Perfused Gills Reveal Fundamental Principles of pH Regulation and Ammonia Homeostasis in the Cephalopod Octopus vulgaris

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In contrast to terrestrial animals most aquatic species can be characterized by relatively higher blood NH4+ concentrations despite its potential toxicity to the central nervous system. Although many aquatic species excrete NH3/NH4+ via specialized epithelia little information is available regarding the mechanistic basis for NH3/NH4+ homeostasis in molluscs. Using perfused gills of Octopus vulgaris we studied acid-base regulation and ammonia excretion pathways in this cephalopod species. The octopus gill is capable of regulating ammonia (NH3/NH4+) homeostasis by the accumulation of ammonia at low blood levels (<260 µM) and secretion at blood ammonia concentrations exceeding in vivo levels of 300 µM. NH4+ transport is sensitive to the adenylyl cyclase inhibitor KH7 indicating that this process is mediated through cAMP-dependent pathways. The perfused octopus gill has substantial pH regulatory abilities during an acidosis, accompanied by an increased secretion of NH4+. Immunohistochemical and qPCR analyses revealed tissue specific expression and localization of Na+/K+-ATPase, V-type H+-ATPase, Na+/H+-exchanger 3, and Rhesus protein in the gill. Using the octopus gill as a molluscan model, our results highlight the coupling of acid-base regulation and nitrogen excretion, which may represent a conserved pH regulatory mechanism across many marine taxa.

Keywords: NH3/NH4+ homeostasis, acid-base regulation, cephalopod, invertebrate, Na+/K+-ATPase, Rh-protein

INTRODUCTION

Cephalopods including squid, cuttlefish, and octopods have evolved an active lifestyle and vertebrate like sensory abilities to compete with fish for similar resources in marine habitats. Due to their less efficient swimming mode by jet propulsion, locomotion in cephalopods comes at a higher cost, reflected in very high metabolic rates when compared to other active marine animals including sharks and tunas (Rosa and Seibel, 2008). Despite their relatively short lifespan, usually not exceeding 1–2 years, cephalopods have high growth rates and can reach a body mass of several kilograms within 1 year (Llpiński, 2010). This “live fast and die young” lifestyle is predominantly
fueled by protein metabolism and ammonia is the main end-product of their amino acid metabolism (Boucher-Rodoni and Mangold, 1988, 1989, 1994). Ammonia in its uncharged gaseous form as NH₃ can, to a certain extent passively diffuse across biological membranes, whereas the charged ionic form, NH₄⁺ cannot cross cell membranes without the help of respective transporters. At physiological pH of ~7.2–7.8, 95–99% of total ammonia occurs in the hydrated form (NH₄⁺). High concentrations of ammonia are toxic for organisms as they can cause severe detrimental effects on the central nervous system and can lead to intra- as well as extra-cellular acid-base disturbances (Albrecht, 2007). Studies on ammonia tolerance demonstrated LC50-values for environmental ammonia levels in the micromolar range for most aquatic organisms including fish, crustaceans and cephalopods (Miller et al., 1990; Randall and Tsui, 2002; Camargo and Alonso, 2006). Pelagic squids were demonstrated to be particularly sensitive to elevated seawater ammonia concentrations >10 µM (Hanlon, 1990). Thus, potent ammonia excretion pathways must represent an important evolutionary trait of ammonotelic organisms like cephalopods to control intra- and extra-cellular NH₃/NH₄⁺ homeostasis.

