SEM, $n = 6$



ammonia-N infused, of which J_{Amm} represented 86 % and $J_{\text{Urea-N}}$ accounted for 27 %. In the NH_4Cl -infused fish, the elevation equalled 87 % of the amount infused, of which J_{Amm} represented 51 % and $J_{\text{Urea-N}}$ accounted for 36 %.

Molecular responses

Gills

The branchial mRNA expression levels of both *Rhbg* (Fig. 5a) and *Rhp2* (Fig. 5b) did not respond to either NH_4HCO_3 or NH_4Cl infusions. However, HCl infusion caused a significant 5.5-fold up-regulation of *Rhbg* (Fig. 5a) but had no effect on *Rhp2* expression (Fig. 5b). *CA-IV* did not respond significantly to any of the treatments (Fig. 5c), but there was a marked (>80 %) down-regulation of gill H^+ -ATPase mRNA levels in response to internal ammonia loading with either NH_4HCO_3 or NH_4Cl (Fig. 5d). *NHE2* (Fig. 5e) responded in a similar pattern to *Rhbg* (Fig. 5a) with an approximate doubling of expression in the HCl treatment but no response in the two ammonia

treatments. *NKA* expression levels were variable, with no significant response to any of the infusions (Fig. 5f).

Kidney

The renal mRNA expression levels of *Rhbg* did not respond to any of the treatments (Fig. 6a), but there was a significant up-regulation of *Rhp2* expression (~3-fold) in response to HCl infusion but no change with NH_4Cl or NH_4HCO_3 infusion (Fig. 6b). *UT* expression increased greatly (4-fold to 7-fold) in response to both HCl and NH_4Cl loading but did not change in response to NH_4HCO_3 infusion (Fig. 6c).

Discussion

Overview

The present experiments were primarily motivated by our recent observations on the responses to high environmental ammonia exposure in *Squalus acanthias* (Nawata et al.

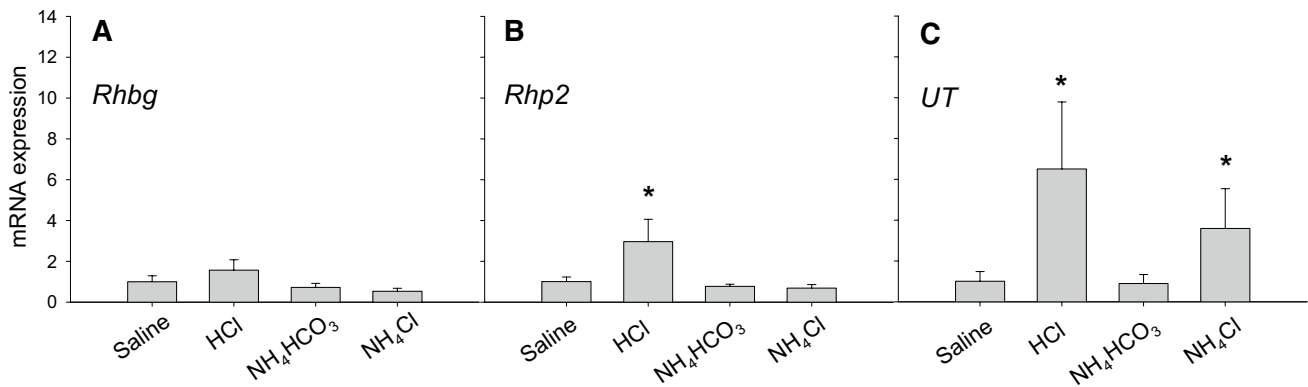


Fig. 6 mRNA expression levels in the kidney of *Squalus acanthias* after 24 h of infusion with saline (NaCl), HCl, NH₄HCO₃, or NH₄Cl. **a** *Rhbq*, **b** *Rhp2*, and **c** *UT*. All saline means were set to 1 in order to

facilitate comparisons with the other treatments. Asterisks indicate a significant difference ($P < 0.05$) from the pre-infusion control levels. Data are mean \pm SEM, $n = 6$

