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RESEARCH ARTICLE

Physiological and molecular responses of the spiny dogfish shark (*Squalus acanthias*) to high environmental ammonia: scavenging for nitrogen

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ABSTRACT

In teleosts, a branchial metabolon links ammonia excretion to Na^+ uptake via Rh glycoproteins and other transporters. Ureotelic elasmobranchs are thought to have low branchial ammonia permeability, and little is known about Rh function in this ancient group. We cloned Rh cDNAs (*Rhag*, *Rhbg* and *Rhp2*) and evaluated gill ammonia handling in *Squalus acanthias*. Control ammonia excretion was <5% of urea-N excretion. Sharks exposed to high environmental ammonia (HEA; 1 mmol $^{-1}$ NH_4HCO_3) for 48 h exhibited active ammonia uptake against partial pressure and electrochemical gradients for 36 h before net excretion was re-established. Plasma total ammonia rose to seawater levels by 2 h, but dropped significantly below them by 24–48 h. Control ΔP_{NH_3} (the partial pressure gradient of NH_3) across the gills became even more negative (outwardly directed) during HEA. Transepithelial potential increased by 30 mV, negating a parallel rise in the Nernst potential, such that the outwardly directed NH_4^+ electrochemical gradient remained unchanged. Urea-N excretion was enhanced by 90% from 12 to 48 h, more than compensating for ammonia-N uptake. Expression of *Rhp2* (gills, kidney) and *Rhbg* (kidney) did not change, but branchial *Rhbg* and erythrocytic *Rhag* declined during HEA. mRNA expression of branchial *Na^+/K^+-ATPase* (*NKA*) increased at 24 h and that of *H^+-ATPase* decreased at 48 h, while expression of the potential metabolon components *Na^+/H^+ exchanger2* (*NHE2*) and *carbonic anhydrase IV* (*CA-IV*) remained unchanged. We propose that the gill of this nitrogen-limited predator is poised not only to minimize nitrogen loss by low efflux permeability to urea and ammonia but also to scavenge ammonia-N from the environment during HEA to enhance urea-N synthesis.

KEY WORDS: Elasmobranchs, Rh glycoproteins, Gene expression, Urea, Gills, Ornithine–urea cycle, Transepithelial potential, P_{NH_3} gradient

INTRODUCTION

The discovery that Rhesus (Rh) glycoproteins are expressed in the gills (Nakada et al., 2007; Nawata et al., 2007) has created a new understanding of the mechanisms of ammonia excretion in teleosts (reviewed by Wright and Wood, 2009; Wright and Wood, 2012; Weihrauch et al., 2009). These channel proteins appear to facilitate the diffusion of NH_3 along P_{NH_3} (partial pressure of NH_3) gradients (Nawata et al., 2010b) and in the gills they function as part of a metabolon which flexibly couples ammonia excretion to Na^+ uptake (Tsui et al., 2009; Ito et al., 2013). The theory proposes that the deprotonation of NH_4^+ as NH_3 enters the apical Rh channel creates a source of protons to fuel Na^+ uptake via Na^+/H^+ exchangers (NHEs) and/or via a coupled Na^+ channel/V-type H^+-ATPase system, while at the same time the NH_3 leaving the Rh channel traps protons, thereby creating an external microenvironment in which $[H^+]$ is less concentrated. This mechanism becomes particularly prominent during chronic exposure to high environmental ammonia (HEA), as evidenced by increased Na^+ uptake, ammonia excretion against the gradient and increased expression of metabolon components at the gills (Nawata et al., 2007; Zimmer et al., 2010; Sinha et al., 2013). To date, most studies have been on freshwater ammoniotelic teleosts, but there is also evidence that some components of the system are present and respond to HEA in marine ammoniotelic teleosts (Nawata et al., 2010a; Wood and Nawata, 2011), and even in a 100% ureotelic tilapia living in hypertonic Lake Magadi (Wood et al., 2013b).

But what about marine elasmobranchs, which funnel most of their N-waste production into the ornithine–urea cycle (OUC) so as to make urea for osmoregulation (Smith, 1931; Wright, 1995)? These fish appear to be nitrogen limited (Haywood, 1973; Wood et al., 2005; Kajimura et al., 2006; Kajimura et al., 2008), exhibiting elaborate urea-retention mechanisms at the gills (Boylan, 1967; Pärt et al., 1998; Fines et al., 2001; Wood et al., 2013a) and kidney (Schmidt-Nielsen et al., 1972; Morgan et al., 2003a; Morgan et al., 2003b). Nevertheless, there is still substantial urea leakage across the gills, so elasmobranchs are predominantly ureotelic, excreting very little ammonia. Rh proteins are known to be expressed in these fish (Nakada et al., 2010; Anderson et al., 2010; Anderson et al., 2012), but their function has been little studied. Early investigations focused on mechanisms of ammonia excretion at the gills and the possible involvement of branchial Na^+/NH_4^+ exchange mechanisms in acid–base balance (Payan and Maetz, 1973; Evans et al., 1979; Evans, 1982; Evans and More, 1988; Claiborne and Evans, 1988). To our knowledge, there has been only one HEA study on a marine elasmobranch (Cameron, 1986), performed long before the involvement of Rh proteins in teleost ammonia excretion was discovered. Cameron (Cameron, 1986) reported that for the same HEA exposure, ammonia built up far more slowly in the plasma of the ray *Raja erinacea* than in a teleost or a crab, and interpreted this as resulting from low gill permeability to ammonia, analogous to that for urea. This conclusion was supported by a later study which reported that gill ammonia permeability (in the efflux direction) in the dogfish shark was about 22-fold lower than that in standard

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List of symbols and abbreviations

CA-IV	carbonic anhydrase IV
$E_{NH_4^+}$	Nernst potential for NH_4^+
$F_{NH_4^+}$	electrochemical gradient for NH_4^+
$[HCO_3^-]a$	arterial bicarbonate concentration
HEA	high environmental ammonia
J_{Amm}	total ammonia flux rate
$J_{Urea\text{-}N}$	total urea-N (nitrogen) flux rate
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase
OUC	ornithine–urea cycle
Pa_{CO_2}	partial pressure of arterial CO_2
Pa_{NH_3}	partial pressure of arterial NH_3
Pa_{O_2}	partial pressure of arterial O_2
pHa	arterial pH
P_{NH_3}	partial pressure of NH_3
Rh	Rhesus
T_{Amm}	total ammonia
TEP	transepithelial potential
$T_{Urea\text{-}N}$	total urea-N (nitrogen)
UT	urea transporter
ΔP_{NH_3}	partial pressure gradient of NH_3

teleosts, even more pronounced than the 13-fold lower urea permeability (Wood et al., 1995). Cameron (Cameron, 1986) also speculated that the ray might convert a substantial proportion of the ammonia entry into urea-N through the OUC, but presented no evidence on this point. This idea is in accord with the results of an NH_4Cl infusion experiment on sharks (Wood et al., 1995) as well as several studies on HEA exposure in two ureotelic teleosts, the Magadi tilapia (Wood et al., 1989; Wood et al., 2013a) and the gulf toadfish (Walsh et al., 1990); in all of these, ammonia loading resulted in a substantial rise in urea-N excretion.

The present study examined the responses to prolonged (48 h) HEA exposure in the dogfish shark, *Squalus acanthias suckleyi* L. Rh cDNAs were cloned, and their mRNA expression tracked in gills, kidney and red blood cells, together with other gill transporters that could be involved directly or indirectly in the metabolon [*Na^+/H^+ exchanger2* (*NHE2*), *carbonic anhydrase IV* (*CA-IV*), *Na^+/K^+-ATPase* (*NKA*), *V-type H^+-ATPase*]. Ammonia and urea-N fluxes with the environment were measured, together with blood gases, acid–base status, major ions, and plasma ammonia and urea-N concentrations. In light of recent evidence that changes in the transepithelial potential (TEP) across the gills play a significant role in the adaptive response of freshwater teleosts to HEA (Wood and Nawata, 2011; Liew et al., 2013), TEP was also monitored so that both P_{NH_3} and NH_4^+ electrochemical gradients across the gills could be calculated.