In most aquatic organisms including fish and crustaceans ammonia is excreted from body fluids into the environment via specialized epithelial cells located on the skin or gills (Randall et al., 2004; Tay et al., 2006; Weihrauch et al., 2009; Wright and Wood, 2009; Wu et al., 2010). The current models denote that besides the direct basolateral transport of NH₃ through Rh-glycoproteins (e.g., Rhbg) the ammonium ion (NH₄⁺) may also be imported on the basolateral side via the Na⁺/K⁺-ATPase. Hydrated NH₃ and K⁺ ions have very similar sizes of ~1.45 Å and due to their K⁺ like behavior NH₄⁺ ions can compete with K⁺ as substrates for transporters like the Na⁺/K⁺-ATPase (Skou, 1957; Leone et al., 2014; Quijada-Rodriguez et al., 2015). Alternatively, the basolateral entry of ammonia can be facilitated through NH₃ channels of the Rh-glycoprotein family that allow the entry and subsequent hydration of NH₃ to form the ammonium ion in the cytosol. At the apical membrane export of ammonia has been proposed to be facilitated through apical ammonium (NH₃) channels in combination with Na⁺/H⁺-exchanger (NHE) or proton pump-based [e.g., V-type H⁺-ATPase (VHA), H⁺/K⁺-ATPase] H⁺ extrusion mechanisms that lead to an acid trapping of NH₃ that has been excreted through the NH₃ channels, in the outer boundary layer of excretory epithelia (Wright and Wood, 2009; Gruswitz et al., 2010; Nawata et al., 2010; Wu et al., 2010). The removal of the proton from the ammonium ion at the apical membrane is potentiated by the deprotonating activity found in NH₃ channel proteins (Javelle et al., 2008). Besides ammonia excretion mechanisms involving transporters, an alternative model has been proposed for the gills of the green shore crab *Carcinus maenas*, where NH₃ excretion has been demonstrated to be highly sensitive to the microtubule inhibitors, colchicine, taxol and thiabendazole, suggesting a vesicular ammonia (NH₃) trapping in acidified vesicles and subsequent transport of the potentially toxic NH₄⁺ ions across the cytosol (Weihrauch et al., 2002).

Similar to the situation in fish and crustaceans the gills of cephalopods are probably also the most important sites for gas exchange and pH regulation (Hu et al., 2010, 2011, 2014b). Earlier studies suggested that despite the existence of other potential excretory organs such as branchial hearts or renal appendages (equivalent to the vertebrate kidneys) gills may represent the most important site for NH₄⁺ excretion in cephalopods (Potts, 1965). High concentrations of Na⁺/K⁺-ATPase localized in basolateral membranes of branchial epithelia of cephalopods underline their predominant role in active ion transport and excretion (Schipp et al., 1979; Hu et al., 2011). Furthermore, the expression of transporters and channels like the teleost orthologs Na⁺/H⁺-exchanger 3 (NHE3) and Rh-proteins (RhP) localized in apical membranes suggest an involvement of gill epithelia in acid-base balance and ammonia transport in cephalopods (Hu et al., 2014b). Recent studies conducted on squid *Sepioteuthis lessoniana*, have demonstrated that acidified seawater stimulates the expression of branchial acid-base transporters including V-type H⁺-ATPase, Na⁺/HCO₃⁻ cotransporter (NBC), NHE3, and one primitive Rh-protein (RhP) suggesting that pH regulation and NH₄⁺ excretion are coupled processes (Hu et al., 2014b).

To date, ammonia excretion mechanisms are still poorly understood for most invertebrate species. Although, some information is available for crustaceans (reviewed in Henry et al., 2012), ammonia excretion mechanisms are virtually unexplored for the entire phylum mollusca. Thus, the exclusively ammonotelic nature of cephalopods and their extensive protein metabolism makes this taxonomic group an interesting specimen to investigate NH₄⁺ excretion pathways.

Gill perfusion experiments were successfully applied in aquatic organisms including fish and crustaceans allowing the examination of transport rates and metabolic demands in an isolated organ (Tresguerres et al., 2008; Deigweiler et al., 2010; Fehsenfeld and Weihrauch, 2013). Using perfused gills of the common octopus, *Octopus vulgaris*, the present work aims to demonstrate that pH regulation and NH₄⁺ transport are coupled processes in complex branchial epithelia of this cephalopod species. The present work introduces a new molluscan model to study acid-base regulation and NH₄⁺ excretion mechanisms. This will help understanding nitrogen excretion pathways in cephalopods, a taxonomic group that has received little attention regarding ionic regulation associated with extracellular pH and ammonia homeostasis.

**MATERIALS AND METHODS**

**Experimental Animals**

Experimental animals, *O. vulgaris*, were obtained from a local dealer in Keelung, Taiwan, and transported in well aerated containers to the Marine Research Station, Institute of Cellular and Organismic Biology, at the Academia Sinica (I-lan, Taiwan). Animals ranging from 300 to 600 g were held in closed circulatory seawater systems of ~600 l for several days before they were used for experimental procedures. Individuals were kept separately in cages at a constant 12/12 h light cycle at 26°C and low seawater ammonia levels <5.6 µmol l⁻¹ and were fed with live clams (*Meretrix spp.*). For experimental procedures including perfusion experiments and blood sampling, animals were anesthetized by cooling below 5°C until full depression of ventilation.
and were killed by decapitation. Best practices for handling cephalopods as experimental animals were followed, including currently discussed ethical standards for anesthesia and killing of cephalopods (Andrews et al., 2013). The experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFIZOOHP2 20782).