2015), and provide clear answers to some of the questions raised by that study (see “Introduction”). Firstly, the down-regulation of the branchial mRNA expression of *Rhbq* seen during HEA does not appear to be a response to internal ammonia parameters. On the other hand, the simultaneous down-regulation of branchial *H⁺-ATPase* expression may well be a response to an internal ammonia signal. Secondly, while there was no evidence that an acid–base signal plays a role in the responses to ammonia, there was an interaction between acid–base status and ammonia in certain molecular responses, inasmuch as *NHE2* and *Rhbq* expression were up-regulated by metabolic acidosis alone (HCl infusion), but not when acidosis was combined with ammonia loading (NH₄Cl infusion). There was also an interaction in physiological responses, in that ammonia excretion was much greater when the ammonia loading caused alkalosis than when it caused acidosis. Thirdly, ammonia infusion resulted in a marked elevation of urea-N excretion similar to that seen during HEA exposure, probably reflecting increased urea-N synthesis by the ornithine-urea cycle (OUC). This occurred regardless of the anion accompanying NH₄⁺ and therefore the resulting acid–base status, providing strong evidence that urea-N synthesis is driven primarily by the availability of ammonia rather than that of HCO₃⁻ in this elasmobranch. Finally, with respect to the function of *Rhp2*, branchial mRNA expression of this “primitive” Rhesus glycoprotein appears to be completely refractory to ammonia and acid–base disturbances as well as to HEA. However, in the kidney, its expression responded to acid–base status, suggesting a possible function (ammonia secretion) alternate or additional to that (ammonia reabsorption) proposed by Nakada et al. (2010).

Blood gases and acid–base status

As expected, HCl infusion caused a classic metabolic acidosis (decreased pH_a and (HCO₃⁻)_a, non-significant rise

in PaCO₂; Fig. 1b) in accord with previous HCl injection or infusion studies in *Squalus acanthias* (Murdaugh and Robin 1967; King and Goldstein 1983; Tresguerres et al. 2005; Wood et al. 2007c). Notably, the acidosis was less intense than with NH₄Cl infusion, but the rate of acid loading by infusion of acidic equivalents (125 mmol l⁻¹ HCl versus 500 mmol l⁻¹ NH₄Cl) was only 25 % as great, which likely explains the more moderate acid–base disturbance. Preliminary experiments with higher HCl loading rates proved fatal, as also reported by Tresguerres et al. (2005). The acid–base response to NH₄Cl infusion (large decreases in [HCO₃⁻]_a and pH_a; Fig. 1d) were also indicative of a primary metabolic acidosis. These changes were similar to those reported in a previous study on *Squalus acanthias* (Wood et al. 1995) where the same NH₄Cl loading rate was maintained for only 6 h rather than 24 h. However, in the present study, there was also a respiratory component to the acidosis (increased PaCO₂) not seen by Wood et al. (1995). In teleost fish, infusion or injection of NH₄⁺ coupled with a non-metabolizable anion (e.g. Cl⁻, SO₄²⁻) generally causes mild metabolic acidosis (Cameron and Kormanik 1982; Cameron and Heisler 1983; McDonald and Prior 1988; Milligan et al. 1991; Wilson et al. 1994; Zhang and Wood, 2009), usually attributed to NH₃ loss across the gills leaving behind a strong mineral acid (e.g. HCl, H₂SO₄). In the elasmobranch, this may be part of the explanation, but the additional removal of NH₃ by stimulated ureagenesis (see below) is probably responsible for the more intense acidosis. As intended, the blood acid–base responses to NH₄HCO₃ infusion comprised a mild metabolic alkalosis with slight respiratory compensation (significant increases in [HCO₃⁻]_a, non-significant increases in pH_a and PaCO₂; Fig. 1c), and were very different from those with NH₄Cl (Fig. 1d). We are aware of no previous NH₄HCO₃ infusion studies in elasmobranchs, but the responses were similar to those seen in comparable investigations on teleosts