Our specific hypotheses were that: (i) ammonia influx and build-up in the blood plasma would both occur more slowly relative to previous studies on teleosts; (ii) HEA exposure would induce a marked increase in urea-N excretion; (iii) Rh proteins would be expressed in gills and other tissues, and their mRNA expression would respond to HEA; and (iv) other potential components of the metabolon would respond in a manner coherent with the Rh mRNA response. Some but not all of these hypotheses were confirmed, and our data point to a surprising conclusion – that the gill of the N-limited elasmobranch may be poised to scavenge ammonia-N from the environment.

RESULTS

All concentrations and flux 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).

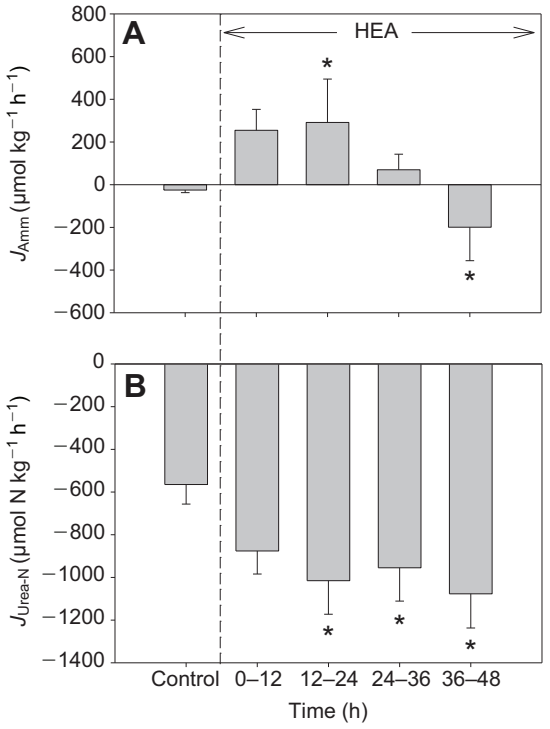

Fig. 1. Net rates of ammonia flux (J_{Amm}) and urea-nitrogen flux ($J_{Urea\text{-}N}$) in dogfish (*Squalus acanthias*) exposed to high environmental ammonia (HEA) for 48 h. (A) Ammonia flux. (B) Urea-N flux. Negative values indicate excretion while positive values indicate uptake. Asterisks indicate a significant difference (P<0.05) from the pre-exposure control levels. Data are means ± s.e.m. (N=8–11).

Series I: effects of HEA on ammonia and urea flux

Under control conditions, dogfish excreted ammonia-N at a very low rate (~25 μmol kg^{-1} h^{-1}) relative to urea-N (~565 μmol kg^{-1} h^{-1}). Exposure to 48 h of HEA resulted in a reversal of J_{Amm} (net ammonia uptake) during the first 36 h and a subsequent re-establishment of net excretion at a rate greater than control values at 36–48 h (Fig. 1A). Urea-N excretion ($J_{Urea\text{-}N}$) was significantly enhanced by about 90% from 12 to 48 h (Fig. 1B).

Terminal blood samples demonstrated that plasma total ammonia (T_{Amm}) was significantly elevated from control levels of 42±23 μmol l^{-1} (N=7) to 1031±111 μmol l^{-1} (N=12) after 48 h of HEA exposure. However, these plasma T_{Amm} levels remained significantly lower than simultaneously measured water T_{Amm} concentrations (1331±113 μmol l^{-1}, N=12) in these same fish. Plasma total urea-nitrogen ($T_{Urea\text{-}N}$) in the HEA-exposed fish was 641±16 mmol l^{-1} (N=12), significantly lower than the 788±18 mmol l^{-1} (N=7) measured in control animals.

Series 2: effects of HEA on arterial plasma ammonia parameters

Plasma T_{Amm} rose significantly to levels that closely matched the water T_{Amm} levels (~1000 μmol l^{-1}) within 2 h of HEA exposure (Fig. 2). From 24 to 48 h, levels in the plasma dropped significantly below those in the water to about 750 μmol l^{-1}, in qualitative agreement with the terminal sampling data of series 1. Nevertheless, plasma T_{Amm} remained greatly elevated relative to pre-exposure control levels (Fig. 2).

Plasma $T_{Urea\text{-}N}$ remained stable across time with the exception of a significant decrease at 36 h (Table 1), similar to the decrease measured in the sharks exposed to HEA for 48 h in series 1. Cl^-, Na^+

The Journal of Experimental Biology (2015) doi:10.1242/jeb.114967

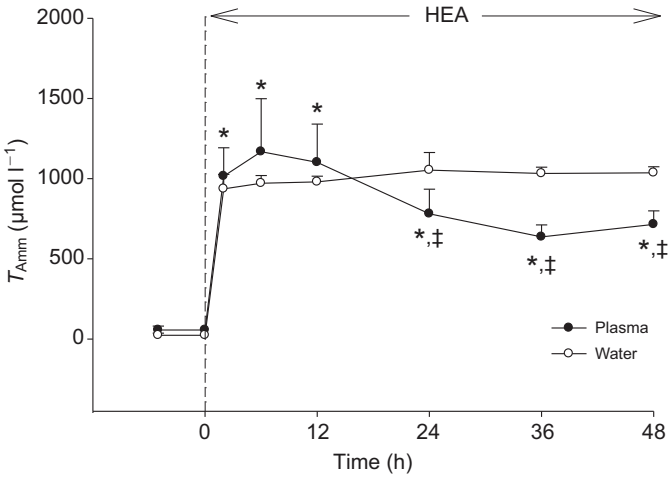

Fig. 2. Total ammonia levels in the water and plasma of *S. acanthias* exposed to HEA for 48 h. Asterisks indicate a significant difference from the pre-exposure control levels and double daggers indicate a significant difference between plasma and water values (P<0.05). Data are means ± s.e.m. (N=6).

and Ca^{2+} levels in the plasma were unchanged throughout the 48 h HEA treatment, but Mg^{2+} increased significantly, almost doubling at 36 h (Table 1).

Arterial pH (pHa) and partial pressure of arterial O_2 (Pa_{O_2}) levels (Fig. 3A,D) did not vary significantly across the entire HEA treatment. Partial pressure of arterial CO_2 (Pa_{CO_2}) was significantly elevated at 2 h (Fig. 3B) and arterial bicarbonate concentration ([HCO_3^-]a) was elevated at 2 and 6 h (Fig. 3C).

Partial pressures of arterial plasma NH_3 (Pa_{NH_3}) and water NH_3 (P_{NH_3}) were calculated from simultaneous measurements of T_{Amm} and pH in the plasma (Fig. 2, Fig. 3A) and water (Fig. 2), respectively. Water pH remained constant at 7.7–7.8 except at 36 h when it fell significantly to 7.53 (data not shown). Both Pa_{NH_3} and water P_{NH_3} increased greatly throughout the period of HEA exposure, and Pa_{NH_3} remained significantly greater than water P_{NH_3} throughout the entire 48 h of HEA exposure (Fig. 4). Therefore, the P_{NH_3} gradient across the gills (ΔP_{NH_3}), which was negative (−11.2 μTorr) under control conditions, became even more negative (up to −263 μTorr at 6 h) from 2 to 36 h of HEA (Table 2), reflecting a tendency for NH_3 to be driven out of the fish. This increase in ΔP_{NH_3} was driven by the overall increases in T_{Amm} and the higher pH (by about 0.35 units) in blood versus external seawater.

The Nernst potential for NH_4^+ ($E_{NH_4^+}$) could also be calculated from these measurements. Under control conditions, $E_{NH_4^+}$ was negative (−23 mV), suggesting a tendency for NH_4^+ to be driven out of the fish, but upon exposure to HEA it increased significantly to positive values (~+10 mV) at 24–48 h of HEA exposure, suggesting a tendency for NH_4^+ to be driven into the fish (Fig. 5). However, this does not take the TEP into account. Indeed, it is the difference between $E_{NH_4^+}$ and TEP, and not the $E_{NH_4^+}$ alone, which is the true net driving force ($F_{NH_4^+}$) for NH_4^+ across the gills. Therefore, changes in TEP were measured in series 3.

