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RESEARCH ARTICLE

Branchial osmoregulation in the euryhaline bull shark, *Carcharhinus leucas*: a molecular analysis of ion transporters

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SUMMARY

Bull sharks, *Carcharhinus leucas*, are one of only a few species of elasmobranchs that live in both marine and freshwater environments. Osmoregulation in euryhaline elasmobranchs is achieved through the control and integration of various organs (kidney, rectal gland and liver) in response to changes in environmental salinity. However, little is known regarding the mechanisms of ion transport in the gills of euryhaline elasmobranchs and how they are affected by osmoregulatory challenges. This study was conducted to gain insight into the branchial ion and acid–base regulatory mechanisms of *C. leucas* by identifying putative ion transporters and determining whether their expression is influenced by environmental salinity. We hypothesised that expression levels of the Na^+/K^+-ATPase (NKA) pump, Na^+/H^+ exchanger 3 (NHE3), vacuolar-type H^+-ATPase (VHA) and anion exchanger pendrin (PDN) would be upregulated in freshwater (FW) *C. leucas*. Immunohistochemistry was used to localise all four ion transporters in gills of bull sharks captured in both FW and estuarine/seawater (EST/SW) environments. NHE3 immunoreactivity occurred in the apical region of cells with basolateral NKA expression whereas PDN was apically expressed in cells that also exhibited basolateral VHA immunoreactivity. In accordance with our hypotheses, quantitative real-time PCR showed that the mRNA expression of NHE3 and NKA was significantly upregulated in gills of FW-captured *C. leucas* relative to EST/SW-captured animals. These data suggest that NHE3 and NKA together may be important in mediating branchial Na^+ uptake in freshwater environments, whereas PDN and VHA might contribute to Cl^-/HCO_3^- transport in marine and freshwater bull shark gills.

Key words: NHE3, elasmobranch, euryhaline.

INTRODUCTION

Elasmobranch fishes reside primarily in marine environments; however, a limited number of species have the capacity to inhabit brackish and freshwater (FW) environments for prolonged periods (Martin, 2005). As seawater (SW) and FW are vastly different osmotic and ionic media, elasmobranchs in FW face opposite osmoregulatory problems from that of marine forms. It is well established that marine elasmobranchs retain large quantities of urea and electrolytes in the body fluids so as to become slightly hyper-osmotic (1000–1150 mOsm), although hypo-ionic to the surrounding SW (~900–1000 mOsm) (Smith, 1931b; Robertson, 1975; Shuttleworth, 1988; Evans et al., 2004). These fish experience a constant influx of water by osmosis and a large diffusional gain of NaCl across the gills. In contrast, the osmolality of euryhaline elasmobranchs in FW (1 mOsm) is considerably reduced relative to marine conspecifics (595–642 mOsm), but remains significantly hyper-osmotic and hyper-ionic to the external milieu (Smith, 1931a; Piermarini and Evans, 1998; Pillans and Franklin, 2004; Anderson et al., 2005; Pillans et al., 2005). Thus, elasmobranchs in FW face a larger osmotic influx of water, whereas Na^+ and Cl^- ions are lost by diffusion and in the urine.

The hyper-osmoregulatory strategy and associated influx of water presents a significant osmoregulatory challenge for elasmobranchs resident in and migrating between FW and SW. The large water gain by osmosis in FW must be compensated for by increased water excretion, and the loss of Na^+ and Cl^- ions must be balanced by active ion uptake mechanisms and/or ingestion of food. In FW teleosts, branchial mitochondrion-rich cells (MRCs) perform uptake of Na^+ and Cl^- from ambient water into the blood, compensating for passive salt efflux down the large internal–external ionic gradient (Garcia Romeu and Maetz, 1964; Perry, 1997; Wilson et al., 2000; Evans, 2008). Osmotic and ionic regulation in elasmobranchs is achieved through the function and integration of multiple organs, including the rectal gland, kidneys, liver and gills (reviewed by Hazon et al., 2003; Evans et al., 2004). The rectal gland of marine elasmobranchs eliminates excess Na^+ and Cl^- acquired as a consequence of living in SW, thereby maintaining plasma ionic homeostasis (Burger and Hess, 1960). In FW-acclimated elasmobranchs, salt excretion by the rectal gland is markedly reduced, as evidenced by a reduction in activity of the ion pump, Na^+/K^+-ATPase (NKA) (Piermarini and Evans, 2000; Pillans et al., 2005). Moreover, in the stenohaline FW stingrays *Potamotrygon* spp., the gland is greatly atrophied and is non-functional (Thorson et al., 1978). The kidneys are important for effective osmoregulation in FW, producing a large volume of urine in response to osmotic water gain, while also facilitating tubular reabsorption of urea and Na^+ and Cl^- ions to reduce urinary osmolyte losses (Payan et al., 1973; Janech and Piermarini, 2002; Janech et al., 2006). The modulation of hepatic urea production is also critical for euryhaline elasmobranchs to maintain plasma osmolality as appropriate with changes in environmental salinity (Tam et al., 2003; Anderson et al., 2005).

Unlike teleost fish, where the mechanisms of ion transport across the gills have been widely studied (for reviews, see Evans et al., 2005; Hwang and Lee, 2007), our understanding of gill ion regulation in elasmobranchs is relatively limited. Although the gills of elasmobranchs do exhibit MRCs, they are distinctly different from those of teleosts (Wilson and Laurent, 2002; Evans et al., 2005). In SW, the gills of elasmobranchs are generally not considered important in the process of unidirectional NaCl efflux, largely because of the presence of the rectal gland (see Shuttleworth, 1988), and have been proposed to be involved in acid–base regulation (Edwards et al., 2002; Tresguerres et al., 2006; Tresguerres et al., 2007). However, for elasmobranchs inhabiting FW, the gills also appear to function more like that of FW teleosts, and actively contribute to ion uptake from the surrounding medium (Evans and Claiborne, 2009).

A series of recent quantitative gene expression and immunological studies on the euryhaline Atlantic stingray *Dasyatis sabina* have identified two distinct types of branchial MRCs, each with their own suite of ion transporters that actively participate in Na^+ and Cl^- uptake when animals are acclimated to FW (Piermarini and Evans, 2000; Piermarini and Evans, 2001; Piermarini et al., 2002; Choe et al., 2005). These include a Na^+/H^+ exchanger (NHE3), the putative ion transporter responsible for Na^+ uptake that is localised to the apical membrane of a subpopulation of MRCs (Choe et al., 2005), and the electrogenic NKA located in the basolateral membrane of the same MRC, which functions to transport Na^+ from the cells into the blood (Piermarini and Evans, 2000). In a separate gill MRC subtype, a specialised apical Cl^-/HCO_3^- exchanger (pendrin protein; PDN) along with a basolateral vacuolar-type H^+-ATPase (VHA) have been identified, and together are thought to mediate Cl^- uptake (Piermarini and Evans, 2001; Piermarini et al., 2002). In this model, VHAs actively transport protons out of the cell, leading to an increase in intracellular [HCO_3^-]. Accordingly, this generates a favourable HCO_3^- gradient, allowing Cl^- entry across the apical surface in exchange for HCO_3^- *via* PDN (Piermarini et al., 2002). Little is understood about gill osmoregulatory function in other euryhaline elasmobranchs.

The euryhaline bull shark, *Carcharhinus leucas*, is circumtropical in distribution, being found in coastal waters, brackish estuaries and FW environments (Thorson, 1971; Montoya and Thorson, 1982; Compagno, 1984; Last and Stevens, 2009). These environments can undergo regular fluxes in salinity; therefore, bull sharks in their natural environment must maintain osmotic and ionic homeostasis over a wide breadth of conditions. In contrast to *D. sabina*, which can reproduce and complete its life cycle in FW (Johnson and Snelson, 1996), *C. leucas* does not usually breed in FW environments (Montoya and Thorson, 1982). Females are thought to give birth to one to 13 pups in estuaries and river mouths, from where the young migrate and may remain far upstream for up to five years (Pillans, 2006; Last and Stevens, 2009). Juvenile bull sharks regularly inhabit the FW reaches of various rivers in northern Australia (Pillans and Franklin, 2004; Thorburn, 2006), necessitating an osmoregulatory strategy that conserves Na^+ and Cl^- ions. Although it is known that the rectal gland, kidneys and liver are vital organs contributing to the osmoregulatory plasticity of *C. leucas* (Anderson et al., 2005; Pillans et al., 2005), little is known regarding branchial osmoregulatory function in this species.