(Claiborne and Evans 1988; Salama et al. 1999; Zhang and Wood 2009). They were also very similar to those seen when sharks were exposed to elevated NH_4HCO_3 in the environment (i.e., HEA; Nawata et al. 2015). The alkalosis must reflect the fact that at least a portion of the ammonia load from NH_4HCO_3 is removed as NH_4^+ . Removal of NH_3 alone would leave behind H^+ and HCO_3^- , forming CO_2 , and would therefore be neutral in terms of its effect on metabolic acid–base status.

Both forms of ammonia infusion tended to lower PaO_2 (Fig. 1c, d), and a similar trend was seen with HEA exposure (Nawata et al. 2015). This is probably not due to ventilatory inhibition, because internal ammonia loading stimulates ventilation in *Squalus acanthias* (Zhang et al. 2014; De Boeck and Wood 2015). Therefore, ammonia may acutely decrease the diffusive conductance of the gill epithelium and/or increase hemoglobin O_2 affinity. The latter could occur simply by an effect of NH_3 in raising the intracellular pH in the red cell, by interactions of either NH_3 or NH_4^+ with heme or the globin chains, or by more complex secondary changes in the intracellular milieu. All of these are interesting topics for future research.

Plasma ammonia, urea-N, and ions

Changes in plasma urea-N concentrations associated with the various infusions were unremarkable, probably mainly due to dilution (Table 2), but there were large differences in plasma ammonia parameters. Despite the same ammonia loading rates, NH_4Cl infusion caused an increase in plasma T_{Amm} that was 2-fold greater than with NH_4HCO_3 infusion (Fig. 2c, d). As elevations in urea-N excretion were similar in the two treatments (Fig. 4c, d), this was not due to differential removal of ammonia by ureagenesis (see below), but rather to a 2-fold greater elevation in ammonia excretion rates (Fig. 3). This higher J_{Amm} was probably due to the much greater increases in PaNH_3 and therefore in the outwardly directed ΔPNH_3 gradient across the gills in the sharks infused with NH_4HCO_3 (Fig. 2c, d), differences attributable to the much higher pH_a in this treatment (Fig. 1c, d). This would cause greater diffusive efflux of NH_3 , but it is unknown whether there was also greater diffusive efflux of NH_4^+ because transepithelial potential (TEP), an important contributor to the net driving force on NH_4^+ (cf. Nawata et al. 2015) was not measured in these experiments.

Changes in plasma ions largely reflected the addition or dilution effects of the infusions (Na^+ , Cl^- , Ca^{2+}), but the increase in plasma Mg^{2+} concentration, which occurred only with NH_4HCO_3 infusion, was remarkable (Table 2). Interestingly, a similar increase was seen when dogfish were exposed to HEA in the form of NH_4HCO_3 added to the external medium (Nawata et al. 2015). The explanation

for this consistent and unexpected effect is not obvious but an interesting topic for future investigation.