Series 3: effects of HEA on branchial transepithelial potential

The TEP was ~0 mV under control conditions, but increased significantly to about +30 mV throughout HEA exposure (Fig. 5). Therefore, this large increase in TEP paralleled and essentially negated the rise in $E_{NH_4^+}$. As a result, $F_{NH_4^+}$ remained negative at about −20 mV throughout the period of HEA, as under control conditions, except at 48 h where it fell to about −10 mV (Table 2). $E_{NH_4^+}$ and TEP were measured on different fish so the changes in $F_{NH_4^+}$ could not be statistically evaluated. Nevertheless, it is clear that the uptake of ammonia during the first 36 h of HEA exposure (Fig. 1) occurred despite the fact that both the partial pressure gradient for NH_3 (ΔP_{NH_3}) and the electrochemical gradient for NH_4^+ ($F_{NH_4^+}$) remained in the outward direction (i.e. negative; Table 2). Thus, ammonia was taken up from the water against both gradients during HEA exposure.

Series 4

Rh cDNA identification

We identified three Rh glycoprotein cDNA sequences in the dogfish; a partial (1117 bp) *Rhag* sequence (GenBank accession no. KJ960196) and full-length sequences of *Rhbg* (GenBank accession no. KJ960197; encoding a 460 amino acid protein) and *Rhp2* (GenBank accession no. KJ960198; encoding a 476 amino acid protein). PCR analyses revealed that the both *Rhbg* and *Rhp2* transcripts are ubiquitously expressed (Fig. 6). Protein BLAST analyses revealed that the partial *Rhag* sequence is most similar (81%) to the holocephalan elephant fish (*Callorhinchis milii*) *Rhag* sequence (GenBank accession no. XP007901455). The *Rhbg* sequence is 70% identical to the Rhbg sequences of the amphibian *Xenopus laevis* (GenBank accession no. NP001087152), the primitive actinopterygian gar (*Lepisosteus oculatus*) (GenBank accession no. XP006641869) and the teleost midshipman (*Porichthys notatus*) (GenBank accession no. A6A93879), and has 12 predicted transmembrane regions (Fig. 7). The *Rhbg* EST of the elasmobranch skate *Leucoraja erinacea* (GenBank accession no. FL670153) was not large enough for meaningful sequence comparison to the *Squalus acanthias Rhbg* sequence, and the same was true for the *Rhbg* fragment identified in the holocephalan ratfish *Hydrolagus colliei* (GenBank accession no. HQ852228) by Anderson et al. (Anderson et al., 2012). *Rhp2* is highly conserved (95% sequence identity) in the dogfish and another elasmobranch, the houndshark (*Triakis scyllium*) (GenBank accession no. AB2877007) (Fig. 8).

Table 1. Mean plasma concentrations of urea-N, Cl^-, Na^+, Ca^{2+} and Mg^{2+} in *Squalus acanthias* exposed to HEA for 48 h

	0 h	2 h	6 h	12 h	24 h	36 h	48 h
Urea-N	736±12 (6)	728±16 (5)	745±15 (6)	733±18 (6)	729±15 (6)	693±13 (6)*	698±6 (6)
Cl^-	267±5 (6)	268±4 (6)	275±7 (6)	274±6 (6)	275±9 (6)	272±13 (6)	281±7 (6)
Na^+	287±5 (6)	293±5 (6)	302±4 (6)	306±4 (6)	289±8 (6)	294±13 (6)	310±8 (6)
Ca^{2+}	3.46±0.23 (6)	3.24±0.25 (6)	3.33±0.15 (6)	3.40±0.23 (6)	3.54±0.17 (6)	3.46±0.11 (6)	3.40±0.28 (6)
Mg^{2+}	1.26±0.10 (6)	1.23±0.09 (6)	1.31±0.13 (6)	1.37±0.16 (6)	1.77±0.30 (6)	2.21±0.30 (6)*	1.95±0.34 (6)

HEA, high environmental ammonia.
Values for plasma concentrations (mmol l^{-1}) are means ± s.e.m. (N).
*Significantly different from control (P<0.05).

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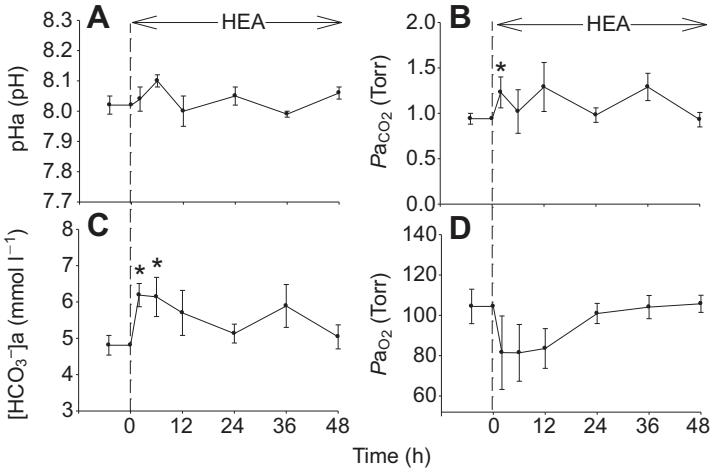

Fig. 3. Arterial blood parameters in *S. acanthias* exposed to HEA for 48 h. (A) Arterial pH (pHa), (B) partial pressure of arterial CO_2 (Pa_{CO_2}), (C) arterial HCO_3^- concentration ($[HCO_3^-]a$) and (D) partial pressure of arterial O_2 (Pa_{O_2}). Asterisks indicate a significant difference (P<0.05) from the pre-exposure control levels. Data are means ± s.e.m. (N=6).

mRNA levels

In the gill, expression of *Rhbg* (Fig. 9E) and *H⁺-ATPase* (Fig. 9B) was significantly downregulated after 48 h of HEA by ~5- and 2-fold, respectively. *Carbonic anhydrase IV* (*CA-IV*; Fig. 9A), *Na⁺/H⁺ exchanger 2* (*NHE2*; Fig. 9C) and *Rhp2* (Fig. 9F) expression levels remained unchanged while *Na⁺/K⁺-ATPase* (*NKA*; Fig. 9D) expression was significantly elevated by almost 4-fold at 24 h, but fell back to control levels at 48 h.

In erythrocytes, *Rhag* mRNA levels decreased significantly by about 2.5- to 3-fold after 24–48 h of HEA (Fig. 10A). In the kidney, *Rhbg* and *Rhp2* mRNA levels were stable during HEA while urea transporter (*UT*) levels increased significantly by about 5-fold at 24 h (Fig. 10B).

DISCUSSION

Overview

This is the first detailed investigation of the responses of a marine elasmobranch to prolonged HEA exposure. As such, it has confirmed some of our original hypotheses, disproven others, and also provided some completely unexpected findings. In contrast to our first hypothesis, ammonia influx and build-up in the blood plasma of the dogfish were not markedly slower than in comparable previous studies on teleosts, despite the fact that the efflux permeability to ammonia is very low under control conditions. This relates to the unexpected finding that the effective gill permeability to ammonia appears to be strongly rectified, such that permeability in the uptake direction is much greater than permeability in the efflux direction. Thus, ammonia uptake is favoured even in the presence of outwardly directed partial pressure (ΔP_{NH_3}) and electrochemical gradients ($F_{NH_4^+}$), such that the gill appears to be set up to scavenge ammonia when it is elevated in the environment. However, our second hypothesis was confirmed: ammonia uptake and the associated rise in plasma T_{Amm} resulted in a marked elevation of urea-N excretion, probably reflecting increased urea-N synthesis by the OUC. In accord with our third prediction, two Rh proteins (*Rhbg* and *Rhp2*) were expressed at the mRNA level in the gill and other tissues and a third (*Rhag*) was expressed in the red blood cells. Furthermore, branchial *Rhbg* and erythrocytic *Rhag* responded to HEA exposure, but with a downregulation rather than the expected upregulation seen in most previous studies on teleosts. Finally, other components of the metabolon involved in the coupling of ammonia excretion and Na^+ uptake in freshwater fish gills did respond at the mRNA level to HEA exposure, but not in a manner comparable with previous findings on teleosts. Clearly, the responses of this marine elasmobranch to HEA are fundamentally different from those in freshwater teleosts, and also appear to differ from the limited data available to date on seawater teleosts.

