

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

**C. Michele Nawata, Patrick J. Walsh &  
Chris M. Wood**

**Journal of Comparative Physiology B**  
Biochemical, Systems, and  
Environmental Physiology

ISSN 0174-1578  
Volume 185  
Number 5

J Comp Physiol B (2015) 185:511-525  
DOI 10.1007/s00360-015-0898-4



**Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at [link.springer.com](http://link.springer.com)".**

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

C. Michele Nawata<sup>1,2,3</sup> · Patrick J. Walsh<sup>1,4</sup> · Chris M. Wood<sup>1,2,5</sup>

Received: 23 November 2014 / Revised: 18 January 2015 / Accepted: 10 March 2015 / Published online: 21 March 2015  
 © Springer-Verlag Berlin Heidelberg 2015

**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<sup>-1</sup> h<sup>-1</sup> with isosmotic NaCl (500 mmol l<sup>-1</sup>, control), NH<sub>4</sub>HCO<sub>3</sub> (500 mmol l<sup>-1</sup>), NH<sub>4</sub>Cl (500 mmol l<sup>-1</sup>), or HCl (as 125 mmol l<sup>-1</sup> HCl + 375 mmol l<sup>-1</sup> NaCl). While NaCl had no effect on arterial acid–base status, NH<sub>4</sub>HCO<sub>3</sub> caused mild alkalosis, NH<sub>4</sub>Cl caused strong acidosis, and HCl caused lesser acidosis, all predominantly metabolic in nature. Total plasma ammonia (T<sub>Amm</sub>) and excretion rates of ammonia (J<sub>Amm</sub>) and urea-N (J<sub>Urea-N</sub>) were unaffected by NaCl or HCl. However, despite equal loading rates, plasma T<sub>Amm</sub> increased to a greater extent with NH<sub>4</sub>Cl, while J<sub>Amm</sub> increased to a greater extent with NH<sub>4</sub>HCO<sub>3</sub> due to much greater increases in blood-to-water PNH<sub>3</sub> gradients. As with HEA, both treatments caused large (90 %) elevations

of J<sub>Urea-N</sub>, indicating that urea-N synthesis by the ornithine-urea cycle (OUC) is driven primarily by ammonia rather than HCO<sub>3</sub><sup>-</sup>. Branchial mRNA expressions of *Rhbg* and *Rhp2* were unaffected by NH<sub>4</sub>HCO<sub>3</sub> or NH<sub>4</sub>Cl, but *v-type H<sup>+</sup>-ATPase* was down-regulated by both treatments, and *Rhbg* and Na<sup>+</sup>/H<sup>+</sup> 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 NH<sub>4</sub>Cl. 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 · NH<sub>4</sub>Cl · NH<sub>4</sub>HCO<sub>3</sub> · 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 HCO<sub>3</sub><sup>-</sup> 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

Communicated by G. Heldmaier.

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

- <sup>1</sup> Bamfield Marine Sciences Centre, 100 Pachena Road, Bamfield, BC V0R 1B0, Canada
- <sup>2</sup> Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON L8S 4K1, Canada
- <sup>3</sup> Department of Physiology, University of Arizona, 1501 N. Campbell Ave., Tucson, AZ 85724, USA
- <sup>4</sup> Department of Biology and Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON K1N 6N5, Canada
- <sup>5</sup> 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 [ $\text{HCO}_3^-$ ] 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  $\text{NH}_4\text{HCO}_3$ , which raises plasma [ $\text{HCO}_3^-$ ], and as  $\text{NH}_4\text{Cl}$ , which lowers plasma [ $\text{HCO}_3^-$ ]. 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  $\text{HCO}_3^-$ ) 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 ( $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, *v*-type  $\text{H}^+$ -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  $\text{Cl}^-$  rises more than plasma  $\text{Na}^+$  (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  $\text{NH}_4\text{Cl}$  infusion) or not coupled (by  $\text{NH}_4\text{HCO}_3$  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 ( $30 \pm 2$  ‰), pH ( $7.90 \pm 0.15$ ), 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  $1 \text{ l min}^{-1}$  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.  $\text{ml}^{-1}$  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 ( $0.15 \text{ g l}^{-1}$ ; 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.  $\text{ml}^{-1}$  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)  $500 \text{ mmol l}^{-1}$  NaCl (control), (ii)  $125 \text{ mmol l}^{-1}$  HCl +  $375 \text{ mmol l}^{-1}$  NaCl, (iii)  $500 \text{ mmol l}^{-1}$   $\text{NH}_4\text{HCO}_3$ , and (iv)  $500 \text{ mmol l}^{-1}$   $\text{NH}_4\text{Cl}$ , 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  $\mu\text{l}$ ) 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 ( $\text{PaO}_2$ ). 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  $\text{CO}_2$  concentration; the other two were flash frozen in liquid  $\text{N}_2$  and then stored at  $-80$  °C for subsequent analyses of (i) plasma total ammonia ( $T_{\text{Amm}}$ ) 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/aatgaaatcaggacggttgc	KJ960196
Rhbg	gctcatctgcctctgttg/tgggatacctgaagtagaactcg	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 N<sub>2</sub>, taking care to avoid contamination with white blood cells. The fish was then anesthetized (0.15 g l<sup>-1</sup> 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<sup>-1</sup> NaCl containing 10 i.u. ml<sup>-1</sup> 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<sup>-1</sup>, and a stroke volume scaled to the body weight such the output was approximately 20 ml kg<sup>-1</sup> min<sup>-1</sup>. 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<sub>Amm</sub> 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<sub>Amm</sub> 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 (PNH<sub>3</sub>) and ammonium ion concentrations ([NH<sub>4</sub><sup>+</sup>]) were calculated from the respective T<sub>Amm</sub> 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<sub>NH3</sub>, pK<sub>amm</sub>) were taken from the study of Cameron and Heisler (1983) on trout, using an ionic strength equivalent to 500 mmol l<sup>-1</sup> NaCl and their adjustments for plasma. The PNH<sub>3</sub> gradient across the gills (ΔPNH<sub>3</sub>) was calculated as the difference between simultaneous measurements of water PNH<sub>3</sub> and arterial blood plasma PaNH<sub>3</sub>.

Arterial blood oxygen tensions (PaO<sub>2</sub>), pHa, and water pH were measured using Radiometer electrodes (Copenhagen, Denmark; E5036 for PO<sub>2</sub>, 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 CO<sub>2</sub> concentrations. Arterial blood carbon dioxide tensions (PaCO<sub>2</sub>) and bicarbonate concentrations ([HCO<sub>3</sub><sup>-</sup>]<sub>a</sub>) were calculated using rearrangements of the Henderson–Hasselbalch equation and coefficients for the solubility of carbon dioxide (α<sub>CO2</sub>) and the apparent pK (pK<sub>app</sub>) in dogfish plasma from Boutilier et al. (1984).