**Determination of Blood pH and NH$_4^+$ Concentrations**

Blood samples were collected from the *vena cava* via a gas-tight Hamilton syringe by dissecting the funnel and mantle from the ventral side. Determination of extracellular pH (pH$_e$) and NH$_4^+$ were conducted as previously described (see also Supplementary Information for Materials and Methods) using a WTW 340 pH meter (precision ± 0.01 units) equipped with a microelectrode (WTW Mic-D). The pH meter was calibrated with Radiometer precision buffers 7 and 10 (S11M44, S11 M007) and pH-values are provided using the NBS (National Bureau of Standards) scale.

**Perfusion Experiments**

For perfusion experiments gills were carefully dissected from the mantle along the branchial gland, and two cuts at the 1st order efferent and afferent vessels were made before the gill was carefully detached from the mantle and intestinal sac. All dissection procedures were conducted while the tissues were immersed in seawater. Five centimeters of long polyethylene tubes with an outer diameter of 1.52 mm and an inner diameter of 0.86 mm that were tapered toward the end, were inserted into the 1st order efferent and afferent vessels of the octopus gill. Synthetic woven threads of 0.2 mm diameter were used to tie branchial vessels to the PVC tubes. PVC tubes were connected to a peristaltic pump, pumping perfusion saline adjusted to the ionic composition of seawater. Five centimeters of long polyethylene tubes with an outer diameter of 2 mm and the afferent tube was connected to a gas-tight Hamilton syringe by dissecting the funnel and mantle from the ventral side. Determination of extracellular pH (pH$_e$) and NH$_4^+$ were conducted as previously described (also see Supplementary Information for Materials and Methods) using a WTW 340 pH meter (precision ± 0.01 units) equipped with a microelectrode (WTW Mic-D). The pH meter was calibrated with Radiometer precision buffers 7 and 10 (S11M44, S11 M007) and pH-values are provided using the NBS (National Bureau of Standards) scale.

**Immunohistological Staining**

Immunohistochimical analyses of acid-base transporters in *O. vulgaris* gills were conducted as previously described (Hu et al., 2010, 2014b). Gill tissues were fixed by direct immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. After fixation, the tissues were transferred to a PBS solution containing 5% bovine serum albumin (BSA) for 30 min to block non-specific binding. The sections were then washed in PBS and incubated for 12 h at 4°C that continuously irrigated the gill with oxygenated saline (see Figure S1 for a schematic illustration of the setup).

Before the start of experiments using perfusion salines with different NH$_3$-NH$_4^+$ concentrations (prepared by the addition of NH$_3$ to perfusion saline), pH (adjusted with HCl and NaOH) or pharmacological compounds (e.g., 10 µM KH7 and 75 µM cAMP) gills were perfused with the respective saline for 10 min to achieve full exchange of fluids within the tubes and gill vessels. 8-Bromoadenosine 3',5'-cyclic monophosphate (cAMP) was dissolved in perfusion saline to a final concentration of 75 µmol l$^{-1}$ and a stock solution of the specific soluble adenylyl cyclase (sAC) inhibitor KH7 (Bitterman et al., 2013) was dissolved in DMSO and was diluted to 10 µmol l$^{-1}$ with <0.1% DMSO in the final perfusion saline. The duration of perfusion experiments never exceeded 1.5 h to assure full vitality of the examined tissues.

For perfusion experiments gills were carefully dissected from the mantle along the branchial gland, and two cuts at the 1st order efferent and afferent vessels were made before the gill was immersed in an aerated bathing solution (same as perfusion saline, without addition of ammonia) in a volume of 50 ml at 25°C that continuously irrigated the gill with oxygenated saline (see Figure S1 for a schematic illustration of the setup).