The goal of this study was to determine the role and plasticity of the gills of *C. leucas* in osmoregulation. It was hypothesised that the gills of *C. leucas* would exhibit the putative ion transporters NHE3, NKA, VHA and PDN, which might function to maintain ionic homeostasis when animals are resident in FW, and that levels of gene expression of these transporters would be upregulated or downregulated in sharks inhabiting FW or saline environments, respectively. To address these hypotheses, we determined the plasma osmolyte concentrations (which provided an indication of the shark's environmental history) and the relative gene expression levels of *slc9a3* (NHE3), *atp1a1* (NKA), *atp6v1b* (VHA) and *slc26a4* (PDN) in the gills of juvenile *C. leucas* captured in FW and estuarine/seawater (EST/SW) habitats. We also examined the distribution and specific cellular location of the aforementioned ion transporters in gills using immunohistochemistry.

MATERIALS AND METHODS

Animals and tissue sampling

Twelve juvenile bull sharks, *Carcharhinus leucas* (J. P. Müller and Henle 1839) [total length (TL)=690–1000 mm], were captured using rod and reel in FW regions (0–5 ppt) of both the Wenlock (12°31.0′S, 142°10.0′E; *N*=6) and Brisbane Rivers (27°32.0′S, 152°50.0′E; *N*=6), Queensland, Australia. Six sharks (TL=830–2100 mm; five juvenile, one sub-adult) were also captured from lower EST/SW environments (21–32 ppt) approximately 4 and 7 km from the mouth of the Pine (27°17.0′S, 153°02.0′E; *N*=3) and Mission Rivers (12°35.0′S, 141°53.0′E; *N*=3), Queensland, respectively. An additional sub-adult animal was sampled in June 2010 from Underwater World aquarium, Sunshine Coast, Queensland, Australia. This shark had been acclimated to SW (1042 mOsm; 35 ppt) for an extended period (>12 months).

Immediately after capture, animals were placed on their side onto a large wet towel and a small biopsy of gill filament tissue was cut from just above the interbranchial septum of the first holobranch (see McCormick, 1993) using instruments pre-treated with RNAse *AWAY*™ (Invitrogen, Mulgrave, VIC, Australia) to eliminate RNase contamination. To preserve cellular RNA, tissue biopsies were placed immediately into tubes containing 1 ml of RNA*later*® (Applied Biosystems, Scoresby, VIC, Australia) and stored on ice. Additional gill biopsies were also collected and fixed in 10% neutral-buffered formalin (NBF) for subsequent immunohistochemistry. After tissue sampling, sharks were placed ventral side up for venipuncture. Blood samples (1–3 ml) were collected from the caudal vein using a heparinised 3 ml syringe with a 22 gauge hypodermic needle, transferred to tubes and stored on ice. All sharks were released post-sampling. At all capture locations, an optical refractometer was used to measure environmental salinity, and additional water samples were taken and stored for measurement of environmental osmolality. Upon return to the laboratory, gill biopsies in RNA*later*® were stored at 4°C overnight before being transferred to –20°C until required for molecular analysis. Biopsies for immunohistochemistry were kept in 10% NBF overnight at 4°C before being transferred to 70% ethanol and stored at –20°C.

Plasma analysis

Blood samples were centrifuged at 10,000 ***g*** for 3 min and the plasma was collected and stored at –20°C until subsequent analysis. A Vapro 5520 vapor pressure osmometer (Wescor, Logan, UT, USA) was used to determine plasma osmolality in triplicate. Plasma urea, Na^+, Cl^-, K^+, Mg^{2+} and Ca^{2+} concentrations were analysed by IDEXX Laboratories Pty Ltd (East Brisbane, QLD, Australia).

Gene expression

RNA extraction

Total RNA was extracted from preserved gill tissue using an Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Gladesville, NSW, Australia) according to the manufacturer's

Table 1. Sequence information on which degenerate primers were designed

Primer name	Amino acid sequences used for degenerate primer design (GenBank accession number)	Orientation	Primer (5′ to 3′)	Amplicon size (bp)
NHE3	*Homo sapiens* (AAI43329); *Dasyatis sabina* (AAT45738); *Danio rerio* (AAI71608); *Gallus gallus* (XP_418895)	Sense	GAR GAR GTB CAT GTS AAY GAR GT	864
		Antisense	GMD AGD ATR TGR TCR AAW GC	
PDN	*H. sapiens* (NP_000432); *D. rerio* (NP_001159387); *G. gallus* (XP_425419); *Xenopus laevis* (NP_001089008)	Sense	CAY ATH TCH GTK GGH CCW TTY C	537
		Antisense	GTC ACA ATH ACT TCT ATW GGW AT	
VHA	*H. sapiens* (AAP36494); *Squalus acanthias* (ABS71821); *D. rerio* (AAH59455); *G. gallus* (AAF73735)	Sense	ATH ATG GGT CAG CCH ATM AAY CC	422
		Antisense	ACR TGY TTY TCA CAY TGR TAN GC	
NKA*	*C. leucas*	Sense	CTG TCC CTC ATC CTT GGA TAC A	429
		Antisense	AGC ATC TCC AGC AAC ACT TCG	
L8	*H. sapiens* (AAH93064); *D. rerio* (AAH59473); *G. gallus* (XP_416772); *X. laevis* (NP_001080465)	Sense	GGC TAC ATC AAG GGH ATY GTS AAR GAC AT	509
		Antisense	TCH ACA GGR TTC ATR GCC ACA CC	

L8, ribosomal protein L8; NHE3; Na^+/H^+ exchanger 3; NKA, Na^+/K^+-ATPase (α1-subunit); PDN, pendrin protein; VHA, vacuolar-type H^+-ATPase (B-subunit).
*Gene-specific primers used (Meischke, 2006).

guidelines. Briefly, gill tissue (~10–100 mg) was homogenised in 1 ml of PureZOL™ (Bio-Rad) reagent using an Ultra-turrax (IKA, Staufen, Germany) disperser before centrifugation (4°C) in 0.2 ml chloroform. The aqueous phase containing RNA was mixed with approximately 0.5 ml of 70% ethanol and collected by means of a nucleic acid binding column. A DNase digestion step was incorporated to eliminate genomic DNA. RNA was eluted into nuclease-free water, aliquotted and stored at –80°C. RNA yield and purity were determined on an ND-1000 spectrophotometer (A_{260}/A_{280} ratio; NanoDrop Technologies, Inc., Wilmington, DE, USA) and visualised on a 1.6% agarose gel.

cDNA synthesis

RNA was reverse transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad), with a blend of oligo (dT) and random primers. A set amount of total RNA (2 µg) per individual was used for each reaction protocol. A 20 min incubation of the product (37°C) with 0.5 µl RNase H removed residual RNA, before samples were purified using a QIAQuick PCR Purification Kit (Qiagen, Doncaster, VIC, Australia) and resuspended in 50 µl RNase-/DNase-free water.

Degenerate primer design, gene amplification and sequencing

Degenerate primers were designed to amplify conserved regions of the vertebrate genes *slc9a3* (NHE3), anion exchanger *Slc26a4* (PDN), *atp6v1b* (VHA) and, as a reference gene, ribosomal protein L8 (Table 1). Known protein sequences from four vertebrates (human, elasmobranch, teleost and another representative) were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST). Protein sequences were aligned using the program ClustalW (www.genome.jp/tools/clustalw/), before degenerate primers were designed from highly conserved regions of amino acid sequence within the alignment. The NKA (α1-subunit) primers used in this study were specific for *C. leucas* (Meischke, 2006). Using gill cDNA, the target genes were amplified using standard PCR in 20 µl reactions. The stringency of the reaction was adjusted for the degenerate primers by reducing the annealing temperature from 60 to 50°C. PCR products were size separated by 1.6% agarose gel electrophoresis, gel isolated and purified (Freeze 'N Squeeze DNA Gel Extraction Spin Columns, Bio-Rad). Using both the forward and reverse degenerate primers, purified products were directly sequenced at the Australian Genome Research Facility (AGRF) in Brisbane, Australia.

Quantification of mRNA using real-time PCR (qRT-PCR)

Sequence-specific primers for qRT-PCR (size 170- to 230-bp amplicons) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/; Table 2). The specificity of primers was validated by direct sequencing of amplified products (AGRF). All qRT-PCR reactions contained 4 µl of a 1:10 dilution of cDNA, 100 nmol l^{-1} of each primer, 10 µl of iQ SYBR Green supermix (Bio-Rad) and 5.6 µl of RNase-free water in a total volume of 20 µl. All reactions were run in triplicate and performed on a sequence detection thermal cycler (MiniOpticon™, Bio-Rad). Cycling parameters were: initial denaturation for 3 min at 95°C, followed by 40 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. Melt curve analysis was conducted after each reaction to verify that there was only a single product in the reaction. Each assay also included a no-reverse transcriptase and a no-template control.