Urea-N concentrations (T<sub>Urea-N</sub>) 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<sub>Amm</sub>) and urea-N (J<sub>Urea-N</sub>) 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

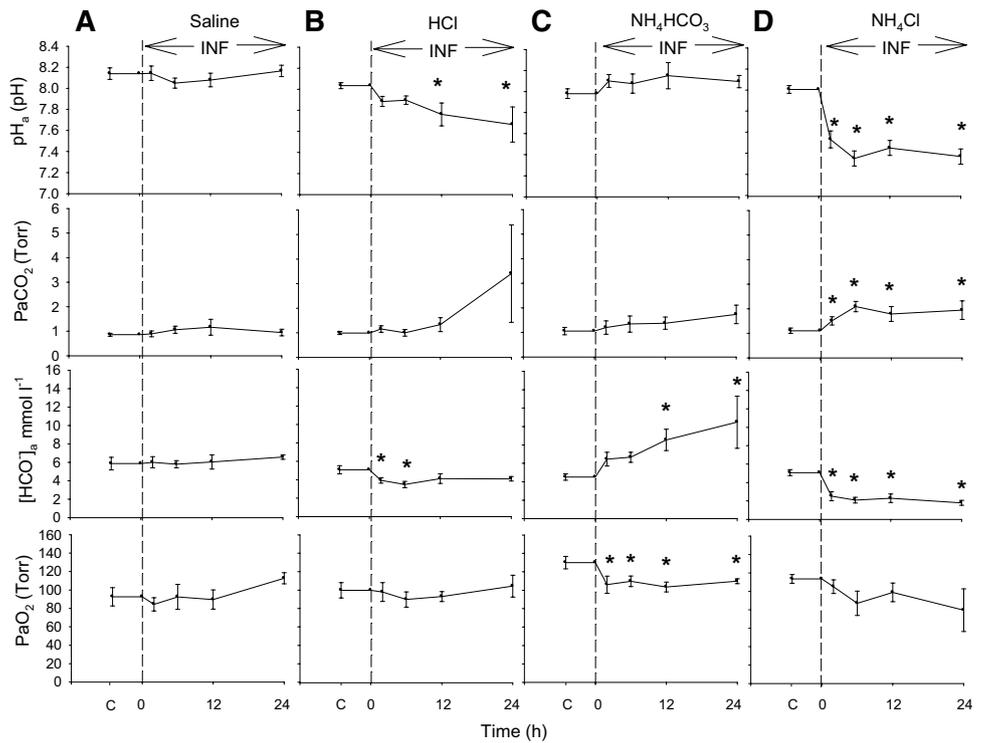
First-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) using 1 μg of DNaseI-treated total RNA (gill and kidney) that was extracted with Trizol (Invitrogen).

mRNA levels of *carbonic anhydrase IV (CA-IV)*, *Rhbg*, *Rhp2*, *v-type H<sup>+</sup>-ATPase (B subunit) (H<sup>+</sup>-ATPase)*, *Na<sup>+</sup>/H<sup>+</sup> exchanger type 2 (NHE2)*, and *Na<sup>+</sup>,K<sup>+</sup>-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).

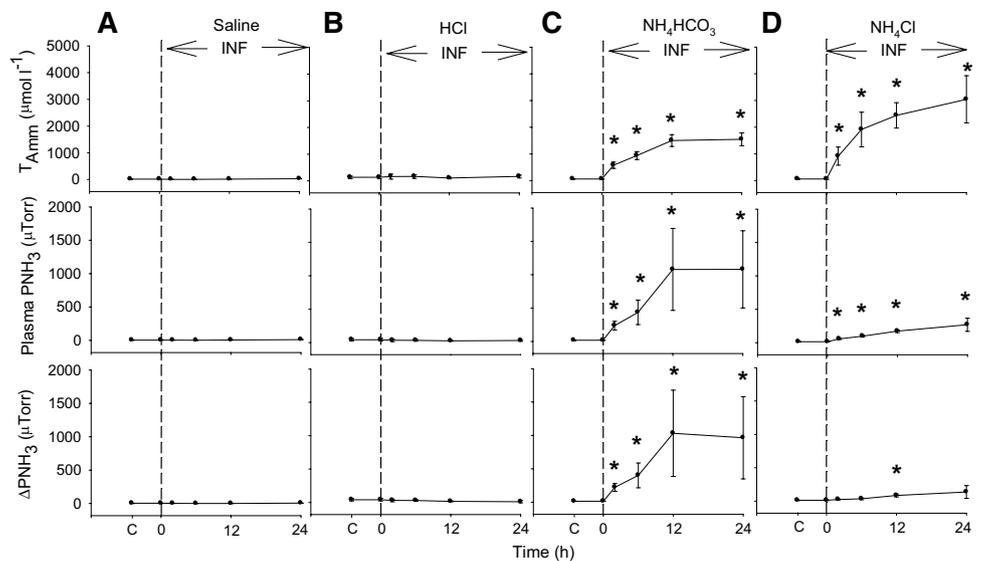
### Statistics

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,  $\text{PCO}_2$  (Torr),  $\text{HCO}_3^-$  ( $\text{mmol l}^{-1}$ ) and  $\text{PO}_2$  (Torr) in the arterial blood of *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c**  $\text{NH}_4\text{HCO}_3$ , or **d**  $\text{NH}_4\text{Cl}$  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 ( $T_{\text{Amm}}$ ;  $\mu\text{mol l}^{-1}$ ),  $\text{PNH}_3$  ( $\mu\text{Torr}$ ), and  $\Delta\text{PNH}_3$  ( $\mu\text{Torr}$ ) in *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c**  $\text{NH}_4\text{HCO}_3$ , or **d**  $\text{NH}_4\text{Cl}$  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