Ammonia and urea-N flux rates

HCl infusion had no effect on J_{Amm} (Fig. 3b). Therefore, metabolic acidosis was not accompanied by an increase in branchial ammonia excretion, in contrast to previous reports on respiratory acidosis (hypercapnia) in this same species where elevated J_{Amm} , presumably as $\text{Na}^+/\text{NH}_4^+$ exchange (Payan and Maetz 1973), seemed to play a role in acid–base compensation (Evans 1982; Claiborne and Evans 1988), though see Evans and More (1988) for opposing evidence. However, there were substantial increases in both J_{Amm} (Fig. 3c, d) and $J_{\text{Urea-N}}$ (Fig. 4c, d) with infusions of either NH_4Cl or NH_4HCO_3 . Clearly the complex retention mechanisms which normally serve to minimize losses of ammonia (Wood et al. 1995; Nawata et al. 2015) and urea-N (Boylan, 1967; Wood et al. 1995; Pärt et al. 1998; Fines et al. 2001; Wood et al. 2013a) at the gills were overwhelmed. Similar large increases in J_{Amm} and $J_{\text{Urea-N}}$ were recorded by Wood et al. (1995) in dogfish infused with NH_4Cl for only 6 h. As noted above, the greater elevations in J_{Amm} and ΔPNH_3 with NH_4HCO_3 versus NH_4Cl point to an important role for NH_3 efflux in this response. Using a perfused head preparation of *Squalus acanthias* ‘pups’, Evans and More (1988) concluded that NH_3 efflux was much more important than NH_4^+ efflux when internal T_{Amm} levels were elevated.

As plasma $T_{\text{Urea-N}}$ levels were not differentially affected by either NH_4Cl or NH_4HCO_3 relative to saline infusion (Table 2), we interpret the observed increases in $J_{\text{Urea-N}}$ (Fig. 4c, d) as increases in urea-N production rates by the OUC. The fact that these increases were very similar with the two different NH_4^+ salts indicates that urea-N synthesis is driven much more by ammonia availability than by HCO_3^- availability and indeed is relatively insensitive to acid–base status. This is reinforced by the fact that HCl-induced acidosis also did not inhibit baseline $J_{\text{Urea-N}}$ (Fig. 4b). These findings oppose the ideas of Atkinson and colleagues (Atkinson and Bourke 1984; Atkinson 1992) who earlier argued that urea synthesis in vertebrates (including elasmobranchs) is geared primarily to the needs of acid–base regulation (i.e., metabolic removal of HCO_3^-) rather than to the needs of N-homeostasis. However, they are in accord with the conclusions of Wood et al. (1995) who compared the responses to NH_4Cl versus NaHCO_3 infusion in dogfish, and with Wood et al. (2008) who assayed the effect of NaHCO_3 infusion on OUC enzymes. Indeed, by the direct comparison of two NH_4^+ salts, the current results provide more convincing evidence that the Atkinson hypothesis is not applicable to the operation of the OUC in elasmobranchs.

HEA exposure also caused elevated $J_{\text{Urea-N}}$ in *Squalus acanthias* (Nawata et al. 2015), as well as in two ureotelic teleosts, the Magadi tilapia (Wood et al. 1989, 2013b), and the gulf toadfish (Walsh et al. 1990). In all cases, this was similarly interpreted as an increase in urea-N production by the OUC, but for the teleosts, ureagenesis may serve primarily for ammonia detoxification whereas in the elasmobranch, it may be part of an overall nitrogen scavenging and conservation strategy (Wood et al. 2005; Kajimura et al. 2006; Nawata et al. 2015). The uptake of ammonia from the medium by *Squalus acanthias* during HEA exposure appears to be active, occurring against prevailing P_{NH_3} and electrochemical gradients (Nawata et al. 2015), suggesting the acquisition of a valuable resource in these nitrogen-limited predators (see “Introduction”).

The increased production of urea-N in response to ammonia loading could simply reflect increased supply of substrate to the OUC, without elevation of activities of key enzymes, or additionally it could reflect increased activities of these enzymes. There is some precedent for the latter inasmuch as feeding, which is also a form of N-substrate supply, markedly stimulated the activities of key OUC enzymes in the liver and muscle of *Squalus acanthias* (Kajimura et al. (2006), and HEA exposure had a similar effect in whole bodies of early life stages of the ureotelic gulf toadfish (Barimo et al. 2004).