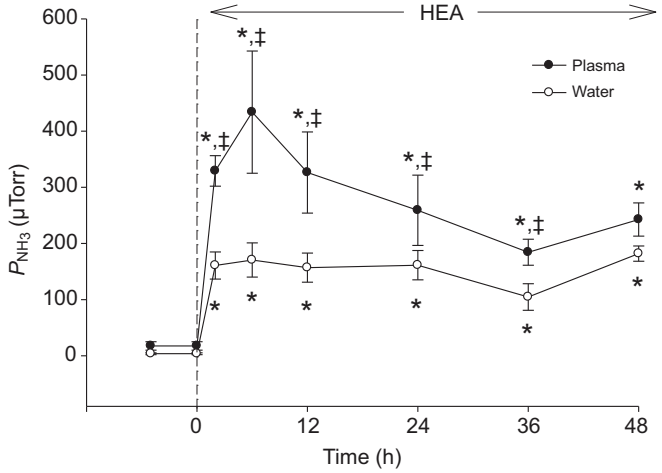

Fig. 4. Plasma and water partial pressure of NH_3 (P_{NH_3}) in *S. acanthias* exposed to HEA for 48 h. Asterisks indicate a significant difference from the pre-exposure control values and double daggers represent a significant difference between plasma and water values (P<0.05). Data are means ± s.e.m. (N=6).

Ammonia uptake and gill ammonia permeability during HEA

Within 2 h of acute HEA exposure, plasma T_{Amm} in the dogfish shark rose to levels equal to water T_{Amm} (Fig. 2). This was very different from the response seen in the only previous HEA study on a marine elasmobranch, the little skate (*R. erinacea*), where plasma T_{Amm} rose very slowly, reaching only about 50% of seawater T_{Amm} levels by 11 h (Cameron, 1986); Cameron attributed this pattern to low gill permeability to ammonia. In comparison to previous HEA studies on a variety of freshwater and marine teleosts (Cameron and Heisler, 1983; Cameron, 1986; Claiborne and Evans, 1988; Wilson and Taylor, 1992; Wilson et al., 1994; Nawata et al., 2007; Nawata et al., 2010a; Zimmer et al., 2010; Wood and Nawata, 2011; Liew et al., 2013), the rate of plasma T_{Amm} rise in the dogfish was comparable or faster, but the subsequent pattern was different. In the teleosts, plasma T_{Amm} never reached external water T_{Amm} levels, but rather was maintained at a plateau or slow decline significantly below the latter. In some of these teleost studies, flux rates of ammonia (J_{Amm}) and/or the P_{NH_3} gradients across the gills (ΔP_{NH_3}) were also measured, showing that net ammonia excretion was re-established relatively rapidly (within 3–12 h) despite the fact that

The Journal of Experimental Biology (2015) doi:10.1242/jeb.114967

Table 2. ΔP_{NH_3} and F_{NH4^+} gradients in *S. acanthias* exposed to HEA for 48 h

	0 h	2 h	6 h	12 h	24 h	36 h	48 h
ΔP_{NH_3} (μTorr)	−13.9±6.2 (6)	−168.6±49.5 (6)	−263.3±63.9 (6)*	−169.4±70.6 (6)*	−97.8±46.2 (6)*	−79.7±15.0 (6)*	−60.7±27.9 (5)
F_{NH4^+} (mV)	−22	−22.4	−29.7	−26.4	−18.3	−20.1	−10.5

Values for the partial pressure gradient of NH_3 (ΔP_{NH_3}) are means ± s.e.m. (*N*). There are no s.e.m. values or statistical tests for the electrochemical gradient for NH_4^+ (F_{NH4^+}) as values were calculated from the difference between the mean values of its two components [the Nernst potential for NH_4^+ (E_{NH4^+}) and transepithelial potential (TEP)] measured in separate series on two different sets of animals.
Values are all negative for both ΔP_{NH_3} and F_{NH4^+}, indicating that the net gradients are outwardly directed, tending to drive both NH_3 and NH_4^+ out of the fish.
*Significantly different from control (P<0.05).

ΔP_{NH_3} remained reversed, suggesting active ammonia excretion across the gills. In only one study (Wood and Nawata, 2011) was the electrochemical gradient for NH_4^+ (F_{NH4^+}) across the gills also measured, but the conclusion was the same: J_{Amm} was quickly re-established despite the fact that both F_{NH4^+} and ΔP_{NH_3} remained reversed during HEA exposure. In contrast, the dogfish shark continued to take up ammonia for 36 h and net outward J_{Amm} was only re-established at 36–48 h (Fig. 1A), yet both ΔP_{NH_3} and F_{NH4^+} were not reversed and remained negative (i.e. outwardly directed) throughout the entire HEA period (Table 2). Cardiovascular parameters were not measured in this study, so it is possible that some of the temporal changes and/or differences from teleosts could reflect responses in gill blood flow rate or perfusion pattern.

Thus, while ammonia efflux permeability of the dogfish gill is low (Wood et al., 1995), and the influx permeability of the skate gill may also be low (Cameron, 1986), the influx permeability of the dogfish gill during HEA exposure certainly is not. Indeed, ammonia is taken up for 36 h under circumstances where this should not occur passively (i.e. both ΔP_{NH_3} and F_{NH4^+} are outwardly directed; Table 2). We speculate that active ammonia uptake across the gills (i.e. 'ammonia scavenging') may occur when environmental ammonia levels are sufficiently high, and that this is a manifestation of a system which is normally used for active ammonia retention under control conditions. This system would be adaptive for an animal that needs to continuously make urea and that appears to be nitrogen limited in nature (Haywood, 1973; Wood et al., 2005; Kajimura et al., 2006; Kajimura et al., 2008). In this respect, sharks, which encounter and ingest pelagic prey only at irregular intervals, may differ from skates, which can continuously browse on benthic invertebrates. While this idea of active ammonia uptake by a fish, and therefore rectified branchial ammonia permeability, is unusual, there is a precedent in the urea-N retention mechanism at elasmobranch gills (Boylan, 1967; Wood et al., 1995; Pärt et al., 1998; Wood et al., 2013a). Urea-N retention appears to involve an active transporter, and Fines et al. (Fines et al., 2001) have presented evidence in *Squalus acanthias* for a Na^+ versus urea exchanger powered by the electrochemical Na^+ gradient across the basolateral membrane of gill cells, which would scavenge urea as it diffuses out. A comparable mechanism for ammonia scavenging such as a basolateral Na^+ versus NH_4^+ exchanger (NHE?) would minimize ammonia excretion under normal circumstances, thereby explaining the low efflux permeability yet high influx permeability, and would be recruited to actively take up ammonia from HEA water. An alternative or additional possibility would be active apical NH_4^+ uptake via substitution on the K^+ site of H^+/K^+-ATPase, which has been identified in elasmobranch gills (Choe et al., 2004). NH_4^+ is well known to be able to substitute for K^+ in a variety of transport systems, including members of this family of transport ATPases (Codina et al., 1999; Cougnon et al., 1999; Crambert, 2014).

How relevant is the present ammonia exposure scenario to real world conditions? The water T_{Amm} level used in our tests (1000 μmol l^{-1}) was chosen for comparison to the many previous HEA studies on teleosts using a similar level, as cited above. Water quality objectives for marine waters set by regulatory authorities are typically about half this value (reviewed by Ip et al., 2001). In the open oceans, ammonia concentrations are in the low micromolar range, but in coastal waters and estuaries may rise to the concentration used here as a result of anthropogenic input (Eddy, 2005), and these inputs are steadily rising as a result of non-point source runoff from agriculture (Howarth et al., 2002) and intensive aquaculture operations (Tovar et al., 2000). Furthermore, sharks appear to be attracted to ammonia (Mathewson and Hodgson, 1972; Gilbert, 1977). Thus, while the present HEA exposure may represent an extreme, it is not unrealistic, and elasmobranchs may well exhibit ammonia scavenging at lower water T_{Amm} levels.