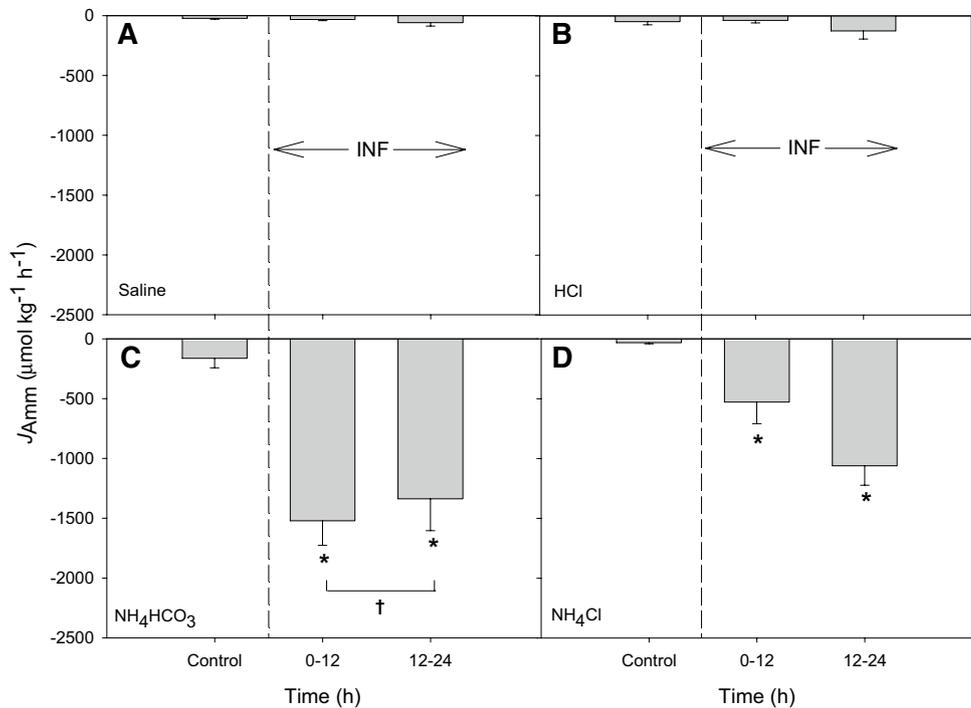
NaCl infusion had no effect on  $\text{pH}_a$ ,  $[\text{HCO}_3^-]_a$ ,  $\text{PaCO}_2$ , or  $\text{PaO}_2$  over the 24-h period (Fig. 1a). However, HCl infusion caused significant decreases in  $\text{pH}_a$  at 2 and 6 h, and significant decreases in plasma  $[\text{HCO}_3^-]_a$  at 12 and 24 h (Fig. 1b).  $\text{PaO}_2$  and  $\text{PaCO}_2$  did not change, though the latter became highly variable by 24 h. The acid–base responses

**Table 2** Mean plasma concentrations of urea-N,  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ( $\text{mmol l}^{-1}$ ) in *Squalus acanthias* during 0, 2, 6, 12, and 24 h of infusion of saline (NaCl), HCl,  $\text{NH}_4\text{HCO}_3$ , or  $\text{NH}_4\text{Cl}$

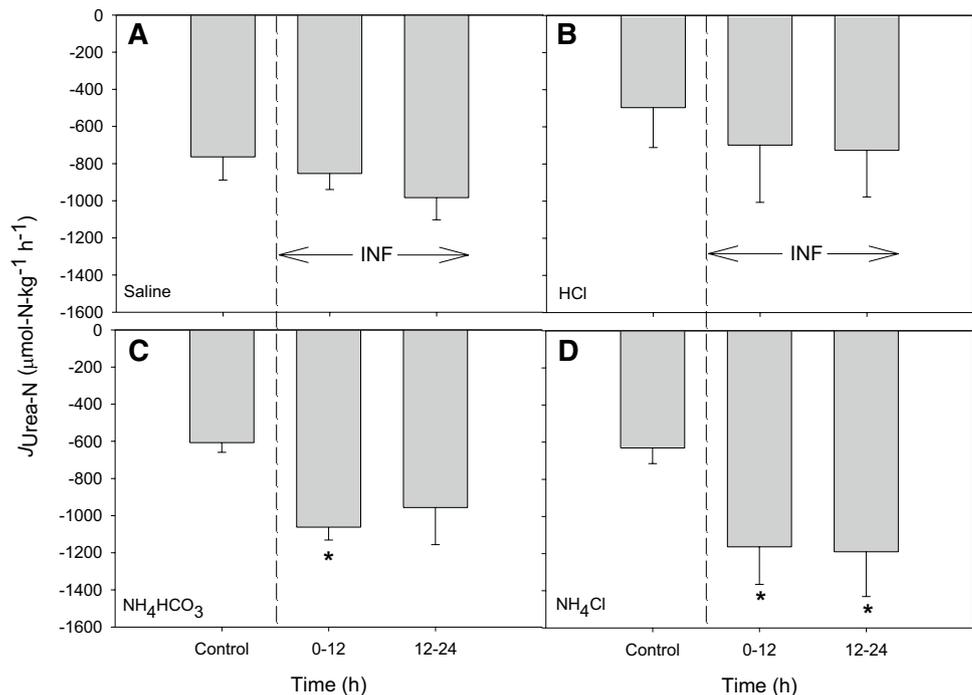
	0 h	2 h	6 h	12 h	24 h
<b>Saline</b>					
Urea-N	687.32 ± 14.04	677.18 ± 2.48	668.08 ± 25.34	652.50 ± 38.56	650.90 ± 31.76
$\text{Cl}^-$	285.25 ± 7.07	294.00 ± 10.44	309.00 ± 11.44*	307.33 ± 14.53*	310.29 ± 12.06*
$\text{Na}^+$	289.46 ± 5.42	288.87 ± 6.52	303.96 ± 13.34	311.25 ± 13.86	310.08 ± 11.23
$\text{Ca}^{++}$	4.74 ± 0.15	4.43 ± 0.12	4.32 ± 0.24	4.38 ± 0.15	4.18 ± 0.16
$\text{Mg}^{++}$	2.42 ± 0.40	2.04 ± 0.33	2.06 ± 0.36	2.08 ± 0.45	2.29 ± 0.30
<b>HCl</b>					
Urea-N	681.08 ± 20.16	680.36 ± 19.26	669.12 ± 23.88	657.76 ± 15.58	657.96 ± 10.12*
$\text{Cl}^-$	292.67 ± 6.14	302.11 ± 9.38	314.22 ± 8.27*	310.75 ± 5.05*	310.17 ± 5.50*
$\text{Na}^+$	313.59 ± 9.95	302.83 ± 10.89	322.37 ± 10.06	313.27 ± 7.77	308.3 ± 7.66
$\text{Ca}^{++}$	4.72 ± 0.28	4.76 ± 0.43	4.49 ± 0.29	4.40 ± 0.40	4.54 ± 0.41
$\text{Mg}^{++}$	2.65 ± 0.66	2.69 ± 0.81	2.45 ± 0.67	1.98 ± 0.24	1.73 ± 0.24
<b><math>\text{NH}_4\text{HCO}_3</math></b>					
Urea-N	698.62 ± 13.22	706.80 ± 15.34	706.86 ± 26.8	694.50 ± 22.40	671.54 ± 15.48
$\text{Cl}^-$	287.00 ± 7.72	282.63 ± 10.19	288.71 ± 10.02	288.75 ± 8.53	276.17 ± 7.42
$\text{Na}^+$	295.94 ± 8.28	302.54 ± 14.24	304.76 ± 9.94	305.76 ± 9.73	290.96 ± 8.04
$\text{Ca}^{++}$	4.93 ± 0.23	4.60 ± 0.22*	4.70 ± 0.18*	4.58 ± 0.14*	4.43 ± 0.15*
$\text{Mg}^{++}$	1.77 ± 0.22	1.86 ± 0.25	2.06 ± 0.22	2.27 ± 0.31*	2.21 ± 0.17*
<b><math>\text{NH}_4\text{Cl}</math></b>					
Urea-N	764.22 ± 15.60	766.36 ± 17.98	750.50 ± 17.80	735.74 ± 16.16*	729.38 ± 35.72*
$\text{Cl}^-$	264.08 ± 3.03	279.67 ± 3.58*	288.83 ± 3.66*	286.50 ± 4.64*	294.00 ± 7.03*
$\text{Na}^+$	290.25 ± 5.05	287.83 ± 4.33	296.48 ± 4.90	293.47 ± 4.31	292.99 ± 6.63
$\text{Ca}^{++}$	4.31 ± 0.29	4.04 ± 0.11	4.19 ± 0.11	4.20 ± 0.13	4.26 ± 0.12
$\text{Mg}^{++}$	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 ( $J_{\text{Amm}}$ ) in *Squalus acanthias* during infusion with **a** saline (NaCl), **b** HCl, **c**  $\text{NH}_4\text{HCO}_3$ , or **d**  $\text{NH}_4\text{Cl}$  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  $\text{NH}_4\text{HCO}_3$  group is significantly greater than that in the  $\text{NH}_4\text{Cl}$  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**  $\text{NH}_4\text{HCO}_3$ , or **d**  $\text{NH}_4\text{Cl}$  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  $\text{NH}_4\text{HCO}_3$  versus  $\text{NH}_4\text{Cl}$  infusions were very different.  $\text{NH}_4\text{HCO}_3$  infusion caused significant increases in plasma  $[\text{HCO}_3^-]_a$  by 12 h and an almost doubling by 24 h; small increases in  $\text{pH}_a$  and  $\text{PaCO}_2$  were not significant, but  $\text{PaO}_2$  was significantly depressed by about 20 % at all time points (Fig. 1c). In contrast,  $\text{NH}_4\text{Cl}$  infusion caused significant 50–70 % losses of plasma  $[\text{HCO}_3^-]_a$  at all time points, accompanied by significant decreases in  $\text{pH}_a$  of 0.5–0.6 units, and increases in  $\text{PaCO}_2$  which approximately doubled from 6 h onwards (Fig. 1d). Decreases in  $\text{PaO}_2$  were comparable to those with  $\text{NH}_4\text{HCO}_3$  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  $\text{NH}_4\text{HCO}_3$  versus  $\text{NH}_4\text{Cl}$  infusions. The ammonia loading rates with these two treatments were the same, but the resulting increases in plasma  $T_{\text{Amm}}$ , which were significant in both by 2 h, were approximately twice as large in the  $\text{NH}_4\text{Cl}$  treatment by 24 h (Fig. 2c, d). Despite this, the accompanying significant increases in  $\text{PaNH}_3$ , and therefore in the  $\Delta\text{PNH}_3$  gradient across the gills, were substantially higher in the sharks infused with  $\text{NH}_4\text{HCO}_3$ . As a result of the Henderson–Hasselbalch relationship, these  $\text{PNH}_3$  differences reflected the large differences in  $\text{pH}_a$  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