Relative quantification

Relative gene expression ratios were calculated using the mathematical model of Pfaffl (Pfaffl, 2001), where the ratio is equal to: $E_{\text{target}}^{\Delta C_{\text{t,target}}}/E_{\text{reference}}^{\Delta C_{\text{t,reference}}}$. The expression ratio of a given target gene was determined on the basis of its qRT-PCR efficiency (E) and the threshold cycle difference (ΔC_t) of the test sample (FW) *versus* that of the control (EST/SW). For each gene, standard curves (10-fold serial dilutions) were generated using standard PCR amplicons as the template, and were used to calculate the individual qRT-PCR efficiencies [$E=10^{(-1/\text{slope})}$]. The amplification efficiencies of all four PCR reactions were >90%, with the expression of each gene normalised to that of the reference gene, ribosomal protein L8.

Antibodies

The rat polyclonal antibody for NHE3 (R1B2) was made against a 212-amino-acid region of NHE3 from Atlantic stingray, *D. sabina*, and has been used to identify NHE3 in elasmobranch gills (Choe et al., 2005; Choe et al., 2007). This antibody was a gift from Dr

Table 2. *Carcharhinus leucas* specific primers used in quantitative PCR

Primer name	Orientation	Primer (5′ to 3′)
NHE3	Sense	AAG TTT GGG TGG AAC TGC TG
	Antisense	CGG TGG TGA TAG ACT GCT CA
PDN	Sense	TGC TGG CAT TAT TCA GCT TG
	Antisense	TTT GTG GAA TGT TCC GGA GT
VHA	Sense	GCA GGT CTG CCA CAT AAT GA
	Antisense	TGG GTC ATT TGC AAG ATT CA
NKA	Sense	CGA ATG ACT GTC GCT CAC AT
	Antisense	TGG CAC ATT CTC TTG ACC TG
L8	Sense	GGC AAG AAG GCT CAG TTG AA
	Antisense	CTC TGT TGG CCG AGG AAA TA

Keith Choe (Department of Biology, University of Florida, Gainesville, FL, USA). NKA was detected using the α5 mouse monoclonal antibody developed by Dr Douglas Fambrough and was acquired from the Developmental Studies Hybridoma Bank, produced under the auspices of the National Institute of Child Health and Human Development (The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA). This antibody recognises fish NKA, and is commonly used in studies on fish gills (Witters et al., 1996; Choe et al., 1999; Piermarini and Evans, 2000; Catches et al., 2006). To detect VHA, an affinity-purified rabbit polyclonal antibody was made against the B subunit of eel (*Anguilla anguilla*) VHA (see Wilson et al., 2007; Reis-Santos et al., 2008). Rabbit polyclonal antibodies were also made against a synthetic peptide of the same conserved region (amino acid #630-643) of mammalian PDN (Royaux et al., 2000) used by Piermarini et al. (Piermarini et al., 2002) in *D. sabina*. VHA and PDN antibodies were produced by Davids Biotechnologie GmbH (Regensburg, Germany).

Immunohistochemistry

Fixed gill filaments (FW-captured, *N*=4; EST/SW-captured, *N*=3; SW-acclimated, *N*=1) were removed from alcohol, dehydrated, cleared and embedded in paraffin wax (Histoplast, Thermo Fisher Scientific, Sydney, Australia). Paraffin-embedded filaments were serially sectioned at 5 μm and collected onto poly-L-lysine coated slides. Sections were left to completely air dry before being dewaxed in xylene and rehydrated. Sections were washed in buffer [0.01 mol l^{-1} phosphate buffered saline (PBS), 0.05% Tween-20, pH 7.2] before endogenous avidin/biotin activity was inhibited with avidin/biotin block (0.001% avidin in 0.01 mol l^{-1} PBS and 0.001% biotin in 0.01 mol l^{-1} PBS). Following avidin/biotin blocking, gill sections were incubated for 30 min at room temperature in normal goat serum [2% serum, 1% bovine serum albumin (BSA), 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween-20, and 0.05% thimerosal in 0.01 mol l^{-1} PBS, pH 7.2]. Sections were then incubated overnight at 4°C with primary antibody, diluted in 1% BSA, 0.1% cold fish skin gelatin and 0.05% thimerosal in 0.01 mol l^{-1} PBS. Primary antibodies were used at the following concentrations: NHE3, 1:1250; α5, 1:200; PDN, 1:100; and VHA, 1:900. Following incubation with primary antibodies, the slides were rinsed in washing buffer, blocked for 10 min in peroxidase blocking solution (3% H_2O_2 in 0.01 mol l^{-1} PBS) and incubated for 30 min at room temperature with either goat anti-rat, anti-mouse or anti-rabbit IgG secondary antibodies depending on the primary antibody used (1:500 in PBS; Antibodies Australia, Clayton, VIC; Invitrogen). A horseradish-peroxidise-labelled streptavidin solution (1:1000 in PBS; Vector Laboratories, Burlingame, CA, USA) was applied to sections for 30 min, followed by chromogenic detection using the 3,3′–diaminobenzidine tetrahydrochloride (DAB) liquid substrate system for immunohistochemistry (Sigma-Aldrich, Sydney, NSW, Australia). Sections were rinsed, dehydrated in ethanol and cleared with xylene before coverslips were mounted using DPX medium (ProSciTech, Thuringowa, QLD, Australia). Sections were viewed with an Olympus BH-2 microscope and images were captured with a Leica DCF280 digital firewire camera (QLab, Brisbane, QLD, Australia). For each antibody, at least five tissue sections were stained and photographed per shark. Controls consisted of the omission of primary and secondary antibodies (NKA), substitution with pre-immune sera (NHE3 and PDN), and a preabsorption control with excess peptide (VHA) to confirm antibody specificity.

Double-labelling immunohistochemistry

Double labelling of gills was conducted using NHE3 or VHA antibodies in combination with α5, and the PDN antibody in conjunction with VHA. Double labelling was performed using a method slightly modified from that of Piermarini and Evans (Piermarini and Evans, 2001). Briefly, sections were dewaxed, rehydrated, blocked and immunostained as above. However, following chromogenic detection with the brown substrate, sections were rinsed in distilled water for 10 min and re-blocked in goat serum (30 min). Sections were then incubated with either α5 (4°C overnight) or the VHA antibody (1 h at room temperature). Concentrations of primary antibodies in these experiments were: NHE3, 1:2000–1:3000; α5, 1:30; PDN, 1:800; and VHA, 1:200 or 1:900. Rinsing and detection was conducted as described above except a blue reaction product was obtained using Vector SG chromogen (Vector Laboratories).

Statistical analysis

Differences in plasma solutes between FW- and EST/SW-captured sharks were determined using a Student's *t*-test, with sample variances assessed with Fisher's *F*-test, in the statistical program R (www.r-project.org). Relative gene expression ratios were tested for significance using Wilcoxon rank-sum tests (expression data did not fit the assumptions of a parametric test). Furthermore, a non-parametric Spearman's rank correlation (r_S) was used to analyse relative expression data from 15 individual sharks to ascertain dependence among the expression levels of ion transporters. For all data, α was set at 0.05.

RESULTS

Plasma osmolytes

Juvenile *C. leucas* caught in FW and EST/SW environments were hyper-osmotic to the ambient environment (Table 3). Plasma osmolality was not significantly different between FW *C. leucas* captured from the Wenlock and Brisbane Rivers (*P*=0.54), thus the data from these animals were combined into a single series. The

Table 3. Environmental and haematological variables from *Carcharhinus leucas* caught in freshwater (0–5 ppt) and estuarine/seawater (21–32 ppt) environments

Environmental and plasma osmolalities and plasma ion concentrations	Freshwater (*N*=11)	Estuarine/seawater (*N*=6)
Environmental osmolality (mOsm kg^{-1})	3.2±0.6	705.8±114.3*
Plasma osmolality (mOsm kg^{-1})	639.7±14.1	797.5±15.6*
Urea (mmol l^{-1})	168.0±6.9	278.1±12.2*
Na^+ (mmol l^{-1})	234±1.7	247.5±4.1*
Cl^- (mmol l^{-1})	230.7±1.6	242.8±4.5*
K^+ (mmol l^{-1})	4.1±0.1	4.5±0.4
Mg^{2+} (mmol l^{-1})	1.3±0.04	1.5±0.3
Ca^{2+} (mmol l^{-1})	3.1±0.07	3.4±0.2

Values represent means ± s.e.m. *P<0.05, significant difference between freshwater- and estuarine/seawater-captured sharks.