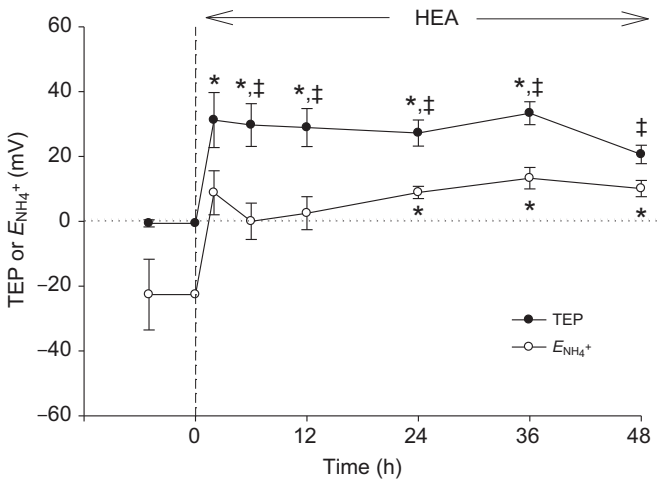

Fig. 5. Transepithelial potential (TEP) and Nernst potential for NH_4^+ (E_{NH4^+}) across the gills in *S. acanthias* exposed to HEA for 48 h. Asterisks indicate a significant difference from the pre-exposure control values and double daggers represent a significant difference between the TEP and E_{NH4^+} values at the same time point (P<0.05). Data are means ± s.e.m. (N=6).

Fig. 6. Tissue distribution of *Rhbg* and *Rhp2* expression in *S. acanthias* as determined by PCR. Rh glycoprotein expression was assessed in red blood cells (Blood), cardiac stomach (Stomach 1) and pyloric stomach (Stomach 2). *β-actin* expression was used as a positive control.

The Journal of Experimental Biology (2015) doi:10.1242/jeb.114967

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MSNASTNMRLKLPLICLLLQLALIILFAIFVRYDGDTDPKEWFRRKAQENVTNAENEFYFRYPSFQDVHV 70
MIFVGFGFLMTFLKKYGYSSVGFNFVLASFSLQWATLVQGWLHGMKEDGKIHIGIESMINADFCTGSVLI 140
SFGALLGKCSPVQMLVMTIFESALFPINEFILLSLLQIKDAGGSMTIHTFGAYFGLVIARVLYRPQLDKS 210
RHREGSVYHSDLFAMIGAIYLWMFWPSFNSAVTTLGSAQHRTALNTYYSLAACTLATFATSSAVNEEGKL 280
DMVHVQNAALAGGVALGTAGEMMLTPYGAMIVGFIAGVISTLGFKYLTPILDSKLKIQDTCGVHNLHGMP 350
GILGGLVGAILAAMATKEVYGDGLQDVFPLLDNERSAKQQGMYQALGLAVTLGIALIGGTIVGFILKLPI 420
WGAPADTQCFSDQPYWELPDDDWGHGEMVPVKIEEVEKLT 460
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Fig. 7. Amino acid sequence of *S. acanthias* Rhbg. Twelve predicted transmembrane domains are underlined. Arrow indicates a predicted *N*-glycosylation site.

Response of TEP to HEA

Under control conditions, TEP was close to 0 mV in *S. acanthias*, in agreement with previous measurements in marine elasmobranchs (Bentley et al., 1976; Evans, 1982; Evans et al., 1982; Evans and More, 1988). A large and persistent rise (approximately +30 mV) in TEP during HEA exposure paralleled the simultaneous rise in $E_{NH_4^+}$, such that $F_{NH_4^+}$ remained virtually unchanged (Fig. 5, Table 2). This phenomenon of a more positive TEP (inside) during HEA has now been seen in three freshwater teleosts [trout, goldfish and carp (Wood and Nawata, 2011; Liew et al., 2013)], but curiously did not occur in seawater trout (Wood and Nawata, 2011). However, the rise in TEP in the dogfish in seawater was greater than that in any of these studies. Potential mechanisms behind the phenomenon have been discussed (Wright and Wood, 2012) but the proximate cause remains unknown. Had this not occurred, $F_{NH_4^+}$ would have favoured NH_4^+ uptake to an even greater extent during HEA. Therefore, it may be interpreted as a mechanism to prevent NH_4^+ uptake in teleosts and to modulate it in this elasmobranch so that plasma T_{Amm} levels do not overwhelm the capacity of the OUC to consume ammonia.

Conversion of ammonia to urea-N in response to HEA

In accord with our prediction, HEA exposure induced a marked increase in $J_{Urea\text{-}N}$ (Fig. 1B). Similar responses have been seen in two ureotelic teleosts which express the OUC (see Introduction) and in an earlier study in which *S. acanthias* were infused with NH_4Cl (Wood et al., 1995). This response became significant at 12–48 h of HEA exposure and probably represents an increase of urea production rate by the OUC. It may be the reason why plasma T_{Amm} levels were subsequently reduced significantly below water T_{Amm} concentrations (Fig. 2). This response was also suggested, but not measured, by Cameron (Cameron, 1986) in his HEA studies on *R. erinacea* as an explanation (alternative or additional to low gill ammonia permeability) for the blunted rise in plasma T_{Amm} seen in that species. The very high affinity of shark glutamine synthetase for ammonia, together with its intramitochondrial location in association with carbamoyl phosphate synthetase III (Shankar and Anderson, 1985) would allow it to act as an ammonia trap, funnelling this valuable nitrogen source directly into the OUC for urea-N synthesis.

The amount of 'extra' urea-N excreted over the 48 h period (Fig. 1B) was about 2.5-fold greater than the amount of ammonia-N taken up (Fig. 1A). Why does the shark not save this urea-N if it is so valuable? One answer may be that its storage anywhere in the body would have unfavourable osmotic effects, raising osmolality substantially above environmental levels, and inducing unwanted water influxes. An alternative possibility is that not all of this 'extra' urea-N originated from new synthesis, but rather from a decline in body stores. The terminal samples of series 1 indicated a drop of 147 mmol l^{-1} of plasma $T_{Urea\text{-}N}$, whereas in series 2 the drop was a more modest 43 mmol l^{-1} (Table 1). If distributed throughout the whole body water pool, even this smaller decrease would be almost large enough to explain the 2.5-fold discrepancy. Overall, we conclude that plasma T_{Amm} and therefore intramitochondrial T_{Amm} levels during HEA are reset to levels where urea-N synthesis is over-stimulated, resulting in extra urea-N being released to the environment, presumably through a downregulation of the retention mechanisms at the gills and kidney. A similar 'over-release' of urea-N by both these routes was seen in dogfish infused with NH_4Cl

```
S. acanthias  MSAIRCRLPFLVLFLEGILLVLIALFVTYDEHSDAAAQNNETVHNHNQLY 50
T. scyllium   ............I.........................S...D.K.....

S. acanthias  AIFPIFQDIQVMIFVGFGLLMGFLKKYGYGGIAFNFMIAVFSVQWALLVQ 100
T. scyllium   ....V...V.............................L.............

S. acanthias  GWFYHFHHGKIHIGVYNLLTAETACATVMISFGAVLGKTSPVQILILALL 150
T. scyllium   .........H.....................................S..

S. acanthias  EVPIFTATEWVIMELLKIKDVGGSITIHLFACYFGLSVTTVLYRPGLKDG 200
T. scyllium   ..........I.....................................G.

S. acanthias  HKDEGADYNSDKLAMLGTLLLWVFWPSFNSVFASTGDGQHRAVIHTYIGL 250
T. scyllium   ..................................S.H......L......

S. acanthias  SSCTLTTFAISSLLDKRGKINIAHIQNAALAGGVAVGSAADMMVTPAGAF 300
T. scyllium   ..................................................

S. acanthias  TLGCIASVLCTVGFKYLTPFLAKKLKIQDVCGINNLHGIPGFIGAIAGIA 350
T. scyllium   ........................R..................V......V

S. acanthias  TILLTADERYGQGLYDTFPERVPKEGDRKLVELVRLLPQLKAGGGRSAWD 400
T. scyllium   ....A...S..H..................A..........P........

S. acanthias  QAQYQAAAIGVCLGIAVLGGTITGFILKLPFLAQPKDEYCFNDDPYFEVP 450
T. scyllium   .....................V..........................

S. acanthias  EVEEKEEFEFTNKNNANNNQRLKLPV 476
T. scyllium   ............S............
```

Fig. 8. Amino acid alignment of Rhp2 of *S. acanthias* and the houndshark (*Triakis scyllium*). Dots represent identical amino acids.