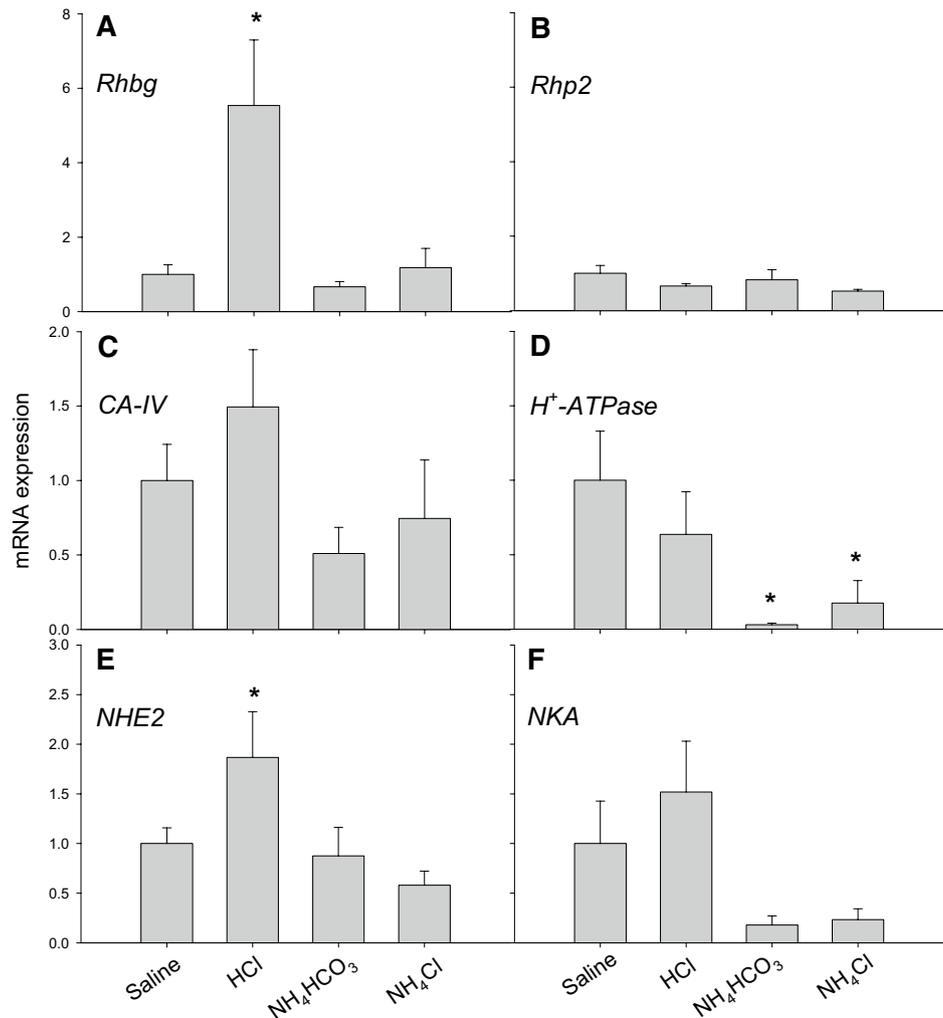
significant at 12 or 24 h in the HCl and  $\text{NH}_4\text{Cl}$  infusions (Table 2). Plasma  $\text{Cl}^-$  increased from 6 h onwards only in the NaCl and HCl treatments, reflecting the  $\text{Cl}^-$  loading (Table 2).  $\text{NH}_4\text{HCO}_3$  infusion caused significant decreases in plasma  $\text{Ca}^{2+}$  from 2 h onwards and significant increases in plasma  $\text{Mg}^{2+}$  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,  $\text{NH}_4\text{HCO}_3$  versus  $\text{NH}_4\text{Cl}$  infusions caused qualitatively similar but quantitatively different responses in these parameters.  $J_{\text{Amm}}$  increased greatly in the two treatments at both 0–12 h and 12–24 h, but the elevation was 2-fold greater in the  $\text{NH}_4\text{HCO}_3$ -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  $\text{NH}_4\text{HCO}_3$  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,  $\text{NH}_4\text{HCO}_3$ , or  $\text{NH}_4\text{Cl}$ . **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  $J_{\text{Amm}}$  represented 86 % and  $J_{\text{Urea-N}}$  accounted for 27 %. In the  $\text{NH}_4\text{Cl}$ -infused fish, the elevation equalled 87 % of the amount infused, of which  $J_{\text{Amm}}$  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  $\text{NH}_4\text{HCO}_3$  or  $\text{NH}_4\text{Cl}$  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  $\text{NH}_4\text{HCO}_3$  or  $\text{NH}_4\text{Cl}$  (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).