Molecular responses in the gills

During HEA exposure, the branchial mRNA expression of *Rhbg* was down-regulated (Nawata et al. 2015), but in the present study, this did not occur with infusions of either NH_4Cl or NH_4HCO_3 (Fig. 5a), both of which increased plasma T_{Amm} and P_{NH_3} to levels equal to or higher (Fig. 2c, d) than those seen with HEA. Thus, *Rhbg* down-regulation appears to be triggered by elevations in external rather than internal ammonia. Rather than it being a response to retain ammonia, as for example might occur after feeding, it is more probably a response to prevent the homeostatic systems being overwhelmed in the face of increased ammonia scavenging during HEA (Nawata et al. 2015). A comparable down-regulation of this basolateral ammonia transporter was seen in the marine pufferfish exposed to HEA and was similarly interpreted as a mechanism to reduce the permeability of the gills to further ammonia influx (Nawata et al. 2010a).

On the other hand, the decrease in branchial H^+ -ATPase expression seen during HEA exposure (Nawata et al. 2015) may well be a response to an internal ammonia signal because it occurred with both types of ammonia infusions (Fig. 5d). Notably, it occurred irrespective of very different acid–base status in the three treatments, so it does not appear to be a response to acid–base disturbance (Fig. 1c,

d). In elasmobranchs, branchial H^+ -ATPase is restricted to specialized base-excreting ionocytes (Piermarini and Evans, 2001; Piermarini et al. 2002; Tresguerres et al. 2005; Reilly et al. 2011). In contrast to teleosts where the enzyme functions apically to excrete H^+ ions to the external water, in elasmobranchs, it functions basolaterally to return H^+ ions to the extracellular fluid when HCO_3^- is excreted apically at times of blood alkalosis (Tresguerres et al. 2005, 2006, 2007; Roa et al. 2014). In the present study, it responded to high internal ammonia regardless of alkalosis or acidosis, suggesting that H^+ -ATPase may have an additional role that of modulating the NH_3 concentration at the extracellular side of the basolateral membrane, and therefore the P_{NH_3} gradient across the *Rhbg* channel. In future studies, it will be of interest to see if H^+ -ATPase and *Rhbg* are co-localized in the base-excreting ionocytes.

In elasmobranchs, NHE2 not only appears to be apically located in the other type of specialized ionocyte (acid-excreting, distinguished by basolateral Na^+ , K^+ -ATPase; Edwards et al. 2002; Claiborne et al. 2008), but also occurs in the base-excreting ionocytes (distinguished by basolateral H^+ -ATPase; Claiborne et al. 2008), so it may have multiple roles. Indeed another isoform (NHE3) appears to be exclusive to the acid-excreting ionocytes (Choe et al. 2005). Up-regulation of *NHE2* mRNA expression during metabolic acidosis caused by HCl infusion is perhaps not surprising, and Tresguerres et al. (2005) reported increased branchial NHE2 protein levels (detected by heterologous antibody) in a similar HCl infusion experiment in *Squalus acanthias*. Curiously, however, this *NHE2* up-regulation was not seen in another HCl infusion experiment on the same species by Claiborne et al. (2008) using either qPCR for mRNA or a homologous antibody for the protein; they proposed that reciprocal up-regulation and down-regulation in the two cell types might have resulted in no overall changes. In the present experiments, it is also curious that a more intense metabolic acidosis caused by NH_4Cl infusion (Fig. 1d versus b) did not up-regulate *NHE2* expression (Fig. 5e). The mRNA expression of *Rhbg* exhibited the same differential response to HCl versus NH_4Cl infusion (Fig. 5a). We speculate that this interaction between acid–base status and ammonia occurs due to effects on the base-excreting ionocytes where H^+ -ATPase was down-regulated by internal ammonia loading (Fig. 5d).