Before the start of experiments using perfusion salines with different NH$_3$-NH$_4^+$ concentrations (prepared by the addition of NH$_3$ to perfusion saline), pH (adjusted with HCl and NaOH) or pharmacological compounds (e.g., 10 µM KH7 and 75 µM cAMP) gills were perfused with the respective saline for 10 min to achieve full exchange of fluids within the tubes and gill vessels. 8-Bromoadenosine 3',5'-cyclic monophosphate (cAMP) was dissolved in perfusion saline to a final concentration of 75 µmol l$^{-1}$ and a stock solution of the specific soluble adenylyl cyclase (sAC) inhibitor KH7 (Bitterman et al., 2013) was dissolved in DMSO and was diluted to 10 µmol l$^{-1}$ with <0.1% DMSO in the final perfusion saline. The duration of perfusion experiments never exceeded 1.5 h to assure full vitality of the examined tissues.
Molecular Cloning
Acid-base transporters’ peptide sequences from cephalopods and other species (aquatic animals were given the highest priority) were used to BLAST the *O. vulgaris* expressed sequence tag (EST) and octopus genome databases in NCBI. Based on those putative sequences assembled from collected tags, gene specific primers (listed in Supplemental Table 2) were designed for the reverse transcription polymerase chain reaction (RT-PCR) analysis. Detailed cloning procedures and further phylogenetic analysis are presented in the Supplementary Information for Material and Methods.

Real-Time Quantitative PCR (qPCR)
The mRNA expressions of target genes were measured by qPCR with the Roche LightCycler® 480 System (Roche Applied Science, Mannheim, Germany). The sequence accession numbers and primers are depicted in Supplemental Table 3. qPCR assay procedures are described in the Supplementary Information for Materials and Methods.

Statistical Analyses
Statistical analyses were performed using Sigma Stat 3.0 (Systat) software. Statistical differences between blood pH and NH$_4^+$ levels at different transit stations as well as the effects of cAMP, KH7 and pH on NH$_4^+$ excretion rates were analyzed by one-way ANOVA followed by Tukey’s post-hoc test. A Student’s t-test was used to compare blood pH and NH$_4^+$ concentrations before and after gill passage in perfusion experiments. Two-way ANOVA was used for analyzing differences between H$^+$ loss and NH$_4^+$ excretion rates with perfusion salines of different pH and NH$_4^+$ concentrations. Data sets were normally distributed (Kolmogorov-Smirnov test). Equal variance was tested using the Levene median test. The significance levels were set to $p < 0.05$ and 0.01.

RESULTS
Regulation of pH and NH$_4^+$ Homeostasis by Branchial Epithelia
The major excretory organs of *O. vulgaris*, such as gills and renal appendages, are connected by blood vessels that direct the blood flow from the vena cava through these organs to the systemic heart (Figure 1A). Blood returning from the body through the vena cava (station 1) enters the lateral vena cava and is distributed to the renal appendages from where the urine is secreted into the renal sac (station 4). After passage of the renal appendages the blood flow reaches the branchial heart by passing the branchial heart appendages. From here the blood is pumped through the gill via the 1st order afferent vessels (station 2) and leaves the gill via the efferent 1st order vessels (station 3) from where it enters the systemic heart. From here the oxygenated blood is pumped back into the body.

Sampling of blood from different blood transit stations before and after passage through major excretory organs, including gills and renal appendages, demonstrated changes in blood NH$_4^+$ concentrations and pH (Figures 1A, B). Blood pH-values in *O. vulgaris* ranged from pH 7.4 to 7.6 depending on the sampling site. Determination of blood pH at different transit stations demonstrated a decrease in pH by $\sim$0.1 pH units between blood samples from the vena cava (station 1) and after gill
passage (station 3). Blood NH$_4^+$ levels ranged from 240 to 300 µM, depending on the sampling site. NH$_4^+$ concentrations were significantly decreased by 60 µM after gill passage (station 3) compared to blood samples taken from the afferent vessel (station 2). The renal sac fluid (urine) was highly acidic with a pH-values being as low as 5.8 and is characterized by very high NH$_4^+$ concentrations of 3,192 ± 146 µM. Determination of pH and NH$_4^+$ concentrations before and after gill passage in perfused gills could demonstrate a drop in pH by 0.2 pH units associated with a drop in perfusion saline NH$_4^+$ levels of 40 µM (Figure 1C). The stability of this decrease in pH after gill passage served as a reliable indication for the viability of the perfused gill. During perfusion experiments exceeding 2 h the difference in pH between saline before and after gill passage decreased indicating a progressively reduced viability of gill tissues.