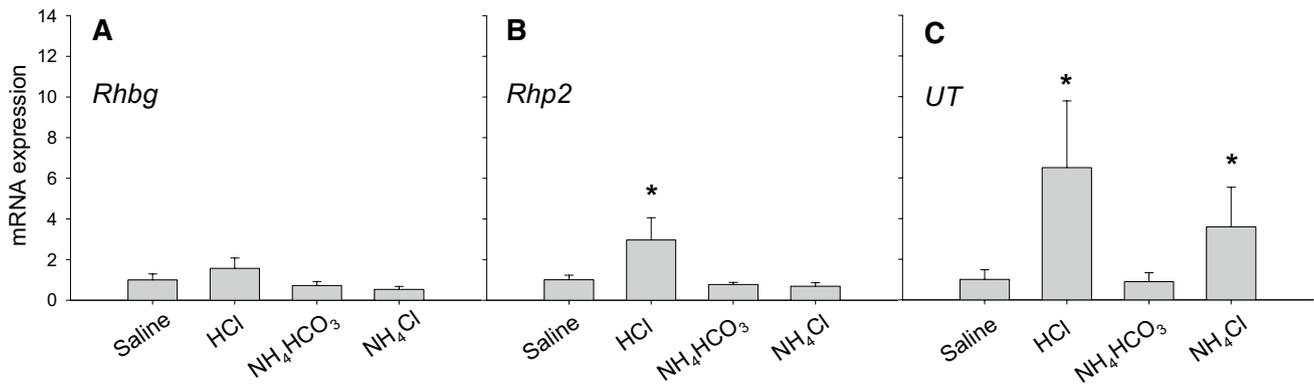
### Kidney

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  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{HCO}_3$  infusion (Fig. 6b). *UT* expression increased greatly (4-fold to 7-fold) in response to both HCl and  $\text{NH}_4\text{Cl}$  loading but did not change in response to  $\text{NH}_4\text{HCO}_3$  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, NH<sub>4</sub>HCO<sub>3</sub>, or NH<sub>4</sub>Cl. **a** *Rhb*, **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 *Rhb* 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<sup>+</sup>-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 *Rhb* expression were up-regulated by metabolic acidosis alone (HCl infusion), but not when acidosis was combined with ammonia loading (NH<sub>4</sub>Cl 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 NH<sub>4</sub><sup>+</sup> 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 HCO<sub>3</sub><sup>-</sup> 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<sub>a</sub> and (HCO<sub>3</sub><sup>-</sup>)<sub>a</sub>, non-significant rise

in PaCO<sub>2</sub>; 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 NH<sub>4</sub>Cl infusion, but the rate of acid loading by infusion of acidic equivalents (125 mmol l<sup>-1</sup> HCl versus 500 mmol l<sup>-1</sup> NH<sub>4</sub>Cl) 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 NH<sub>4</sub>Cl infusion (large decreases in [HCO<sub>3</sub><sup>-</sup>]<sub>a</sub> and pH<sub>a</sub>; 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 NH<sub>4</sub>Cl 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 PaCO<sub>2</sub>) not seen by Wood et al. (1995). In teleost fish, infusion or injection of NH<sub>4</sub><sup>+</sup> coupled with a non-metabolizable anion (e.g. Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>) 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 NH<sub>3</sub> loss across the gills leaving behind a strong mineral acid (e.g. HCl, H<sub>2</sub>SO<sub>4</sub>). In the elasmobranch, this may be part of the explanation, but the additional removal of NH<sub>3</sub> by stimulated ureagenesis (see below) is probably responsible for the more intense acidosis. As intended, the blood acid–base responses to NH<sub>4</sub>HCO<sub>3</sub> infusion comprised a mild metabolic alkalosis with slight respiratory compensation (significant increases in [HCO<sub>3</sub><sup>-</sup>]<sub>a</sub>, non-significant increases in pH<sub>a</sub> and PaCO<sub>2</sub>; Fig. 1c), and were very different from those with NH<sub>4</sub>Cl (Fig. 1d). We are aware of no previous NH<sub>4</sub>HCO<sub>3</sub> 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  $\text{NH}_4\text{HCO}_3$  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  $\text{NH}_4\text{HCO}_3$  is removed as  $\text{NH}_4^+$ . Removal of  $\text{NH}_3$  alone would leave behind  $\text{H}^+$  and  $\text{HCO}_3^-$ , forming  $\text{CO}_2$ , and would therefore be neutral in terms of its effect on metabolic acid–base status.

Both forms of ammonia infusion tended to lower  $\text{PaO}_2$  (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  $\text{O}_2$  affinity. The latter could occur simply by an effect of  $\text{NH}_3$  in raising the intracellular pH in the red cell, by interactions of either  $\text{NH}_3$  or  $\text{NH}_4^+$  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,  $\text{NH}_4\text{Cl}$  infusion caused an increase in plasma  $T_{\text{Amm}}$  that was 2-fold greater than with  $\text{NH}_4\text{HCO}_3$  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  $J_{\text{Amm}}$  was probably due to the much greater increases in  $\text{PaNH}_3$  and therefore in the outwardly directed  $\Delta\text{PNH}_3$  gradient across the gills in the sharks infused with  $\text{NH}_4\text{HCO}_3$  (Fig. 2c, d), differences attributable to the much higher pH<sub>a</sub> in this treatment (Fig. 1c, d). This would cause greater diffusive efflux of  $\text{NH}_3$ , but it is unknown whether there was also greater diffusive efflux of  $\text{NH}_4^+$  because transepithelial potential (TEP), an important contributor to the net driving force on  $\text{NH}_4^+$  (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 ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ), but the increase in plasma  $\text{Mg}^{2+}$  concentration, which occurred only with  $\text{NH}_4\text{HCO}_3$  infusion, was remarkable (Table 2). Interestingly, a similar increase was seen when dogfish were exposed to HEA in the form of  $\text{NH}_4\text{HCO}_3$  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  $J_{\text{Amm}}$  (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  $J_{\text{Amm}}$ , 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  $J_{\text{Amm}}$  (Fig. 3c, d) and  $J_{\text{Urea-N}}$  (Fig. 4c, d) with infusions of either  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{HCO}_3$ . 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  $J_{\text{Amm}}$  and  $J_{\text{Urea-N}}$  were recorded by Wood et al. (1995) in dogfish infused with  $\text{NH}_4\text{Cl}$  for only 6 h. As noted above, the greater elevations in  $J_{\text{Amm}}$  and  $\Delta\text{PNH}_3$  with  $\text{NH}_4\text{HCO}_3$  versus  $\text{NH}_4\text{Cl}$  point to an important role for  $\text{NH}_3$  efflux in this response. Using a perfused head preparation of *Squalus acanthias* ‘pups’, Evans and More (1988) concluded that  $\text{NH}_3$  efflux was much more important than  $\text{NH}_4^+$  efflux when internal  $T_{\text{Amm}}$  levels were elevated.