Table 4. Identity and functional homology of *Carcharhinus leucas* partial sequences

Gene name	Highest annotated BLAST hit	
	Returned nucleotide sequence	Predicted protein sequence
NHE3	Na^+/H^+ exchanger type 3; *Dasyatis sabina*; AY626250 (85%; 900; 0)	Na^+/H^+ exchanger type 3; *Dasyatis sabina*; AAT45738 (90%, 470, 1e–130)
PDN	Anion exchanger *slc26a4* (pendrin); *Xenopus laevis*; BC169728 (66%; 211; 3e–52)	Anion exchanger *slc26a4* (pendrin); *Xenopus laevis*; AAI69728 (66%; 180; 5e–44)
VHA	Vacuolar H^+-ATPase B subunit; *Squalus acanthias*; EU004205 (93%; 553; 1e–153)	Vacuolar H^+-ATPase B subunit; *Squalus acanthias*; ABS71821 (97%; 236; 3e–60)
NKA	Na^+/K^+-ATPase α-subunit; *Squalus acanthias*; AJ781093 (91%; 542; 1e–150)	Na^+/K^+-ATPase α1-subunit; *Dasyatis sabina*; AAU29551 (95%; 212; 5e–53)
L8	Ribosomal protein L8; *Squalus acanthias*; EU004204 (93%; 579; 7e–162)	60S ribosomal protein L8; *Danio rerio*; NP957007 (94%; 233; 9e–60)

Numbers in parentheses represent identity (%), score (bits) and *E*-value, respectively.

plasma osmolality of EST/SW *C. leucas* was significantly higher than that of FW animals (Table 3). FW *C. leucas* maintained their osmolality at a level 200-fold greater than that of the environment, whereas the osmolality of EST/SW *C. leucas* was only 1.1 times higher than the external medium. Circulating urea, Na^+ and Cl^- concentrations in EST/SW *C. leucas* were significantly greater than those of FW sharks, with urea making the largest contribution towards their elevated plasma osmolality (Table 3). Of the other osmolytes, there was no difference in plasma concentrations of K^+, Ca^{2+} and Mg^{2+} between FW and EST/SW sharks.

Molecular identification and gene expression of gill ion transporters

Sequencing of PCR products revealed the presence of the orthologous genes *slc9a3* (NHE3), *atp1a1* (NKA), *slc26a4* (PDN), *atp6v1b* (VHA) and ribosomal protein L8 in bull shark gills (GenBank accession nos JN190348, JN190346, JN190349, JN190347 and JN190350, respectively). Overall, the genes isolated showed very high nucleotide and amino acid sequence similarity to those of other elasmobranch species (Table 4). The four target genes were analysed relative to the expression of the reference gene, ribosomal protein L8, in sharks captured from the Wenlock (FW), Mission and Pine Rivers (EST/SW) only. Ribosomal protein L8 did show some minor inter-individual variation, but its expression was not affected by environmental salinity. There was no difference in mRNA levels for any of the ion transporters in the gills of EST/SW *C. leucas* caught from the Mission and Pine Rivers (P=0.100). The expression of NHE3 mRNA from the gills of FW-captured *C. leucas* was more than sixfold greater than in gills from EST/SW *C. leucas* (P=0.004; Fig. 1A). Additionally, FW-captured sharks showed a modest, but significant (>2.5-fold) relative increase in gill NKA α1 gene expression compared with EST/SW-captured *C. leucas* (P=0.026; Fig. 1B). Curiously, branchial VHA gene expression was downregulated in FW-captured animals (P=0.004; Fig. 1C) whereas there was no effect of environmental salinity on the expression of PDN mRNA (P=0.394; Fig. 1D). A significant correlation was detected among the relative expression levels of NHE3 and NKA α1 mRNA in the gills of bull sharks (r_S=0.93, P<0.0001; Fig. 2A). Conversely, relative VHA gene expression was not significantly correlated with that of PDN (r_S=0.44, P=0.098; Fig. 2B).

Distribution of ion transporters in the branchial epithelium

Immunohistochemistry was used to localise NHE3, NKA, PDN and VHA in the gills of *C. leucas*. NHE3 was visualised using the stingray-specific R1B2 antibody, which reacted predominantly with lamellar epithelial cells in FW-captured sharks (Fig. 3A). Some cells within interlamellar regions also displayed NHE3 immunoreactivity in FW-captured animals (Fig. 3A). In EST/SW-captured and the SW-

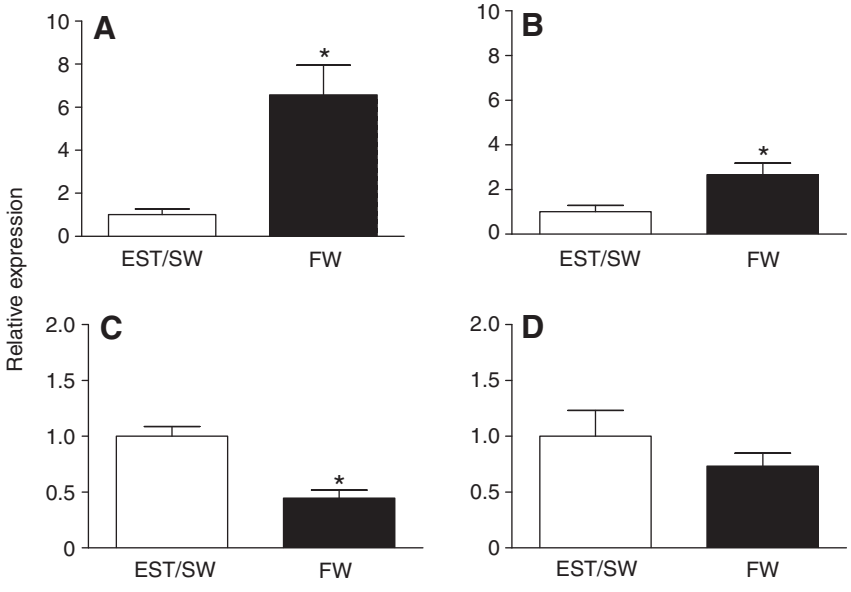

Fig. 1. Relative mRNA expression of (A) Na^+/H^+ exchanger 3 (NHE3), (B) Na^+/K^+-ATPase (α1-subunit) (NKA), (C) vacuolar-type H^+-ATPase (B-subunit) (VHA) and (D) pendrin (Cl^-/HCO_3^- exchanger) (PDN) in the gills of *Carcharhinus leucas* captured from freshwater (FW) and estuarine/seawater (EST/SW) environments. Data are presented as mean ± s.e.m. relative expression ratios after normalisation to the reference gene, ribosomal protein L8; N=6 for both FW and EST/SW animals; *P<0.05.

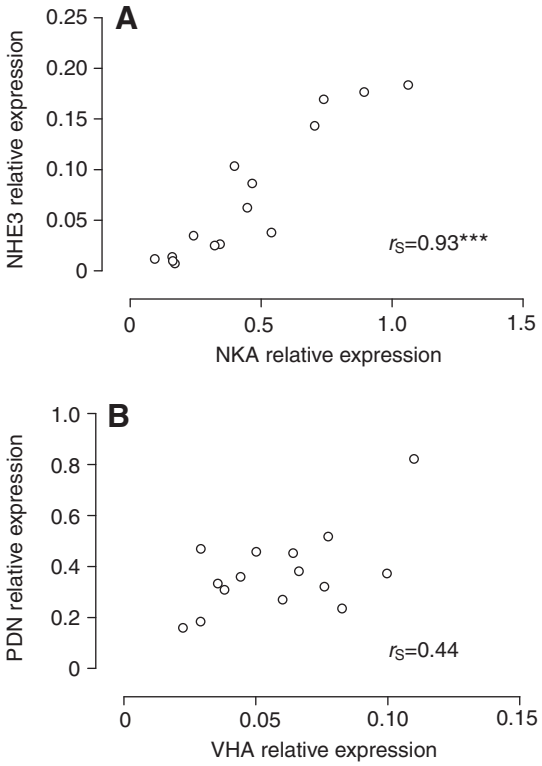

Fig. 2. Correlations between (A) NHE3 and NKA and (B) PDN and VHA mRNA in the gills of *C. leucas*. There was a significant correlation between NHE3 and NKA expression patterns (***P<0.0001), but no correlation between PDN and VHA expression patterns.

acclimated *C. leucas*, NHE3 immunoreactivity occurred in a subpopulation of cells within interlamellar regions and at the base of lamellae, but qualitatively appeared less abundant on lamellae than those of FW-captured sharks (Fig. 3B). Regardless of salinity, the NHE3 signal was confined to the apical region of immunoreactive cells (Fig. 4A). NHE3 immunoreactivity was absent in gill sections incubated with pre-immune serum, whilst negligible background staining remained (not shown). The NKA α5 antibody reacted with cells (likely MRCs) both in gill interlamellar regions and on lamellae in FW-captured sharks (Fig. 3C). The NKA immunoreactivity was always confined to the basolateral membrane of cells (Fig. 4B). There appeared to be comparatively more NKA-immunoreactive cells in the gills of FW-captured relative to EST/SW-captured *C. leucas*, particularly on the more distal regions of lamellae (Fig. 3C,D). No staining was observed when primary or secondary antibodies were omitted from the labelling protocol.