Perfusion experiments using artificial blood salines with different NH$_4^+$ concentrations ranging from 0 to 5,000 µM were used to test the NH$_4^+$ regulatory capacity of O. vulgaris gills. Surprisingly, an enrichment of ammonia was observed in the post branchial fluid at initial NH$_4^+$ concentrations below 300 µM, whereas at higher concentrations a loss of ammonia was detected in the post-branchial fluids (Figure 2A). This reduction in blood NH$_4^+$ was most evident at 5,000 µM with only 2,700 µM NH$_4^+$ remaining after a single gill passage leading to a non-linear flattened curve using blood salines with NH$_4^+$ concentrations >300 µM (Figure 2A). This bi-phasic regulation was also evident when looking at NH$_4^+$ excretion rates into the bathing saline where NH$_4^+$ excretion was maintained at a constant rate of ~1.5 µmol NH$_4^+$·h$^{-1}$·g$^{-1}$ at blood NH$_4^+$ concentrations between 0 and 600 µM (Figure 2B). At blood (perfusion solution) NH$_4^+$ concentrations >600 µM an increased excretion rate was observed with 1.89 ± 0.49, 4.21 ± 1.48, and 5.14 ± 0.93 µmol NH$_4^+$·h$^{-1}$·g$^{-1}$ at initial perfusion solution NH$_4^+$ concentrations of 1,200, 2,500, and 5,000 µM, respectively.

A gain of ammonia was observed in the perfusate, when gills were perfused with solutions initially containing NH$_4^+$ concentrations below 300 µM (Figures 2A,C). At 300 µM or higher NH$_4^+$ concentrations in the initial perfusion solution a positive rate of NH$_4^+$ loss from the blood was observed that increased in a linear fashion as a function of blood NH$_4^+$ levels up to 2,400 µM. At a blood NH$_4^+$ concentration of 5,000 µM the rate of NH$_4^+$ removal from the blood after gill passage was not further increased but was maintained at a rate of ~3 µmol NH$_4^+$·h$^{-1}$·g$^{-1}$ similar to excretion rates determined for 2,400 µM NH$_4^+$, a linear regression analysis for blood NH$_4^+$ concentrations ranging from 0 to 600 µM shows the blood NH$_4^+$ concentration of 287 µM at which no net excretion of NH$_4^+$ was measured (Figure 2C, inset).

**Effects of cAMP, KH7, and pH on Branchial Ammonia Regulation**

At a blood NH$_4^+$ concentration of 0 µM the metabolically produced NH$_4^+$ by the gill led to a total excretion of 4.55 ± 1.73 µmol NH$_4^+$·h$^{-1}$·g$^{-1}$ of which 84% were excreted across the apical side into the bath and 16% being transported into the blood (Figure 3A). Addition of cAMP had no significant effect ($p = 0.098$) on relative NH$_4^+$ transport rates toward both, the apical and the basolateral side regardless whether initial perfusate NH$_4^+$ concentrations were set to 0 and 300 µM (Figures 3B,C). However, the specific soluble adenyl cyclase (sAC) inhibitor KH7 significantly (One-way ANOVA

**FIGURE 2** Determination of NH$_4^+$ transport in perfused Octopus vulgaris gills. (A) NH$_4^+$ concentrations in the perfusate after gill passage as a function of different blood NH$_4^+$ concentrations ranging from 0 to 5,000 µM. Insert showing the natural range of in vivo blood NH$_4^+$ concentrations (gray bar) in the range of 0–600 µM NH$_4^+$ (B) NH$_4^+$ excretion rates (presented as µmol NH$_4^+$·h$^{-1}$·g$^{-1}$, fresh mass: FM) in perfused gills measured in the bath as a function of blood NH$_4^+$ levels ranging from 0 to 6,000 µM (C) Transport rates of NH$_4^+$ from the perfusion saline after gill passage as a function of blood NH$_4^+$ levels. Note the negative transport rates below blood NH$_4^+$ levels of 287 µM indicated by dashed lines (inset: ammonia transport rates at blood NH$_4^+$ concentrations ranging from 0 to 600 µM). Values are given as mean ± SE ($p = 4–7$).
measured at an initial blood NH$_4^+$ concentration of 300 µM (Figure 3C).