The Journal of Experimental Biology (2015) doi:10.1242/jeb.114967

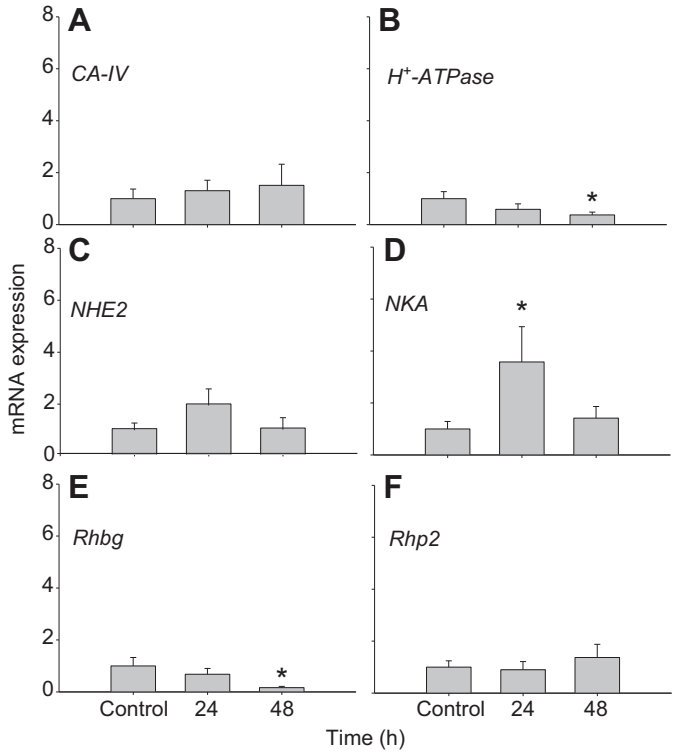

Fig. 9. mRNA expression levels in the gills of *S. acanthias* exposed to HEA for 48 h. (A) *Carbonic anhydrase IV* (*CA-IV*), (B) *H*$^+$*-ATPase*, (C) *Na*$^+$*/H*$^+$ *exchanger 2* (*NHE2*), (D) *Na*$^+$*/K*$^+$*-ATPase* (*NKA*), (E) *Rhbg* and (F) *Rhp2*. Asterisks indicate a significant difference from the control. Data are means ± s.e.m. (*N*=6).

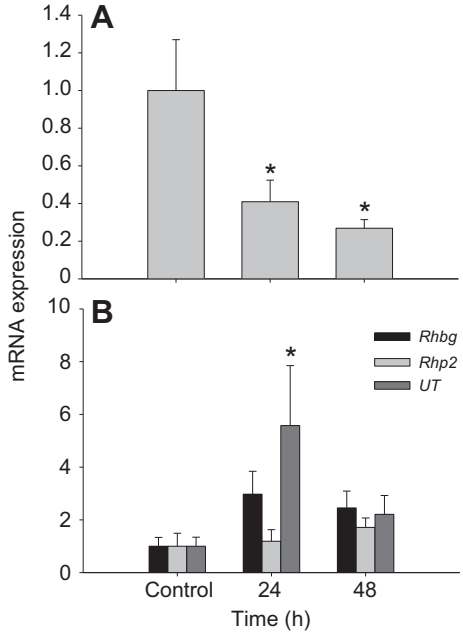

Fig. 10. mRNA expression levels in red blood cells and kidney of *S. acanthias* exposed to HEA for 48 h. (A) *Rhag* in red blood cells and (B) *Rhbg*, *Rhp2* and urea transporter (*UT*) in the kidney. Asterisks represent a significant difference from the control. Data are means ± s.e.m. (*N*=6).

(Wood et al., 1995). The observed increase in *UT* levels in the kidney (Fig. 10A) may have contributed to this response.

Molecular responses to HEA

Three Rh cDNAs (*Rhbg*, *Rhp2*, *Rhag*) were cloned. *Rhbg* and *Rhp2* mRNA were expressed in every tissue examined – red blood cells, brain, gill, intestine, kidney, liver, muscle, rectal gland, cardiac stomach and pyloric stomach (Fig. 6) – and *Rhag* was expressed in red blood cells. In elasmobranchs, there are only two previous reports of Rh proteins – *Rhbg* in the intestine, rectal gland and kidney (the only tissues examined) of the little skate, *Leucoraja erinacea* (Anderson et al., 2010), and *Rhp2* in the kidney of the houndshark, *T. scyllium* (Nakada et al., 2010). In contrast to the present findings, Nakada et al. (Nakada et al., 2010) reported that *Rhp2* was present only in the kidney, but this conclusion was based on northern blot screening, which is less sensitive than the qPCR employed here.

As branchial *Rhp2* expression did not respond to HEA exposure (Fig. 9F), it is difficult to draw any conclusions on its function. However, the widespread tissue distribution of *Rhp2* suggests that it could be involved in processes other than ammonia reabsorption from the urine in the kidney tubules suggested by Nakada et al. (Nakada et al., 2010). Based on analogy to other fish (Wright and Wood, 2009; Wright and Wood, 2012; Weihrauch et al., 2009), dogfish *Rhbg* is probably a basolateral ammonia transporter in the gills and elsewhere. *Rhbg* was expressed at high levels in the gut of the skate, and Anderson et al. (Anderson et al., 2010) speculated that it is involved in taking up ammonia generated in the chyme for urea-N synthesis by the intestine. While we were unsuccessful in finding a sequence for *Rhcg* (the putative apical ammonia transporter), subsequent searches of the *Squalus acanthias* transcriptome suggest that it is present (A. Clifford and G. Goss, personal communication). Rh proteins are bidirectional transporters (e.g. Nawata et al., 2010b). Thus, in the shark, their major roles may not be in ammonia excretion, but rather in ammonia scavenging at the gills and gut, transport through the bloodstream to internal sites of urea-N synthesis (liver, muscle and intestine), and transcellular or intracellular transport at these sites to intramitochondrial glutamine synthetase (Shankar and Anderson, 1985) for conversion into urea-N by the OUC.

Apart from *NKA* (Fig. 9D), components of the metabolon that flexibly couple branchial ammonia excretion to Na^+ uptake in teleost fish (see Introduction) did not exhibit the expected upregulation during HEA exposure in the shark (Fig. 9), in contrast to findings in the former (Nawata et al., 2007; Zimmer et al., 2010; Wood and Nawata, 2011; Wood et al., 2013b; Sinha et al., 2013). The transient *NKA* upregulation at 24 h in the shark (Fig. 9D) could be associated with maintaining the Na^+ electrochemical gradient for ammonia scavenging by a basolateral Na^+ versus NH_4^+ exchanger, as speculated earlier.

It is particularly notable that branchial *Rhbg* expression was progressively downregulated during HEA exposure (Fig. 9E), and the same trends were seen in branchial *H*$^+$*-ATPase* (Fig. 9B) and erythrocytic *Rhag* expression (Fig. 10A). This pattern of downregulation of *Rhbg* and *Rhag* has been seen previously in only one teleost exposed to HEA, the marine pufferfish (*Takifugu rubripes*), but in that study gill *H*$^+$*-ATPase* was upregulated (Nawata et al., 2010a). In the pufferfish study, the *Rh* responses were interpreted as a mechanism to reduce ammonia loading, and the same may be true in the shark. As with the rise in TEP (Fig. 5), had these changes not occurred, ammonia loading might have overwhelmed the capacity of the OUC to consume ammonia, and it might have been impossible to reduce plasma T_{Amm} concentration during the later phases of HEA exposure (Fig. 2). With respect to the *H*$^+$*-ATPase* response, this enzyme does not appear to be apically

located, in contrast to teleosts. Instead, all current models for the elasmobranch gill indicate that H^+-ATPase functions basolaterally in those ionocytes, which excrete base (Piermarini and Evans, 2001; Piermarini et al., 2002; Tresguerres et al., 2005; Reilly et al., 2011), so that H^+ ions are returned to the blood when HCO_3^- is excreted apically at times of blood alkalosis (Tresguerres et al., 2005; Tresguerres et al., 2006; Tresguerres et al., 2007; Roa et al., 2014). In the present study, there was a slight tendency towards alkalosis during HEA exposure, with a significant rise in plasma [HCO_3^-] (Fig. 3), so downregulation of *H^+-ATPase* expression would not be expected. We speculate therefore that H^+-ATPase may have an additional role, that of diffusion trapping of NH_3 at the extracellular side of the basolateral membrane. In future studies, it will be of interest to see whether H^+-ATPase and Rhbg are co-localized in the base-excreting ionocytes.