PDN- and VHA-immunoreactive cells were localised to both interlamellar and lamellar regions of the gills in FW-captured, EST/SW-captured and SW-acclimated *C. leucas* (Fig. 5). PDN immunoreactivity was restricted to the apical membrane of immunoreactive cells in bull sharks (Fig. 6A) whereas VHA immunoreactivity usually occurred throughout the cytoplasm but was sometimes confined to the basolateral membrane (Fig. 6B,C). Gills of EST/SW-captured animals showed staining patterns comparable to those of FW-captured individuals, with no clear qualitative differences in terms of the number of PDN- and VHA-positive cells stained (Fig. 5). VHA and PDN staining was absent in sections incubated with VHA antibody preabsorbed with blocking peptide and pre-immune serum, respectively.

Double-labelling experiments were performed to ascertain whether any of the ion transporters colocalised to the same cell type. In the gills of bull sharks, apical NHE3 immunoreactivity occurred in cells with NKA immunoreactivity, which was limited to the basolateral region (Fig. 7A,B). Additionally, apical PDN immunoreactivity was observed in cells that also exhibited cytoplasmic/basolateral VHA (Fig. 7C,D). Within these double-labelled sections, a minority of cells stained only for VHA. Finally, immunoreactivity for VHA generally occurred in cells distinct from those with NKA immunoreactivity (Fig. 8).

DISCUSSION

In contrast to the extensive literature regarding mechanisms of osmotic and ionic regulation in the gills of teleosts (reviewed by Evans et al., 2005; Hwang and Lee, 2007), much less is known about branchial ion and acid–base regulatory systems in elasmobranchs. In the present study, we have demonstrated that bull shark gills possess the ion transporters NHE3, NKA, PDN and VHA. Furthermore, this is the first study to identify PDN cDNA in the gills of an elasmobranch. Immunostaining demonstrated the presence of two types of putative ion-regulatory cells, NKA-rich and VHA-rich cells, which coexpressed NHE3 and PDN, respectively. The gills of FW-captured *C. leucas* showed significant increases in both NHE3 and NKA α1 gene expression, indicating that these transporters may play a significant role in Na^+/H^+ exchange when bull sharks are resident in FW environments. Contrary to expectations, branchial VHA mRNA was significantly downregulated in FW-captured *C. leucas*, whereas PDN gene expression appeared unaffected by environmental salinity. Overall, our data are consistent with the hypothetical model of NaCl and acid–base transport in the gills of elasmobranchs (Evans and Claiborne, 2009), whereby NKA-rich and VHA-rich cells act as primary sites for Na^+/H^+ and Cl^-/HCO_3^- exchange, respectively.

Plasma osmolality, urea and electrolytes

Plasma osmolality values obtained for FW-captured *C. leucas* were comparable to those of previous studies on bull sharks and Atlantic stingrays in FW (Thorson et al., 1973; Piermarini and Evans, 1998; Pillans and Franklin, 2004). The lower plasma osmolality of FW-captured *C. leucas* was largely accounted for by a decrease in plasma urea content, which was 40% lower than that of EST/SW-captured sharks.

EST/SW-captured *C. leucas* were hyper-osmotic regulators, maintaining a plasma osmolality in excess of that of the environment. Plasma osmolality, urea, Na^+ and Cl^- concentrations of EST/SW-captured sharks in this study resembled those from a previous study where juvenile *C. leucas* had been acclimated to 75% SW (Pillans et al., 2006). There was a similar, small degree of variability in the plasma osmolyte concentrations of FW- and EST/SW-captured sharks. Relatively stable plasma osmolalities may reflect a long-term residence in a particular osmotic environment (Pillans and Franklin, 2004). Tracking data indicate that most bull sharks tagged in the upper FW regions of the Brisbane and Wenlock Rivers inhabit these upper reaches for an extended period of time (months to years) (Pillans, 2006) (H.A.C., unpublished). The significantly lower Na^+ and Cl^- concentrations in plasma of FW-captured *C. leucas* likely reflects both the lower concentrations of these ions in dilute media and the passive efflux of ions from the animal. Thus, for *C. leucas* in FW environments, the combined effect of high gill permeability and the large internal–external osmotic gradient would lead to increased water gain by osmosis and increased urinary output.

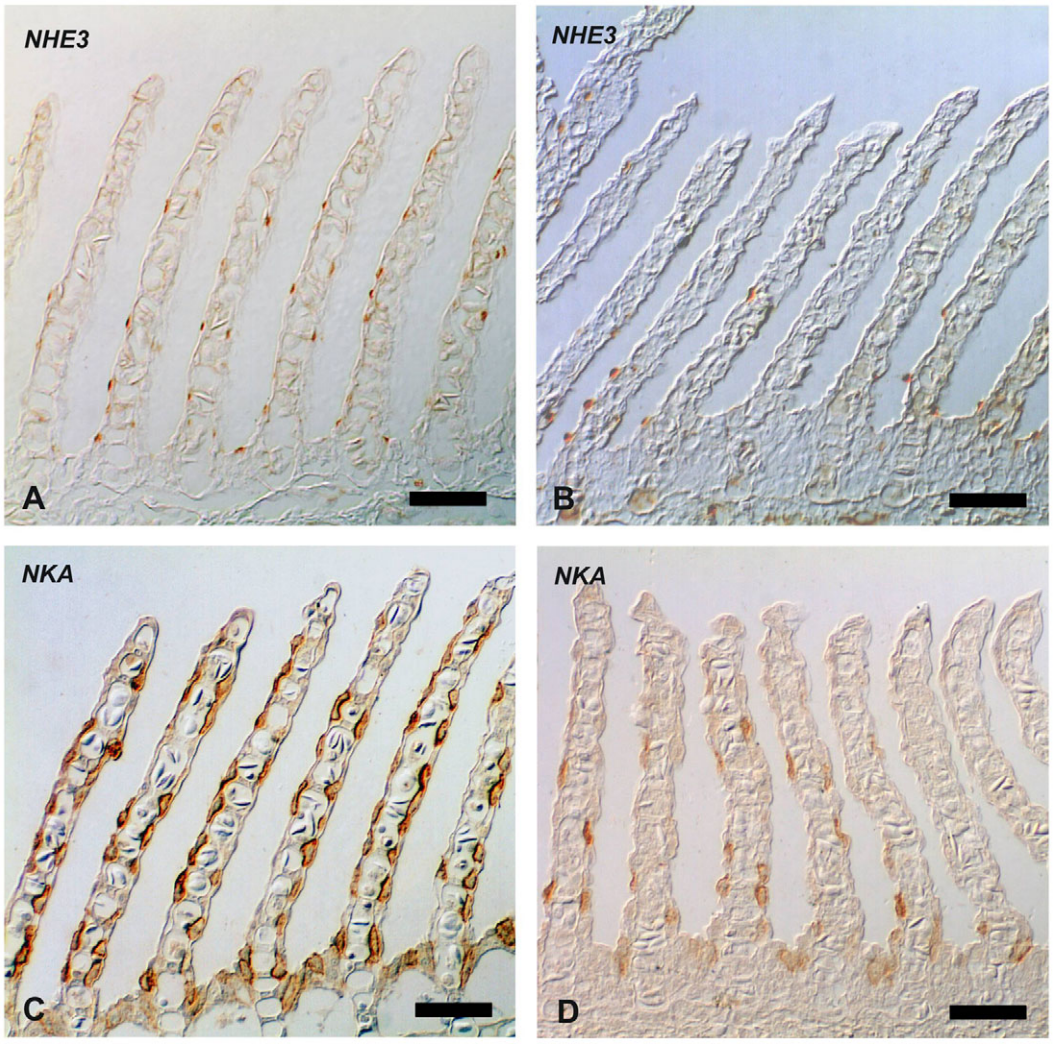

Fig. 3. Representative images of (A,B) NHE3 and (C,D) NKA immunostaining in the gills of (A,C) FW- and (B,D) EST/SW-captured *C. leucas*. Note the profusion of NKA-rich cells in gills of FW-captured *C. leucas* (C) relative to EST/SW-captured *C. leucas* (D). Scale bars, 40 μm.

Moreover, given the large ionic gradients that exist between the environment and sharks living in FW, *C. leucas* must possess the appropriate mechanisms to maintain and decrease losses of ions, and have pathways that facilitate the uptake of Na^+ and Cl^-.