In a second set of experiments we investigated the effects of an extracellular acidosis (pH 7.2) on the branchial pH regulatory capacities and epithelial NH$_4^+$ transport (Figure 4). The effects of an acidosis were combined with the initial blood NH$_4^+$ concentrations of 0 and 300 µM, respectively. The pH levels for the two NH$_4^+$ concentrations before gill passage were compared to those after gill passage. At an initial (before gill passage) blood pH of ∼7.6 a significant decrease in blood pH after gill passage was observed for 0 µM NH$_4^+$ (Figure 4A). Under initial conditions of ∼pH 7.2, an elevation of blood pH back to control levels ranging between pH 7.4 and 7.5 was measured for both blood NH$_4^+$ concentrations, 0 and 300 µM (Figure 4A). This compensation reaction toward an initially induced acidosis was also indicated by a significantly increased transport rate of protons from the blood during gill passage (Figure 4B). Here an increase in transport rate of H$^+$ from the blood was measured for blood containing initially 300 µM NH$_4^+$ (9.52 ± 1.21 pmol h$^{-1}$ g$^{-1}$FM) compared to blood containing 0 µM NH$_4^+$ (5.33 ± 0.72 pmol h$^{-1}$ g$^{-1}$FM). A higher rate of proton removal from the blood under acidified conditions was accompanied by increased NH$_4^+$ excretion rates compared to control (pH 7.6) conditions (Figure 4C). Compared to control saline of pH 7.6 NH$_4^+$ excretion rates increased by 6-fold and by 16-fold when perfused with acidified saline of 7.2 initially containing either 0 or 300 µM NH$_4^+$.

### Tissue Expressions of VHA, NHE3, NKA, and RhP

Tissue expression levels of VHA demonstrated highest abundance of transcripts in brain (B) tissues (Figure 5A and Figure S2). Relatively similar mRNA expression levels were detected in gills, renal appendages (RA), branchial heart appendages (BA), and optical lobes (OL). Low expression levels of VHA were determined for systemic heart (SH), gill hearts (GH), and mantle (M) tissues. Statistical analyses demonstrated significant differences in the expression level between SH vs. GH; p = 0.04, SH vs. OL; p = 0.03 and between GH vs. M; p = 0.04). PCR as well as qPCR analyses demonstrated highest NKA expression levels in RA, followed by neurons (B + OL) and other excretory organs including gills (G) and BA (Figure 5B). Statistical analyses demonstrated significant differences in the expression level between SH vs. G; p = 0.04, SH vs. BHA; p = 0.04 and between BHA vs. M; p = 0.03). Expression levels of RhP are highest in neurons (B + OL) followed by tissues of the circulatory system including branchial gland (BG) and excretory organs including RA, BA, and G (Figure 5C). Lowest transcript levels of RhP were detected in SH and M tissues. Statistical analyses demonstrated significant differences in the expression level between RA vs. M; p = 0.04). Highest expression levels for NHE3 that belongs to the invertebrate clade of NHE3 (see Figure S3 for phylogenetic analysis) were detected in GH and G, whereas the remaining tissues had relatively similar expression levels (Figure 5D). Very low expression levels for this transporter were measured in the systemic heart (SH).

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Gill Morphology and Localization of Branchial Acid-Base Transporters

Immunohistochemical analyses were used to describe the morphology of the octopus gill and to identify epithelia that are rich in acid-base transporters relevant for pH and NH$_4^+$ regulation (Figure 6A). The morphology of the octopus gill differs from that of most cephalopods (e.g., squid and cuttlefish) by having a less organized hierarchy in folding pattern of 1st to 3rd order lamellae. Instead of continuous fan like folds as found in squid and cuttlefish the 2nd order lamellae of octopods are joined and the 3rd order lamellae are branching folds (Figure 6A). Immunohistochemical stainings demonstrated a predominant signal of the NKA in the blood vessels with a predominant basolateral localization in membranes of the outer epithelium. While some larger blood vessels face the seawater, others are located within the gill. All blood vessels branch out toward the periphery of the gill where the blood is merely separated by the thin branchial epithelium from the surrounding seawater. Additionally, branchial blood vessels show positive immunoreactivity for VHA. The VHA antibody shows weaker immunoreactivity in basolateral membranes compared to the NKA but shows a distinct localization of the VHA in the cytosol of endothelial cells of blood vessels. However, this antibody shows a strong signal in endothelial cells lining the inner side of the blood vessel (Figure 6A). Distinct NHE3 and RhP immunoreactivity was observed in apical membranes of blood vessel epithelial cells. Additionally, endothelial cells facing the lumen of the blood vessel also show a weak immunoreactivity of NHE3 and RhP in basolateral membranes. Besides expression of NHE3 and RhP in blood vessels, a distinct positive immunoreactivity was also found in apical membranes of gill epithelial cells facing the seawater. Peptide compensation assays using antibody-specific peptides demonstrated full abolishment of the immunoreactivity on gill section (Figure 6B). Western blot analysis demonstrated distinct immunoreactivity with proteins in the predicted size range (Figure 6C).