As plasma  $T_{\text{Urea-N}}$  levels were not differentially affected by either  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{HCO}_3$  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  $\text{NH}_4^+$  salts indicates that urea-N synthesis is driven much more by ammonia availability than by  $\text{HCO}_3^-$  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  $\text{HCO}_3^-$ ) 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  $\text{NH}_4\text{Cl}$  versus  $\text{NaHCO}_3$  infusion in dogfish, and with Wood et al. (2008) who assayed the effect of  $\text{NaHCO}_3$  infusion on OUC enzymes. Indeed, by the direct comparison of two  $\text{NH}_4^+$  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  $P_{\text{NH}_3}$  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  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{HCO}_3$  (Fig. 5a), both of which increased plasma  $T_{\text{Amm}}$  and  $P_{\text{NH}_3}$  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  $\text{HCO}_3^-$  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  $\text{NH}_3$  concentration at the extracellular side of the basolateral membrane, and therefore the  $P_{\text{NH}_3}$  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  $\text{Na}^+$ ,  $\text{K}^+$ -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  $\text{NH}_4\text{Cl}$  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  $\text{NH}_4\text{Cl}$  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  $\text{NH}_4\text{Cl}$  infusions but no change in response to  $\text{NH}_4\text{HCO}_3$  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  $\text{NH}_4\text{Cl}$

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  $\text{NH}_4\text{Cl}$  or  $\text{NH}_4\text{HCO}_3$  infusions (Fig. 6a, b). It is known, however, that renal ammonia excretion increases markedly in sharks infused with  $\text{NH}_4\text{Cl}$ , 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  $T_{\text{Amm}}$ , 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 ( $\text{NH}_4\text{Cl}$  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  $\text{CO}_2$  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.

**Acknowledgments** We thank Bruce Cameron, former Research Coordinator at Bamfield Marine Sciences Centre, for invaluable logistical assistance; Linda Diao and Tania Ng for excellent technical help; Sue Edwards for helpful input; and four anonymous reviewers for constructive comments. We are supported by the Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to CMW and PJW, who were both supported by the Canada Research Chairs Program.

### References

- Anderson WG, Dasiewicz PJ, Liban S, Ryan C, Taylor JR, Grosell M, Weihrauch D (2010) Gastro-intestinal handling of water and solutes in three species of elasmobranch fish, the white-spotted bamboo shark, *Chiloscyllium plagiosum*, little skate, *Leucoraja erinacea*, and the clear nose skate, *Raja eglanteria*. *Comp Biochem Physiol* 155A:493–502
- Anderson WG, Nawata CM, Wood CM, Piercey-Normore MD, Weihrauch D (2012) Body fluid osmolytes and urea and ammonia flux in the colon of two chondrichthyan fishes, the ratfish, *Hydrolagus colliei*, and spiny dogfish, *Squalus acanthias*. *Comp Biochem Physiol* 161A:27–35
- Atkinson DE (1992) Functional roles of urea synthesis in vertebrates. *Physiol Zool* 65:243–267
- Atkinson DE, Bourke E (1984) The role of ureagenesis in pH homeostasis. *Trends Biochem Sci* 9:297–300
- Bakouh N, Benjelloun F, Hulin P, Brouillard F, Edelman A, Cherif-Zahar B, Planelles G (2004)  $\text{NH}_3$  is involved in the  $\text{NH}_4^+$  transport induced by the functional expression of the human Rh C glycoprotein. *J Biol Chem* 279:15975–15983
- Ballantyne JS (1997) Jaws: the inside story. The metabolism of elasmobranch fishes. *Comp Biochem Physiol* 118B:703–742
- Barimo JF, Steele SL, Wright PA, Walsh PJ (2004) The ontogeny of urea synthesis and excretion in the gulf toadfish, *Opsanus beta*. *J Exp Biol* 207:2011–2020