Na^+ uptake

In the present study, mRNA transcripts of two putative ion transporters, which have previously been implicated in branchial Na^+ uptake (Piermarini and Evans, 2000; Choe et al., 2005), were isolated from the gills of FW- and EST/SW-captured *C. leucas* and their abundance was quantified. The partial Na^+/H^+ exchanger (NHE3) sequence identified in the gills of *C. leucas* was found to be strongly homologous with that of the stingray NHE3 (90%). Accordingly, the stingray-specific R1B2 antibody identified NHE3 in gills of bull sharks, where it was localised to the apical region of NKA-rich cells. Na^+/H^+ exchange *via* NHEs is widely considered to be dependent upon activity of the enzyme NKA, and not surprisingly, both transporters have been co-localised to the same cells in a variety of teleosts (Catches et al., 2006; Inokuchi et al., 2008; Ivanis et al., 2008) and elasmobranchs (Edwards et al., 2002; Choe et al., 2005; Claiborne et al., 2008). Moreover, Choe et al. reported qualitative differences following acclimation to either SW or FW in the location and staining intensity of branchial NHE3-immunoreactive cells in the gills of *D. sabina* (Choe et al., 2005). For FW stingrays, NHE3-labelled cells occurred largely in gill lamellae, whereas in marine individuals there were fewer immunoreactive cells that were confined to the gill filaments. This staining pattern is consistent with the results of the present study, although unlike *D. sabina*, NHE3-immunoreactive cells were also evident in lamellae of SW-captured *C. leucas*. This may reflect the fact that bull sharks are capable of movement from hyper- to hypo-saline waters and would need to quickly adopt a FW osmoregulatory strategy (Pillans, 2006). Additionally, NHEs have been shown to play a role in acid excretion during acid–base regulation in some fish models (Parks et al., 2007; Ivanis et al., 2008), and thus maintenance of branchial NHE3 immunoreactivity in *C. leucas* from saline environments may also reflect this function. However, further work concerning branchial NHEs in *C. leucas* is necessary with regard to other NHE isoforms (e.g. NHE2) and their involvement in acid transport (Claiborne et al., 2008). Indeed, in the gills of Atlantic stingray and rainbow trout, *Oncorhynchus mykiss*, NHE3 expression was unaffected by hypercapnia, indicating that at least in these species, NHE3 might not play a role in acid–base balance (Choe et al., 2005; Ivanis et al., 2008).

As hypothesised, the expression of NHE3 mRNA was significantly higher in the gills of FW-captured *C. leucas* relative to EST/SW-captured sharks, which indicates this exchanger has an increased role in FW environments, and we propose that this is for Na^+ uptake. In FW teleosts, there are two current hypotheses for the mechanism of gill Na^+ ion uptake (Hwang and Lee, 2007). One

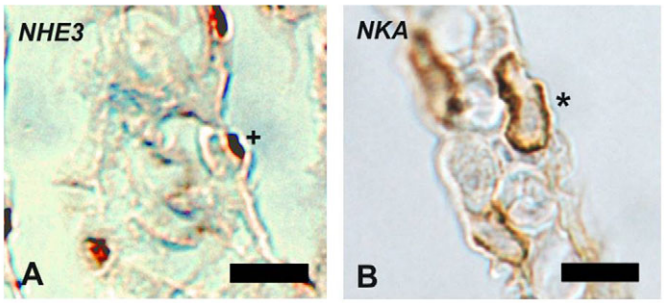

Fig. 4. High-magnification images showing the subcellular localisation of (A) NHE3 and (B) NKA in FW-captured *C. leucas*. NHE3 staining was apical (+) whereas NKA staining was restricted to the basolateral membrane (*) of cells. Scale bars, 10 μm.

proposes that passive Na^+ absorption occurs *via* an epithelial Na^+ channel, with a favourable electrochemical gradient created by apical VHA-driven proton efflux. Parallel to this model is support for electroneutral exchange of Na^+ and H^+ *via* an apical NHE. Quantitative expression data from teleosts suggests that NHEs may play a key ion regulatory role in some FW-acclimated fish species. NHE3 mRNA was significantly upregulated in the gills of Mozambique tilapia, *Oreochromis mossambicus*, and zebrafish, *Danio rerio*, acclimated to low-Na^+ waters (Yan et al., 2007; Inokuchi et al., 2009), whereas NHE2 expression increased in the gills of killifish, *Fundulus heteroclitus*, also when exposed to FW (Scott et al., 2005). Further evidence that NHEs perform Na^+ uptake was provided by Esaki et al. (Esaki et al., 2007), who showed that Na^+ influx across MRCs of zebrafish larvae was severely reduced upon application of a selective NHE inhibitor, EIPA. As in teleosts and *C. leucas*, branchial NHE3 gene expression was found to be much greater in FW-acclimated *D. sabina versus* SW stingrays (Choe et al., 2005), indicating that NHE3 is also of increased importance to (or plays a greater role in) FW-acclimated fish.

Immunostaining of NKA demonstrated that this enzyme was present in NHE3-positive cells in the gills of *C. leucas*, and environmental salinity had a significant effect on gill NKA α1 mRNA abundance, with FW-captured sharks having a higher (~165%) expression than EST/SW-captured sharks. Consistent with other fish models, NKA was expressed largely in the basolateral membrane of cells (Karnaky et al., 1976; Wilson et al., 2002; Hirata et al., 2003). Qualitatively, there appeared to be more NKA-rich cells in the gills of FW-captured sharks, particularly on the lamellae, compared with EST/SW-captured and SW-acclimated sharks. FW-acclimated *D. sabina* have been shown to have elevated levels of branchial NKA mRNA, immunoreactivity and activity relative to stingrays in SW

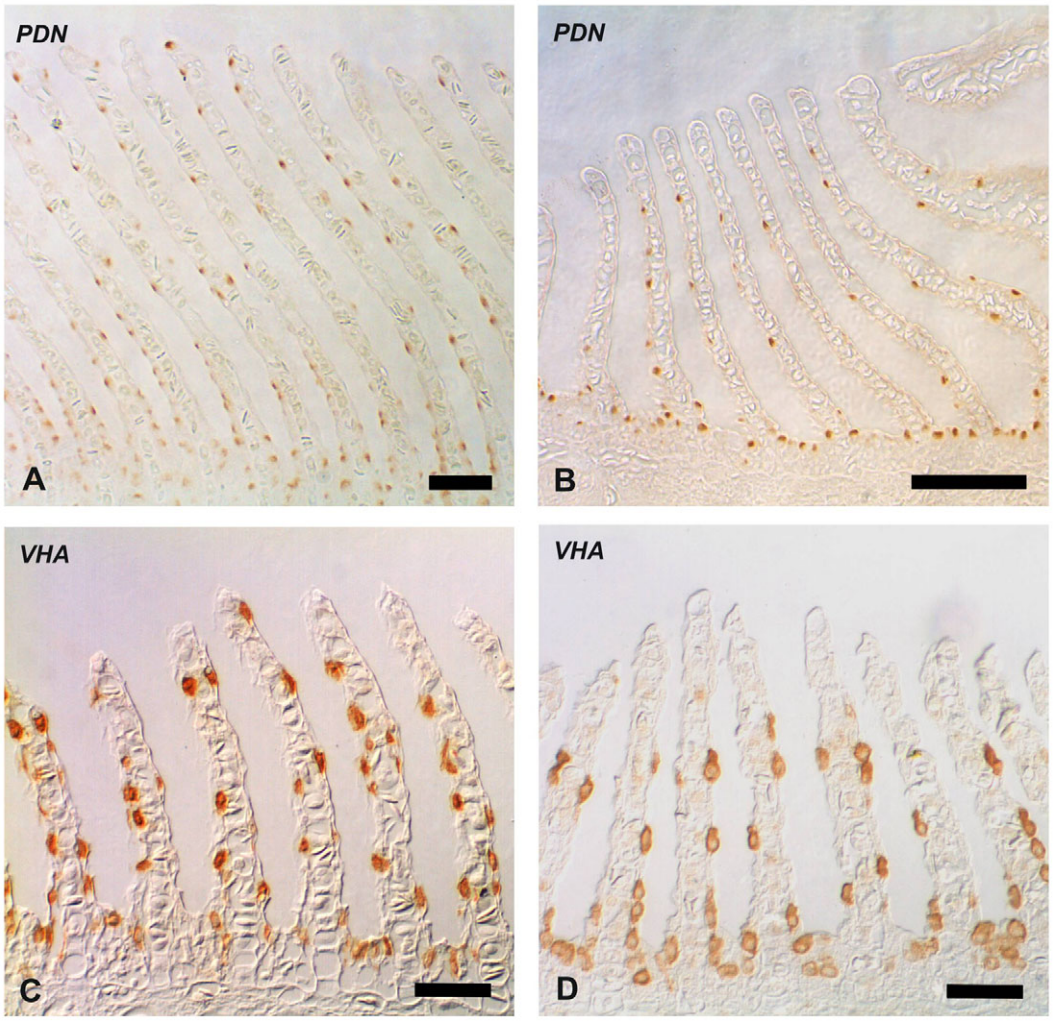

Fig. 5. Representative images of (A,B) PDN and (C,D) VHA immunostaining in gills of (A,C) FW- and (D) EST/SW-captured *C. leucas* and (B) SW-acclimated *C. leucas*. Note the abundance and general cytoplasmic staining of VHA in gills of both FW- (C) and EST/SW-captured *C. leucas* (D). Scale bars, 50 μm (A), 120 μm (B) and 40 μm (C,D).