DISCUSSION

Changes in Blood pH and NH$_4^+$ during Passage of Excretory Organs

In vivo determinations of O. vulgaris blood pH and NH$_3$/NH$_4^+$ are in general in accordance with earlier findings by Potts (1965), demonstrating that during passage through excretory organs, including renal appendages, branchial heart appendages and gills, the majority of NH$_4^+$ is excreted across gill epithelia. The passage of blood through excretory organs is accompanied by a drop in blood pH-values. In contrast, determinations of blood pH during gill passage using implanted catheters demonstrated an increase in blood pH after gill passage in the free swimming octopus (Houlihan et al., 1986). These different findings may be explained by the different sampling methods in anesthetized and free swimming animals and requires further clarification. In the anesthetized animals a slight but significant increase in blood NH$_4^+$ level was observed during blood passage through renal appendages. The renal appendages are enclosed by the renal sacs, filled with an acidic (∼pH 6) urine that contains high concentrations of ammonia (∼3.2 mM). Despite potential ammonia trapping in this acidic urine, a small amount of NH$_3$/NH$_4^+$ may diffuse back into the blood (at regular blood NH$_4^+$ levels below 300 µM) across epithelia of the renal
FIGURE 5 | Tissue specific expression of acid-base transporters. Expression profiles of (A) VHA, (B) NKA, (C) RhP, and (D) NHE3 in various tissues determined by qPCR analysis (for tissue panel see Figure S2). The different tissues were classified into three functional groups circulatory system (hearts and branchial gland), excretory organs, and neurons and muscles. BG, branchial gland; SH, systemic heart; GH, gill heart; G, gill; RA, renal appendages; BA, branchial heart appendages; B, brain; OL, optical lobe; M, muscle.

FIGURE 6 | Localization of branchial acid-base and NH$_3$ transporters. (A) Positive immunoreactivity of important transporters was mainly observed in blood vessels and epithelial cells. The NKA is located in basolateral membranes and the VHA antibody shows positive immunoreactivity in basolateral membranes, the cytosol as well as endothelial cells of blood vessels. Positive NHE3 and RhP immunoreactivity is mainly restricted to apical membranes of blood vessels and branchial epithelia facing the seawater. Autofluorescence of the blood was observed in blood vessels of the gill using 488 nm excitation and the 530 nm emission filter. RhP and NHE immunoreactivity is abolished by incubating the primary antibodies with their specific peptides for 8 h. (B) Negative controls by omitting the primary antibody demonstrate no unspecific binding of the secondary antibodies used. (C) Western blot analysis of antibodies used, indicating specific immunoreactivity with proteins in the predicted size range (indicated by arrows). LU, blood vessel lumen; sw, sea water.

appendages. This would explain the increase in blood NH$_4^+$ levels after passage of excretory organs within the renal sacs. Ex vivo measurements of pH and NH$_4^+$ concentrations within the artificial blood before and after passage through the isolated perfused gill were in accordance to observations made in the intact animal, demonstrating that the isolated gill behaves in a
similar fashion compared to in vivo conditions. Furthermore, contractile movements of the gill supported perfusion of the gill and demonstrated the viability of the isolated perfused gill under ex vivo conditions for up to 2 h. In other aquatic species, isolated gill tissues showed viability and functionality over a time span of several hours. For instance, long-term recordings of oxygen consumption and ion transport capacities in isolated fish gill preparations demonstrated the viability of the perfused organ for at least 1–2 h and for the gills of the blue crab Callinectes sapidus 4 h, respectively (Perry et al., 1984; Burnett and Towle, 1990; Deigweher et al., 2010).