- Boutilier RG, Heming TA, Iwama GK (1984) Appendix: physicochemical parameters for use in fish respiratory physiology. In: Hoar WS, Randall DJ (eds) Fish physiology, vol 10A. Academic Press, Orlando, pp 403–430
- Boylan JW (1967) Gill permeability in *Squalus acanthias*. In: Gilbert PW, Mathewson RF, Rall DP (eds) Sharks, skates and rays. Johns Hopkins Press, Baltimore, pp 197–206
- Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169–193
- Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 29:23–39
- Cameron JN, Heisler N (1983) Studies of ammonia in the trout: physicochemical parameters, acid–base behaviour and respiratory clearance. *J Exp Biol* 105:107–125
- Cameron JN, Kormanik GA (1982) The acid–base responses of gills and kidneys to infused acid and base loads in the channel catfish, *Ictalurus punctatus*. *J Exp Biol* 99:143–160
- Choe KP, Kato A, Hirose S, Plata C, Sindic A, Romero MF, Claiborne JB, Evans DH (2005) NHE3 in an ancestral vertebrate: primary sequence, distribution, localization and function in gills. *Am J Physiol* 289:R1520–R1534
- Claiborne JB, Evans DH (1988) Ammonia and acid–base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). *J Exp Biol* 140:89–105
- Claiborne JB, Choe KP, Morrison-Shetlar AI, Weakley JC, Havird J, Freiji A, Evans DH, Edwards SL (2008) Molecular detection and immunological localization of gill Na<sup>+</sup>/H<sup>+</sup> exchanger in the dogfish (*Squalus acanthias*). *Am J Physiol* 294:R1092–R1102
- Cooper AR, Morris S (2004) Osmotic, sodium, carbon dioxide, and acid–base state of the Port Jackson shark, *Heterodontus portusjacksoni*, in response to lowered salinity. *J Comp Physiol B* 174:211–222
- De Boeck G, Wood CM (2015) Does ammonia trigger hyperventilation in the elasmobranch *Squalus acanthias suckleyi*? *Respir Physiol Neurobiol* 206:25–35
- De Boeck G, Grosell M, Wood CM (2001) Sensitivity of the spiny dogfish (*Squalus acanthias*) to waterborne silver exposure. *Aquat Toxicol* 54:261–275
- Ebert DA, White WT, Goldman KJ, Compagno LJV, Daly-Engel TS, Ward RD (2010) Resurrection and redescription of *Squalus suckleyi* Girard from the North Pacific, with comments on the *Squalus acanthias* subgroup (Squaliformes: Squalidae). *Zootaxa* 2612:22–40
- Edwards SL, Donald JA, Toop T, Donowitz M, Tse CM (2002) NHE-like immunoreactivity in the gills of elasmobranchs. *Comp Biochem Physiol* 131:257–265
- Endeward V, Cartron J-P, Ripoche G, Gros G (2006) Red cell membrane CO<sub>2</sub> permeability in normal human blood and in blood deficient in various blood groups, and effect of DIDS. *Transfus Clin Biol* 13:123–127
- Evans D (1982) Mechanisms of acid extrusion by two marine fishes: the teleost, *Opsanus beta*, and the elasmobranch, *Squalus acanthias*. *J Exp Biol* 97:289–299
- Evans DH, More KJ (1988) Modes of ammonia transport across the gill epithelium of the dogfish pup (*Squalus acanthias*). *J Exp Biol* 138:375–397
- Fines GA, Ballantyne JS, Wright PA (2001) Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. *Am J Physiol* 280:R16–R24
- Haywood GP (1973) Hypo-osmotic regulation coupled with reduced metabolic urea in the dogfish *Poroderma africanum*: an analysis of serum osmolality, chloride, and urea. *Mar Biol* 23:121–127
- Huang C-H, Peng J (2005) Evolutionary conservation and diversification of Rh family genes and proteins. *Proc Natl Acad Sci USA* 102:15512–15517
- Kajimura M, Walsh PJ, Mommsen TP, Wood CM (2006) The dogfish shark (*Squalus acanthias*) activates both hepatic and extrahepatic ornithine urea cycle enzyme activities for nitrogen conservation after feeding. *Physiol Biochem Zool* 79:602–613
- Kajimura M, Walsh PJ, Wood CM (2008) The dogfish shark (*Squalus acanthias*) maintains its osmolytes during long term starvation. *J Fish Biol* 72:656–670
- King PA, Goldstein L (1983) Renal ammoniogenesis and acid excretion in the dogfish, *Squalus acanthias*. *Am J Physiol* 245:R581–R589
- Klein JD, Rouillard P, Roberts BR, Sands JM (2002) Acidosis mediates the upregulation of UT-A protein in livers from uremic rats. *J Am Soc Nephrol* 13:581–587
- Kustu S, Inwood W (2006) Biological gas channels for NH<sub>3</sub> and CO<sub>2</sub>: evidence that Rh (Rhesus) proteins are CO<sub>2</sub> channels. *Transfus Clin Biol* 13:103–110
- Lu Z, Gui H, Yao L, Yan L, Martens H, Aschenbach JR, Shen Z (2014) Short-chain fatty acids and acidic pH up-regulate UT-B, GPR41, and GPR4 in rumen epithelial cells of goats. *Am J Physiol*. doi:10.1152/ajpregu.00323.2014
- Marini A-M, Matassi G, Raynal V, André B, Cartron J-P, Chérif-Zahar B (2000) The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat Genet* 26:341–344
- Maxime V, Peyraud-Waitzenegger M, Claireaux G, Peyraud C (1990) Effects of rapid transfer from sea water to fresh water on respiratory variables, blood acid–base status and O<sub>2</sub> affinity of haemoglobin in Atlantic salmon (*Salmo salar* L.). *J Comp Physiol* 160B:31–39
- McDonald DG, Prior ET (1988) Branchial mechanisms of ion and acid–base regulation in the freshwater rainbow trout *Salmo gairdneri*. *Can J Zool* 66:2699–2708
- Milligan CL, McDonald DG, Prior T (1991) Acid and ammonia fluxes in response to alkalosis and acidosis in two marine teleosts: coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*). *Physiol Zool* 64:169–192
- Mondzac A, Ehrlich GE, Seegmiller JE (1965) An enzymatic determination of ammonia in biological fluids. *J Lab Clin Med* 66:526–531
- Mouro-Chanteloup I, Cochet S, Chami M, Genetet S, Zidi-Yahiaoui N, Engel A, Colin Y, Bertrand O, Ripoche P (2010) Functional reconstitution into liposomes of purified human RhCG ammonia channel. *PLoS One* 5:e8921
- Murdaugh HV, Robin ED (1967) Acid–base metabolism in the dogfish shark. In: Gilbert PW, Mathewson RF, Rall DP (eds) Sharks, skates and rays. Johns Hopkins Press, Baltimore, pp 249–264
- Nakada T, Westhoff CM, Kato A, Hirose S (2007) Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J* 21:1067–1074
- Nakada T, Westhoff CM, Yamaguchi Y, Hyodo S, Li X, Muro T, Kato A, Nakamura Hirose S (2010) Rhesus glycoprotein P2 (Rhp2) is a novel member of the Rh family of ammonia transporters highly expressed in shark kidney. *J Biol Chem* 285:2653–2664
- Nawata CM, Wood CM (2008) The effects of CO<sub>2</sub> and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J Exp Biol* 211:3226–3236
- Nawata CM, Hung CCY, Tsui TKN, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H<sup>+</sup>-ATPase involvement. *Physiol Genomics* 31:463–474
- Nawata CM, Hirose S, Nakada T, Wood CM, Kato A (2010a) Rh glycoprotein expression is modulated in pufferfish (*Takifugu*