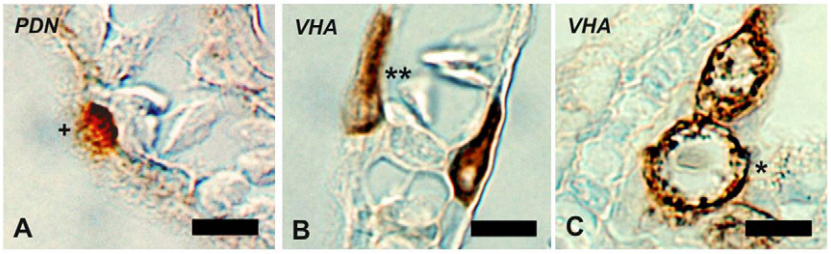

Fig. 6. High-magnification images showing the subcellular localisation of (A) PDN and (B,C) VHA in (A,B) SW-acclimated and (C) FW-captured *C. leucas*. PDN staining was apical (+) whereas VHA staining was primarily cytoplasmic (**) and/or restricted to the basolateral membrane (*) of cells. Scale bars, 10 μm.

(Piermarini and Evans, 2000; Choe et al., 2005), whereas Duncan et al. showed that stenohaline FW stingrays, *Paratrygon aiereba*, in ion-poor environments exhibit significantly greater branchial NKA activity compared with conspecifics in ion-rich waters (Duncan et al., 2009). These data provide strong evidence that gill NKA has a greater ion-regulatory role for elasmobranchs resident in FW. In the gills of FW *C. leucas*, it is possible that basolateral NKA is responsible for maintaining a very low intracellular [Na^+] by pumping cytosolic Na^+ into the blood, thus allowing Na^+ absorption from the environment *via* NHE3. Sustained NKA activity might also create an intracellular negative potential to support electrogenic transport of Na^+ and HCO_3^- *via* a basolateral Na^+-HCO_3^- cotransporter, as has been described in mammalian renal cells (Soleimani and Burnham, 2001; Purkerson and Schwartz, 2007). However, a putative Na^+-HCO_3^- cotransporter has not been identified in the gills of an elasmobranch.

A previous experiment examined NKA in the gills of juvenile *C. leucas* and found no significant difference in NKA enzyme activity between FW- and SW-acclimated animals (Pillans et al., 2005). Although the expression and activity of NKA is often directly correlated with ambient salinity in fishes (Lin et al., 2004; Bystriansky et al., 2007; Wilson et al., 2007; Kang et al., 2008), other studies have shown that NKA abundance does not correlate well with NKA activity (Lin et al., 2006; Sardella and Kultz, 2009). For example, Sardella and Kultz reported a significant increase in NKA abundance within gill MRCs of green sturgeon, *Acipenser medirostris*, acclimated to saline water, but no corresponding increase in NKA activity (Sardella and Kultz, 2009). Sardella and Kultz suggested that the lack of a correlation could be due to the nature of the NKA activity assays themselves, which may not accurately represent functional activity at *in vivo* body temperature and under natural environmental conditions (Sardella and Kultz, 2009). Richards et al. showed that rainbow trout, *Oncorhynchus mykiss*, exhibit differential expression of numerous NKA α1 isoforms in the gills during salinity acclimations, thus suggesting that NKA activity assays may provide an incomplete assessment of the osmoregulatory function of this enzyme (Richards et al., 2003). In FW, *O. mykiss* express high levels of NKA α1a mRNA, but dramatically downregulate this isoform when transferred to SW. In contrast, branchial NKA α1b is upregulated when trout are transferred from FW to SW (Richards et al., 2003). This may well be the case for *C. leucas*, as branchial NKA isoforms have been cloned from this species (Meischke and Cramb, 2005). However, the physiological functions of distinct NKA α1-subunit isoforms in elasmobranch gills have not been examined, and this is certainly an area where future research is warranted.

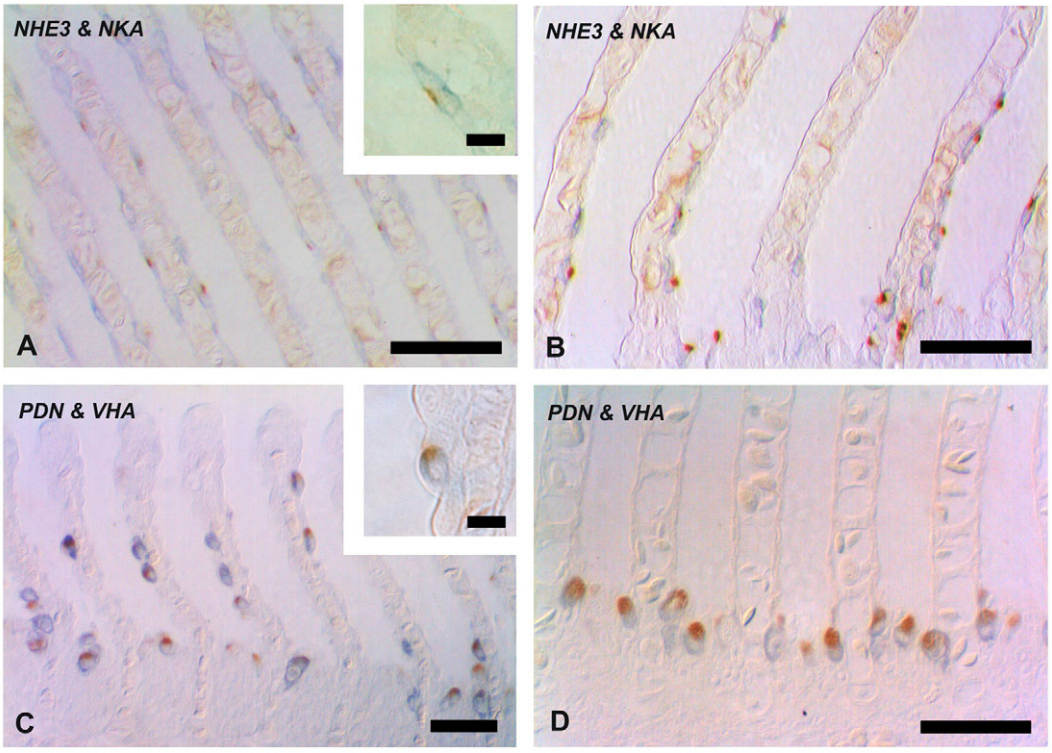

Fig. 7. Representative double-labelled images of (A,C) FW-captured and (B,D) SW-acclimated *C. leucas* gills showing NHE3 (brown) localisation relative to NKA (blue; A,B) and PDN (brown) relative to VHA (blue; C,D). NHE3 immunostaining was apical in cells with basolateral NKA whereas PDN was apical in cells that were positive for VHA. Scale bars, 50 μm (A,B,D), 40 μm (C) and 10 μm (insets).

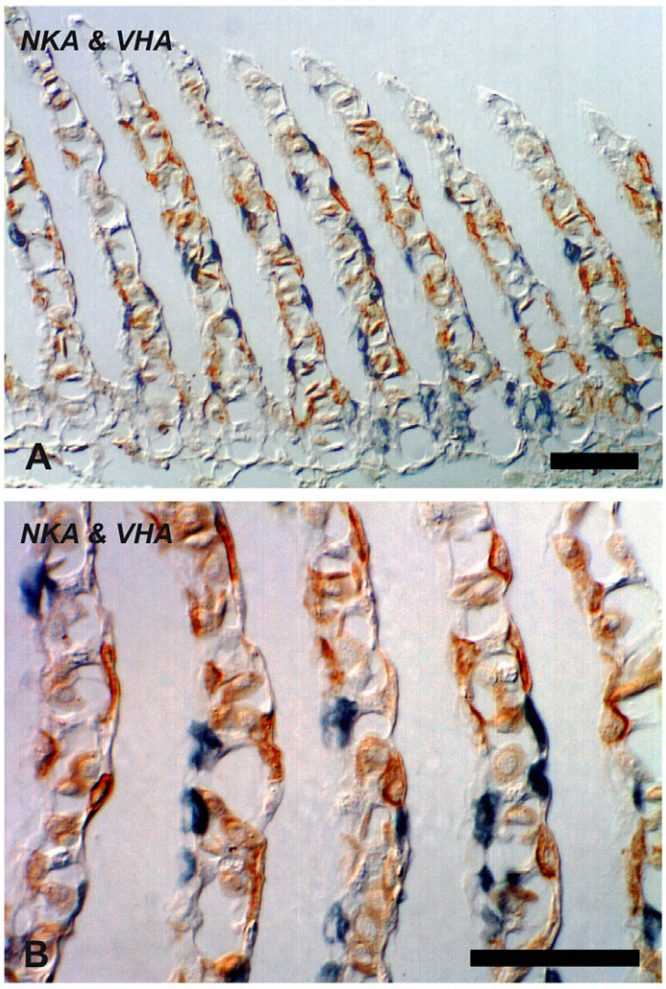

Fig. 8. Representative double-labelled images of (A) a gill filament and (B) gill lamellae from FW-captured *C. leucas* showing NKA (brown) localisation relative to VHA (blue). There was little to no overlap among NKA and VHA immunostaining. Scale bars, 40 μm.