Future directions

We have presented a speculative model in which the gill of this nitrogen-limited predator is poised not only to prevent nitrogen loss but also to scavenge ammonia-N from the environment for the purpose of enhancing urea-N synthesis. However, ammonia uptake against gradients is modulated to prevent the regulatory capacities of the animal being overwhelmed. In future studies, many aspects of this model can be evaluated, such as the concentration-dependent kinetics of ammonia uptake, the identification and localization of the transporters involved, and the signals that control the system. For example, are the molecular responses identified in the present study a response to internal or external ammonia elevation? A recent study of ventilatory control in *S. acanthias* found that the hyperventilatory response to HEA was mediated by internal rather than external ammonia levels (Zhang et al., 2014).

MATERIALS AND METHODS

Experimental animals

Experiments were performed at Bamfield Marine Sciences Centre (BMSC), BC, Canada, in June and July. Pacific spiny dogfish (*S. acanthias suckleyi*, 0.7–2.9 kg) were obtained by trawl netting in nearby Barkley Sound, BC, Canada, in May and June, under a licence from the Department of Fisheries and Oceans, Canada. [Note Ebert et al. (Ebert et al., 2010) have recently proposed that these north-east Pacific dogfish may be a separate species (*Squalus suckleyi*) rather than a subspecies of *Squalus acanthias*.] Only male fish were retained for purposes of population conservation, as females were invariably pregnant. At BMSC they were held in a large 151,000 l circular tank served with running seawater at the experimental temperature (11–12°C), salinity (30±2‰) and pH (7.90±0.15). During this period, the dogfish were fed every 3–4 days with a 3% ration of dead hake (*Merluccius productus*), but removed to smaller 1500 l tanks in batches of 6–10 for 1–2 weeks, fasting under the same conditions, prior to experimentation. All experimental procedures conformed to the guidelines of the Canada Council for Animal Care, and were approved by BMSC and McMaster University Animal Care Committees.

All experiments were performed in covered, polyurethane-coated wooden fish boxes (105 cm length×16.5 cm width×25 cm depth) fitted with perimeter aeration, as described by Wood et al. (Wood et al., 1995). The boxes were served with a flow-through of 1 l min^{-1}, but could be closed for flux measurements, at which time they were bathed in an external running seawater bath to maintain temperature (11–12°C). The fish were placed in the boxes for 24 h before experiments were started.

In series 2 and 3, the dogfish were fitted with indwelling caudal artery catheters as described by De Boeck et al. (De Boeck et al., 2001). These facilitated repetitive blood sampling and measurements of TEP without disturbance. For cannulation, the fish were anaesthetized in MS-222 (0.15 g l^{-1}; Syndel Laboratories, Qualicum, BC, Canada), weighed, and artificially ventilated on an operating table. The vertebral column was exposed by a lateral 5 cm incision through the muscle of the caudal peduncle, and the catheter (Clay-Adams PE50, Becton Dickinson, Franklin Lakes, NJ, USA) was implanted into the artery through a small hole in the haemal canal. The catheter was filled with dogfish saline (see Wood et al., 2013a) containing 50 i.u. ml^{-1} lithium heparin (Sigma-Aldrich, St Louis, MO, USA). A PE160 sleeve, heat-flared at both end, 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. After surgery, the dogfish were revived in anaesthetic-free water and transferred to the wooden fish boxes for 24 h to recover.

For HEA exposures, additions from a 1 mol l^{-1} stock solution of NH_4HCO_3 (pH 7.90) were made, sufficient to raise the total ammonia concentration to nominally 1 mmol l^{-1}.

Experimental series

Series 1

This series (N=8–12) examined the impact of HEA exposure on ammonia (J_{Amm}) and urea-N ($J_{Urea\text{-}N}$) flux rates in non-cannulated dogfish. Fluxes were measured for a 12 h pre-exposure control period, and then over successive 12 h periods of HEA. For flux measurements, flow-through to the box was stopped and the water level set to a mark representing 40 l. Water samples (10 ml) for total ammonia-N (T_{Amm}) and urea-N ($T_{Urea\text{-}N}$) measurements were taken at the start and end of each flux period, up to and including 48 h, and were stored at −20°C. The water in the boxes was renewed every 12 h. After the 48 h water sample, the fish were rapidly anaesthetized by addition of a concentrated solution of neutralized MS-222 (pH 7.9) so as to bring the water concentration to 0.25 g l^{-1}. A terminal blood sample was drawn by caudal puncture into a 5 ml heparinized syringe. Plasma was immediately separated by centrifugation (1 min, 5000 ***g***) and flash-frozen in liquid N_2, then stored at −80°C for later measurement of plasma urea-N and plasma T_{Amm}. An additional water sample was drawn simultaneously for measurement of T_{Amm} in water. This was to determine the plasma-to-water concentration gradient for T_{Amm}. A control group (N=7) was sampled in the same manner. Some of the fish were also sampled for molecular end points (see series 4). All fish were weighed.

Series 2

This series (N=6) examined the impact of HEA exposure on arterial plasma ammonia parameters (T_{Amm}, Pa_{NH3}) relative to the same parameters in the external water, so as to allow precise calculation of trans-branchial P_{NH3} and electrochemical NH_4^+ gradients. Additionally, the blood sampling facilitated the measurement of arterial blood respiratory gases and acid–base status, and plasma urea-N and major electrolyte concentrations. The fish were exposed to HEA in the same manner as in series 1, with renewal of the HEA water at 12 h intervals. Arterial blood samples (600 and 400 µl) were drawn into two ice-cold gas-tight glass syringes (Hamilton, Reno, NV, USA) under pre-exposure control conditions (0 h), and then at 2, 6, 12, 24, 36 and 48 h of HEA exposure. Water samples were drawn simultaneously for pH measurements and then stored at −20°C for later analyses of T_{Amm} and P_{NH3}. The smaller blood sample was analysed for pHa and Pa_{O2}. The larger blood sample was immediately centrifuged (1 min, 5000 ***g***); the plasma was decanted, then divided into three separate aliquots. One was analysed immediately for plasma total CO_2 concentration; the other two were flash-frozen in liquid N_2 and stored at −80°C for subsequent analyses of (i) plasma T_{Amm} and $T_{Urea\text{-}N}$, and (ii) plasma electrolytes. 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.

Series 3

This series (N=6) examined the impact of HEA exposure on branchial TEP in dogfish fitted with arterial catheters. TEP was measured under pre-exposure control conditions (0 h), and then at 2, 6, 12, 24, 36 and 48 h of HEA exposure.

Series 4

Non-cannulated dogfish were sampled for molecular end points at 0 h (pre-exposure control), 24 h and 48 h of exposure to HEA (N=6 at each time) as

in series 1. At sampling, the fish was anaesthetized with 0.25 g l^{-1} MS-222 (pH 7.9), a terminal blood sample was drawn by caudal puncture or via the caudal artery catheter, and 1000 i.u. lithium heparin (in 1 ml saline) was injected. The red blood cells were immediately separated by centrifugation (1 min, 5000 ***g***), and then preserved in liquid N_2, taking care to avoid contamination with white blood cells. The fish was then artificially ventilated on an operating table with HEA or control water, as appropriate, and the ventral aorta was exposed by dissection and cannulated with PE160 tubing, taking care to avoid air bubbles. Perfusion with an ice-cold 500 mmol l^{-1} NaCl solution containing 10 i.u. ml^{-1} of lithium heparin was then commenced, using a Model 1100 cardiac pump (Harvard Apparatus, Holliston, MA, USA) set to a working mode of 1/3 systole, 2/3 diastole, a heart rate of 30 beats min^{-1}, and a stroke volume adjusted so that the output was ~20 ml kg^{-1} min^{-1}. Once the gills and tissues were cleared of blood (2–4 min), samples of gill, kidney, brain, intestine, liver, muscle, rectal gland, cardiac stomach (1) and pyloric stomach (2) were removed and stored in RNAlater (Sigma-Aldrich).