- rubripes*) during high environmental ammonia exposure. *J Exp Biol* 213:3150–3160
- Nawata CM, Wood CM, O'Donnell MJ (2010b) Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. *J Exp Biol* 213:1049–1059
- Nawata CM, Walsh PJ, Wood CM (2015) Physiological and molecular responses of the spiny dogfish shark (*Squalus acanthias*) to high environmental ammonia: scavenging for nitrogen. *J Exp Biol* 218:238–248
- Pärt P, Wright PA, Wood CM (1998) Urea and water permeability in dogfish (*Squalus acanthias*) gills. *Comp Biochem Physiol* 199A:117–123
- Payan P, Maetz J (1973) Branchial sodium transport mechanisms in *Scyliorhinus canicula*; evidence for  $\text{Na}^+/\text{NH}_4^+$  and  $\text{Na}^+/\text{H}^+$  exchanges and for a role of carbonic anhydrase. *J Exp Biol* 58:487–502
- Perry SF, Braun MH, Noland M, Dawdy J, Walsh PJ (2010) Do zebrafish Rh proteins act as dual ammonia– $\text{CO}_2$  channels? *J Exp Zool* 313A:618–621
- Piermarini PM, Evans DH (2001) Immunochemical analysis of the vacuolar proton-ATPase B- subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to  $\text{Na}^+/\text{K}^+$ -ATPase. *J Exp Biol* 204:3251–3259
- Piermarini PM, Verlander JW, Royaux IE, Evans DH (2002) Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch. *Am J Physiol* 283:R983–R992
- Rahmatullah M, Boyde TRC (1980) Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin Chim Acta* 107:3–9
- Reilly BD, Cramp RL, Wilson JM, Campbell HA, Franklin CE (2011) Branchial osmoregulation in the euryhaline bull shark, *Carcharhinus leucas*: a molecular analysis of ion transporters. *J Exp Biol* 214:2883–2895
- Ripoche P, Bertrand O, Gane P, Birkenmeier C, Colin Y, Cartron J-P (2004) Human Rhesus-associated glycoprotein mediates facilitated transport of  $\text{NH}_3$  into red blood cells. *Proc Natl Acad Sci USA* 101:17222–17227
- Roa JN, Munevar CL, Tresguerres M (2014) Feeding induces translocation of vacuolar proton ATPase and pendrin to the membrane of leopard shark (*Triakis semifasciata*) mitochondrion-rich gill cells. *Comp Biochem Physiol* 174A:29–37
- Salama A, Morgan IJ, Wood CM (1999) The linkage between sodium uptake and ammonia excretion in rainbow trout—kinetic analysis, the effects of  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{HCO}_3$  infusion, and the influence of gill boundary layer pH. *J Exp Biol* 202:697–709
- Shankar RA, Anderson PM (1985) Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. *Arch Biochem Biophys* 239:248–259
- Smith HW (1929) The composition of the body fluids of elasmobranchs. *J Biol Chem* 81:407–419
- Smith HW (1936) The retention and physiological role of urea in the elasmobranchii. *Biol Rev* 11:49–82
- Soupe E, Inwood W, Kustu S (2004) Lack of the Rhesus protein Rh1 impairs growth of the green alga *Chlamydomonas reinhardtii* at high  $\text{CO}_2$ . *Proc Natl Acad Sci USA* 101:7787–7792
- Stewart PA (1981) How to understand acid-base: a quantitative acid-base primer for biology and medicine. Elsevier North Holland Inc, New York
- Tresguerres M, Katoh F, Fenton H, Jasinska E, Goss GG (2005) Regulation of branchial  $\text{V-H}^+$ -ATPase,  $\text{Na}^+/\text{K}^+$ -ATPase and NHE2 in response to acid and base infusions in the Pacific spiny dogfish (*Squalus acanthias*). *J Exp Biol* 208:345–354
- Tresguerres M, Parks SK, Katoh F, Goss GG (2006) Microtubule-dependent relocation of branchial  $\text{V-H}^+$ -ATPase to the basolateral membrane in the Pacific spiny dogfish (*Squalus acanthias*): a role in base secretion. *J Exp Biol* 209:599–609
- Tresguerres M, Parks SK, Wood CM, Goss GG (2007)  $\text{V-H}^+$ -ATPase translocation during blood alkalosis in dogfish gills: interaction with carbonic anhydrase and involvement in the post-feeding alkaline tide. *Am J Physiol* 292:R2012–R2019
- Verdouw H, van Eched CJA, Dekkers EMJ (1978) Ammonia determination based on indophenol formation with sodium salicylate. *Water Res* 12:399–402
- Walker RL, Wilkes PRH, Wood CM (1989) The effects of hypersaline exposure on the blood oxygen affinity of the freshwater teleost *Catostomus commersoni*. *J Exp Biol* 142:125–142
- Walsh PJ, Danulat E, Mommsen TP (1990) Variation in urea excretion in the gulf toadfish *Opsanus beta*. *Mar Biol* 106:323–328
- Weihrauch D, Morris S, Towle DW (2004) Ammonia excretion in aquatic and terrestrial crabs. *J Exp Biol* 207:4491–4504
- Weihrauch D, Donini A, O'Donnell MJ (2012) Ammonia transport by terrestrial and aquatic insects. *J Insect Physiol* 58:473–487
- Wilson RW, Taylor EW (1992) Transbranchial ammonia gradients and acid base responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. *J Exp Biol* 166:95–112
- Wilson RW, Wright PM, Munger S, Wood CM (1994) Ammonia excretion in fresh water rainbow trout (*Oncorhynchus mykiss*) and the importance of gill boundary layer acidification: lack of evidence for  $\text{Na}^+/\text{NH}_4^+$  exchange. *J Exp Biol* 191:37–58
- Wood CM, Perry SF, Wright PA, Bergman HL, Randall DJ (1989) Ammonia and urea dynamics in the Lake Magadi tilapia, a ureotelic teleost fish adapted to an extremely alkaline environment. *Respir Physiol* 77:1–20
- Wood CM, Pärt P, Wright PA (1995) Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch, the spiny dogfish (*Squalus acanthias*). *J Exp Biol* 198:1545–1558
- Wood CM, Kajimura M, Mommsen TP, Walsh PJ (2005) Alkaline tide and nitrogen conservation after feeding in the elasmobranch *Squalus acanthias*. *J Exp Biol* 208:2693–2705
- Wood CM, Bucking CP, Fitzpatrick J, Nadella SR (2007a) The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias*. *Respir Physiol Neurobiol* 159:163–170
- Wood CM, Kajimura M, Bucking CP, Walsh PJ (2007b) Osmoregulation, ionoregulation, and acid-base regulation by the gastrointestinal tract after feeding in the dogfish shark. *J Exp Biol* 210:1335–1349
- Wood CM, Munger SR, Thompson J, Shuttleworth TJ (2007c) Control of rectal gland secretion by blood acid-base status in the intact dogfish shark (*Squalus acanthias*). *Respir Physiol Neurobiol* 156:220–228
- Wood CM, Kajimura M, Mommsen TP, Walsh PJ (2008) Is the alkaline tide a signal to activate metabolic or ionoregulatory enzymes in the dogfish shark (*Squalus acanthias*)? *Physiol Biochem Zool* 81:278–287
- Wood CM, Liew HJ, De Boeck G, Walsh PJ (2013a) A perfusion study of the handling of urea and urea analogues by the gills of the dogfish shark (*Squalus acanthias*). *Peer J* 1:e33. doi:10.7717/peerj.33
- Wood CM, Nawata CM, Wilson JM, Laurent P, Chevalier C, Bergma HL, Bianchini A, Maina JN, Johannsson OE, Bianchini LF, Kavembe GD, Papah MB, Ojoo RO (2013b) Rh proteins and  $\text{NH}_4^+$ -activated  $\text{Na}^+$ -ATPase in the Magadi tilapia (*Alcolapia grahami*), a 100% ureotelic teleost fish. *J Exp Biol* 216:2998–3007
- Wright PA, Wood CM (1985) An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of

- environmental pH change and sodium uptake blockade. *J Exp Biol* 114:329–353
- Zhang L, Wood CM (2009) Ammonia as a stimulant to ventilation in rainbow trout *Oncorhynchus mykiss*. *Respir Physiol Neurobiol* 168:261–271
- Zhang L, Nawata CM, Wood CM (2013) Sensitivity in ventilation and brain metabolism to ammonia exposure in rainbow trout, *Oncorhynchus mykiss*. *J Exp Biol* 216:4025–4037
- Zhang L, Nawata CM, DeBoeck G, Wood CM (2014) Rh protein expression in branchial neuroepithelial cells, and the role of ammonia in ventilatory control in fish. *Comp Biochem Physiol A*. doi:[10.1016/j.cbpa.2014.10.004](https://doi.org/10.1016/j.cbpa.2014.10.004)