Discrepancies between NKA α sub-unit expression and activity may also be related to post-transcriptional processing. For example, the family of FXYD proteins are known to interact with and modulate the kinetic properties of Na^+/K^+-ATPase (Garty and Karlish, 2006). FXYD 10 (PLMS) has been demonstrated to regulate NKA activity in the rectal gland of the dogfish *Squalus acanthias* (Mahmmoud et al., 2000; Mahmmoud et al., 2003), and there is increasing evidence to suggest that FXYD proteins have similar functional effects on NKA activity in the gills of teleosts (Tipsmark, 2008; Wang et al., 2008; Tipsmark et al., 2010). Despite low branchial NKA activity in *C. leucas*, the elevated gene expression levels of NKA and NHE3 in FW-captured bull sharks and the colocalisation of these two transporters corroborates findings from *D. sabina*, and is consistent with the proposal that apical NHE3 and basolateral NKA are important for Na^+ uptake from hypo-saline waters.

Cl^- uptake

A putative partial PDN sequence (*slc26* gene family) and PDN immunoreactivity were identified in the gills of *C. leucas*. PDN immunoreactivity in *C. leucas* was clearly apical, and the PDN-positive cells also exhibited basolateral/cytoplasmic immunoreactivity for VHA. In mammals, PDN and VHA are coexpressed at significant levels in acid–base-regulating cells of the kidney, where they are believed to function in Cl^-/HCO_3^- exchange (Royaux et al., 2001; Mount and Romero, 2004). The gills of elasmobranchs are most likely the site of HCO_3^- secretion (Hodler et al., 1955; Swenson and Maren, 1987), yet there has been little research concerning the potential molecular mechanisms responsible for Cl^-/HCO_3^- exchange. The presence of VHA in elasmobranch gills has been known for some time (Wilson et al., 1997), and appears in cells separate to those with NKA (Piermarini and Evans, 2001; Claiborne et al., 2008), which is also consistent with results from the present study. In *C. leucas*, gill VHA staining was typically evident throughout the cell cytoplasm or confined to the basolateral membrane. Our data support earlier studies which have demonstrated that VHA immunostaining in elasmobranch gills is cytoplasmic and/or basolateral (Piermarini and Evans, 2001; Tresguerres et al., 2005; Claiborne et al., 2008). PDN immunoreactivity was reported in the apical membranes of gill epithelial cells in both the marine *S. acanthias* and the euryhaline *D. sabina*, and was also shown to occur in cells containing basolateral VHA (Piermarini et al., 2002; Evans et al., 2004). Branchial PDN was strongly influenced by salinity in *D. sabina*, with FW stingrays showing greater PDN immunoreactivity relative to SW-acclimated rays, which is in agreement with the requirement for increased Cl^- uptake in FW to offset the diffusional loss of ions from the gill and kidney. VHA immunoreactivity in gills of *D. sabina* fluctuates with changes in salinity in concert with PDN (Piermarini and Evans, 2001; Piermarini et al., 2002). This suggests that both transporters are functionally linked and it is likely that VHA provides the driving force necessary for Cl^- uptake and HCO_3^- secretion by PDN (Piermarini and Evans, 2001; Piermarini et al., 2002). Unlike *D. sabina*, there were no obvious differences among FW-captured, EST/SW-captured and SW-acclimated *C. leucas* in either the density or the pattern of distribution of PDN and/or VHA. Despite the occurrence of PDN and VHA largely within the same cells, there was no significant correlation between the two transporters at the transcript level. Additionally, there was no difference in the gene expression of branchial PDN between FW and EST/SW *C. leucas*, whereas VHA gene expression was downregulated in lower salinities.

Given the need to maintain NaCl balance, we hypothesised that the levels of gene expression of PDN and VHA would be significantly higher in gills of *C. leucas* inhabiting FW environments. There are several reasons as to why PDN and VHA expression data appeared incongruous. Firstly, acoustic telemetry tracking of bull sharks in the Brisbane River has shown that some individuals are capable of large-scale movements upstream and downstream with the flood and ebb tides, respectively, and can experience a range of salinities (1–20 ppt) in a very short time (Pillans, 2006). Therefore, interpretation of the branchial gene expression data from *C. leucas* is somewhat complicated given that no information could be acquired as to the recent environmental and osmoregulatory history of the sharks in the days prior to capture. Indeed, a study on striped bass, *Morone saxatilis*, found that expression of gill VHA mRNA can take several days to significantly change during salinity acclimations (Tipsmark et al., 2004). Secondly, two VHA B subunit (B1/B2) isoforms have been identified in different tissues of teleosts (including gills) and appear to be related to differences in physiological function (Niederstätter and Pelster, 2000; Boesch et al., 2003a). For example, in the swim bladder of the eel *A. anguilla*, VHA B1 was localised in the apical region of cells whereas VHA

B2 was confined to the basolateral membrane (Boesch et al., 2003b). In the present study we did not employ exhaustive gene identification methods to isolate VHA B subunit isoforms and therefore it is possible that several isoforms exist in bull shark gills. Additional work is necessary to determine whether branchial VHA B subunit isoforms exist in *C. leucas* and, if so, whether their expression patterns are associated with different physiological roles, for example during salinity and/or acid–base challenges. Thirdly, although branchial VHA is predominantly thought to play a role in FW osmoregulation (Hwang and Lee, 2007; Evans, 2008), it is also involved in acid–base regulation.

Basolateral localisation of VHA in dogfish gill epithelial cells is prominent following intravenous $NaHCO_3^-$ infusion, lending support to the prediction that this ATPase is crucial for driving HCO_3^- secretion (Tresguerres et al., 2005). Moreover, branchial VHA in *S. acanthias* appears to be important for HCO_3^- excretion during an 'alkaline tide' (Tresguerres et al., 2007). This post-feeding condition causes blood pH to increase temporarily, as acid secreted into the stomach for digestion is balanced by HCO_3^- reabsorption into the plasma (Niv and Fraser, 2002). Tresguerres et al. also found that VHA protein abundance was higher in gill cell membranes of naturally fed *S. acanthias* compared with fasted sharks (Tresguerres et al., 2007), indicating that branchial HCO_3^- excretion during an alkaline tide is dependent on an increase in basolateral VHA. In the present study, the majority of bull sharks were captured during spring and summer when water temperatures are warm, and research suggests that sharks eat more readily at warmer water temperatures than at cooler temperatures (Tullis and Baillie, 2005). Because VHA gene expression in the gills of *C. leucas* is inconsistent with models of FW osmoregulation, and given that VHA-immunopositive cells were richly expressed in gills of FW-captured, EST/SW-captured and SW-acclimated *C. leucas*, it is feasible that their expression (and that of PDN) is related to HCO_3^- excretion in response to an alkaline tide. Indeed, it appears that sharks continue to eliminate excess base accumulated *via* metabolic alkalosis more than 24 h post-feeding (Wood et al., 2007). Alternatively, regulation of branchial VHA in response to environmental salinity in bull sharks may involve post-translational modifications of existing VHA rather than *de novo* protein synthesis (Tresguerres et al., 2010). Post-translational regulation of branchial VHA-rich cells in *C. leucas* would not be reflected in changes in the number of VHA-immunopositive cells nor mRNA levels, and therefore may help to explain our surprising VHA data. It should also be recognised that other anion transporters (AE1, Na^+-Cl^- cotransporter and SLC26A3) have been implicated in branchial Cl^- uptake in other fish models (Wilson et al., 2000; Hiroi et al., 2008; Perry et al., 2009) and further investigations are required to ascertain whether similar anion exchange systems exist and play an ion-regulatory role in the gills of elasmobranchs.

In summary, we have identified two types of probable ion-regulatory cells in the gills of *C. leucas*, one with basolateral NKA and apical NHE3 and the other a VHA with apical PDN. These data demonstrate that bull sharks possess the putative ion transporters necessary for Na^+ and Cl^- uptake in FW. Both NHE3 and NKA mRNA were significantly greater in the gills of FW-captured *C. leucas*, which indicates that these transporters play an enhanced osmoregulatory role when sharks are resident in FW environments. Surprisingly, PDN expression in the gills did not change with environmental salinity, and the expression of branchial VHA was downregulated in *C. leucas* caught in FW. Whether these results are associated with environmental salinity or a postprandial alkaline tide remains unclear at present.

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