Renal responses and the role of *Rhp2*

The largest changes in mRNA expression in the kidney were 4-fold to 7-fold increases in *UT* in response to both HCl and NH_4Cl infusions but no change in response to NH_4HCO_3 loading (Fig. 6c). Interestingly, a similar up-regulation of kidney *UT* occurred in dogfish exposed to HEA (Nawata et al. 2015). Both HEA exposure and NH_4Cl

infusion caused large increases in whole animal urea-N excretion in *Squalus acanthias*, and in the latter treatment, about 20 % of the increased excretion was via the kidney (Wood et al. 1995). It is possible that increased *UT* expression helped to facilitate this increased renal urea-N excretion, though it is difficult to see why HCl infusion, which had no effect on urea-N excretion (Fig. 4b), would have a similar effect. However, it may be that acidic conditions stimulate transcription. The mRNA and protein of UT-B, a related mammalian urea transporter, are both up-regulated by acid pH in goat rumen epithelial cells (Lu et al. 2014), and UT-A protein is up-regulated in the liver and kidney of rats fed HCl (Klein et al. 2002).

As with HEA exposure (Nawata et al. 2015), there was no renal response of *Rhbg* or *Rhp2* expression to either NH_4Cl or NH_4HCO_3 infusions (Fig. 6a, b). It is known, however, that renal ammonia excretion increases markedly in sharks infused with NH_4Cl , though this remains quantitatively negligible relative to branchial ammonia excretion (Wood et al. 1995). Possibly, increased urinary ammonia excretion results from increased filtration, reflecting increased plasma T_{Amm} , rather than from changes in tubular secretion. *Rhp2* expression also did not respond in the gills to any treatments (Fig. 5b). *Rhp2* was, however, significantly up-regulated in the kidney by HCl infusion (Fig. 6b), a treatment that is known to elicit increased urinary ammonia excretion in *Squalus acanthias* (King and Goldstein 1983). These authors speculated that the response was due to increased renal ammoniogenesis and resulting ammonia secretion during metabolic acidosis. Yet *Rhp2* did not respond when acidosis was coupled to an internal ammonia load (NH_4Cl infusion; Fig. 6b). In future studies, measurements of glutamate dehydrogenase activity in the kidney, a key enzyme in renal ammonia production, would be helpful here in clarifying whether ammonia secretion was activated. As outlined in “Introduction”, the salinity-dependent *Rhp2* responses reported by Nakada et al. (2010) in the houndshark could alternatively be interpreted as responses to metabolic acidosis and alkalosis. Thus, in the kidney, *Rhp2* may in some way facilitate ammonia secretion during acidosis rather than the ammonia reabsorption proposed by Nakada et al. (2010). Clearly, its function remains elusive, and more work is needed on this primitive glycoprotein, especially coupled to functional measurements of urine flow rate, urine pH, and the rates of glomerular filtration and tubular secretion of ammonia.

Implication and future directions

The present results, together with those of Nawata et al. (2015), Nakada et al. (2010), and Anderson et al. (2010, 2012), illustrate that Rh proteins and the ammonia fluxes, which they likely mediate, probably play critical roles in the

overall N metabolism of elasmobranchs. Current views of this topic tend to focus almost exclusively on urea metabolism, while the present information suggests that a more balanced view considering the importance of both ammonia and urea handling, and particularly their interactions, would be more appropriate. As opportunistic predators with an uncertain food supply, elasmobranchs are probably N-limited in the wild, yet urea-N synthesis is critical for their osmoregulatory homeostasis. Ureagenesis is clearly driven by the availability of ammonia, and the ability of elasmobranchs to store and retain urea-N is somewhat limited. Therefore, continual provision of ammonia, whether obtained from amino acids in the diet, from breakdown of structural protein during fasting, or by scavenging from the external medium, must be carefully matched to urea-N loss rates.

Furthermore, there is evidence that Rh proteins can also facilitate CO_2 transport in a variety of organisms (e.g. Soupene et al. 2004; Kustu and Inwood 2006; Endeward et al. 2006), including teleost fish (Nawata and Wood 2008; Nawata et al. 2010a; Perry et al. 2010). Future analyses of Rh protein function in elasmobranchs should keep this important possibility in mind.

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