Blood and water analyses and calculations

Pa_{O_2}, pHa and water pH were measured using Radiometer electrodes (Copenhagen, Denmark) kept at the experimental temperature with water jackets. Outputs of the electrodes (E5036 for P_{O_2}; GK2401C for pH) were displayed on Radiometer pHM 71 and pHM 72 acid–base analysers. True plasma total CO_2 was measured by a Model 965 CO_2 analyser (Corning, Midland, MI, USA). Pa_{CO_2} and $[HCO_3^-]a$ were calculated using the solubility of carbon dioxide (α_{CO2}), the apparent pK (pK_{app}) for dogfish plasma, and rearrangements of the Henderson–Hasselbalch equation according to Boutilier et al. (Boutilier et al., 1984).

TEP was recorded using 3 mol l^{-1} KCl–agar bridges connected via Ag/AgCl electrodes (WPI, Sarasota, FL, USA) to a high impedance electrometer (Radiometer pHM 84 meter). The reference bridge was placed in the water in the fish chamber, and the measurement bridge was connected to the arterial blood catheter. TEP values (mV) were expressed relative to the water side as 0 mV after correction for junction potential.

Plasma T_{Amm} was measured enzymatically (L-glutamate dehydrogenase method) (Mondzac et al., 1965) using an ammonia reagent kit (Product No. 85446, Raichem Life Sciences, Mumbai, India) on the first thaw of frozen plasma. Water T_{Amm} was measured colorimetrically by the salicylate–hypochlorite method (Verdouw et al., 1978); the two methods were cross-validated in seawater. The colorimetric assay, while economical and very accurate, cannot be used in plasma because of protein interference. Plasma and water ammonia partial pressures (P_{NH_3}) and ammonium ion concentrations ($[NH_4^+]$) were calculated from the respective T_{Amm} and pH measurements, again using the Henderson–Hasselbalch relationship, as detailed by Wright and Wood (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 (Cameron and Heisler, 1983) on trout, with appropriate adjustments for ionic strength. A sensitivity analysis demonstrated that the adjustments used did not affect the conclusions of this study.

The P_{NH_3} gradient across the gills (ΔP_{NH_3}) was calculated as the difference between simultaneous measurements of water P_{NH_3} and arterial blood plasma Pa_{NH_3}. A positive value of ΔP_{NH_3} will tend to drive NH_3 into the fish, while a negative value will drive NH_3 out of the fish. The Nernst potential for NH_4^+ ($E_{NH_4^+}$) was calculated in the standard fashion where $[NH_4^+]_{in}$ is the concentration of NH_4^+ in the arterial blood plasma and $[NH_4^+]_{out}$ is the concentration of NH_4^+ in the outside water. The true electrochemical potential or net driving force ($F_{NH_4^+}$) for NH_4^+ across the gills was calculated according to Kirschner (Kirschner, 1970) as the difference between $E_{NH_4^+}$ and TEP. A positive value of $F_{NH_4^+}$ will tend to drive NH_4^+ into the fish, while a negative value will drive NH_4^+ out of the fish. Equations for ΔP_{NH_3}, $E_{NH_4^+}$ and $F_{NH_4^+}$ have been published previously (Wood and Nawata, 2011).

Plasma and water $T_{Urea\text{-}N}$ concentrations were measured colorimetrically by the diacetyl monoxime method (Rahmatullah and Boyde, 1980). Plasma Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were determined by flame atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Australia) and Cl^- was measured by coulometric titration (Radiometer CMT-10 chloridometer).

Flux rates of total ammonia (J_{Amm}) and urea-N ($J_{Urea\text{-}N}$) were calculated from changes of concentration in the closed chambers, factored by fish mass, volume and time. A negative J_{Amm} indicates a net excretion, and a positive J_{Amm} a net uptake of ammonia into the fish.

Molecular analyses

Total RNA was extracted from dogfish tissues with Trizol (Invitrogen, Burlington, ON, Canada) and quantified spectrophotometrically (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE, USA). First strand cDNA was synthesized from 1 µg of DNaseI-treated total RNA with Superscript II reverse transcriptase (Invitrogen).

cDNA sequences of *Rhag*, *Rhbg* and *Rhp2* were obtained by 5′- and 3′-rapid amplification of cDNA ends (Smart RACE cDNA amplification kit, BD Bioscience Clontech, Mississauga, ON, Canada) using erythrocyte and gill cDNA with cloning primers (Table 3) and the protocol described previously (Nawata et al., 2007).

Hydropathy profile and *N*-linked glycosylation site predictions were made using TMHMM (Krogh et al., 2001; Sonnhammer et al., 1998) and ScanProsite (de Castro et al., 2006), respectively.

Tissue distribution of *Rhbg* and *Rhp2* was determined by PCR with the primer sets listed in Table 3 at 35 cycles for *Rhbg and Rhp2* and 25 cycles for *β-actin*. Products were electrophoresed on 1% agarose gels stained with ethidium bromide.

qPCR was performed using the above synthesized cDNA to determine mRNA levels of *CA-IV*, *Rhbg*, *Rhp2*, *H^+-ATPase* (V-type, B subunit), *NHE2*, *NKA* (α-subunit), *UT* in the gill, *Rhag* in the erythrocytes, and *Rhbg*, *Rhp2* and *UT* in the kidney. All primer sets used (Table 3) were validated and products sequenced beforehand. qPCR reactions were performed on a Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) under conditions detailed previously (Nawata et al., 2007) with melt-curve analysis and no-template controls run in tandem. Values were extrapolated from standard curves and were normalized to ng total RNA.

Statistics

Data are expressed as means ± 1 s.e.m. (N), where N is the number of fish. In series 1, 2 and 3, data were analysed by repeated measures ANOVA, with

Table 3. Primers used for cloning, PCR tissue screen and qPCR (5′→3′)

Primer	Forward	Reverse	Accession no.
Rhag (cloning)	gccatgaaaatgaaggatctgtgtat	cgctccaaatgaaatcaggacggttg	KJ960196
Rhbg (cloning)	cagcgctcaacaccggactgccctcaacacctac	gcatgccgtggaggttgtgcacaccgcacg	KJ960197
Rhp2 (cloning)	aactcggacaagctggccatgctgg	tcaggatctgcaccgggctggttttg	KJ960198
Rhag	ctttgttggatttggctttc	aatgaaatcaggacggttgc	KJ960196
Rhbg	gctcatctgcctcctgttg	tgggatacctgaagtagaactcg	KJ960197
Rhp2	ggagctgctgaagatcaagg	agacccacagcaacaaggtc	KJ960198
β-Actin	tgcactggactttgaacagg	ttccacaggattccataccc	AY581300
CA	caatgcctcacaacacaagg	ggagaagaaaatgcgctctg	DQ092628
HATP	ggaatctcggcaatagatgg	tggcaaaattctctgcactg	EU004205
NHE	ttctggaaacggttcgactc	ccagagggtgatctcaaagg	DQ324545
NKA	tactgctcgaggtgttgtcg	gcttcaagccagctgtatcc	AJ781098
UT	aagaaagcagcaattgttgaag	gcaccacgtaagatccagtc	AF257331

appropriate transformations where necessary to pass normality and variance tests, followed by a Holm–Šidák or Dunnett's multiple comparison test to detect specific differences relative to the pre-exposure control value. Paired Student's *t*-tests were used to evaluate differences between simultaneous measurements in blood plasma versus external water. In series 4, groups were independent, so regular ANOVA followed by the LSD test was used to detect values that were significantly different. All tests were two-tailed and a significance level of 0.05 was used throughout.

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Competing interests
The authors declare no competing financial interests.

Author contributions
The study was jointly conceived by C.M.N., P.J.W. and C.M.W.; C.M.N., C.M.W. and P.J.W. performed the experiments; C.M.N. and C.M.W. analysed the data; C.M.W. and C.M.N. wrote the manuscript; P.J.W. revised the manuscript.

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