**Ammonia Transport in Perfused Gills of Octopus vulgaris**

In order to test the transport properties of ammonia across the gill epithelium we perfused gills with artificial blood containing different concentrations of NH$_4^+$, ranging from 0 to 5,000 µM. In response to an ammonia load exceeding in vivo blood NH$_4^+$ concentrations (≈300 µM) the octopus gill showed an outward directed net transport of NH$_4^+$. This finding is in general accordance with observations made in other aquatic organisms including fish and crustaceans (reviewed by Weihrauch et al., 2009). The magnitude of NH$_4^+$ excretion rates across gill epithelia are in accordance to findings in crustaceans and fish. For example, the green shore crab C. maenas has branchial NH$_4^+$ excretion rates of ca. 35 µmol h$^{-1}$ g$_{BM}$ when gills were perfused with hemolymph-like salines containing 200 µM NH$_4$Cl and excretion rates of 10–20 µmol h$^{-1}$ g$_{BM}$ when symmetrical bath and perfusion salines with NH$_4^+$ concentrations of 100 µM were applied (Weihrauch et al., 1998, 2002). Ammonia excretion rates of perfused gills of marine teleosts demonstrated excretion rates of 0.18–0.3 µmol h$^{-1}$ g$_{BM}$ at relatively high blood NH$_4^+$ concentrations of 1 mM (Goldstein et al., 1982). Interestingly, our results on the octopus gill demonstrated that below in vivo blood NH$_4^+$ levels (<250 µM) the perfused gill responded with an accumulation of ammonia in the perfusate, indicating that the gill itself is capable of generating ammonia (ammoniagenesis) which is used to elevate blood ammonia levels to concentration close to 300 µM. For example, under conditions where the gill was perfused with artificial blood containing 0 mM NH$_4^+$ the gill generated and transported ammonia at a rate of 0.4 µmol h$^{-1}$ g$_{BM}$ into the blood. Although ammonia is generally considered toxic to organisms, our results suggest that gill tissues of O. vulgaris regulate extracellular NH$_4^+$ homeostasis to maintain blood [NH$_4^+$] at levels of 250–300 µM. The physiological reasons for retaining a certain amount of ammonia in the blood remain speculative. Some mid-water cephalopod species accumulate NH$_4^+$ in exchange for Na$^+$ in specialized tissues to improve buoyancy (Seibel et al., 2004). However, since these species accumulate NH$_4^+$ in the mM range in specialized vacuoles it is questionable in how far blood ammonium in the µM range may support buoyancy. In most animals NH$_4^+$ is generated through amino acid metabolism, wherein L-amino acids are first transaminated to form glutamate, which is then deaminated to NH$_4^+$ and α-ketoglutarate by glutamate dehydrogenase (GDH) (Wright, 1995; Nissim, 1999; Weiner and Verlander, 2013). The mammalian kidney is known to be capable of synthesizing NH$_4^+$ from glutamine in order to regulate pH homeostasis (Nissim, 1999; Weiner and Verlander, 2013). Here, a respiratory acidosis stimulates renal H$^+$ secretion, accompanied by an increase in HCO$_3^-$ accumulation and NH$_4^+$ excretion. Since cephalopods are powerful acid-base regulators that accumulate HCO$_3^-$ in the mM range to compensate for an extracellular acidosis (Gutowska et al., 2010; Hu et al., 2014b) it is tempting to speculate that also here branchial ammonia production generates HCO$_3^-$ that can be used to regulate blood pH.

Interestingly, many aquatic organisms including fish (Lin et al., 2012), crustaceans (Fehsenfeld and Weihrauch, 2013), echinoderms (Stumpf et al., 2012; Hu et al., 2014a), and molluscs (Thomsen and Melzner, 2010) respond with increased NH$_4^+$ excretion rates in response to acidified conditions as well. It has been hypothesized that NH$_4^+$ based proton secretion might represent a universal and evolutionary ancient pathway to counter an acidosis in many organisms (Wright, 1995; Hu et al., 2014b). Applying an acidosis by reducing the pH of the artificial octopus blood to pH 7.2 our results clearly showed a substantial capability of the octopus gill to regulate blood pH homeostasis to maintain pH-values around 7.4–7.5. Similar to the situation in teleost fish (Evans et al., 2005) and crustaceans (Henry et al., 2012), the cephalopod gill represents an important site for extracellular pH regulation equipped with an acid-base regulation machinery located in specialized epithelial cells (Hu et al., 2011, 2014b). The present work provides a direct evidence for the coupling of extracellular pH regulation and NH$_4^+$ excretion during an acidosis in a molluscan system.

**Branchial NH$_4^+$ Transport in the Octopus Gill**

The selective soluble adenylyl cyclase (sAC) inhibitor KH7 demonstrated that the excretion of NH$_4^+$ across brachial epithelia is cAMP-dependent. sAC is an evolutionary ancient enzyme that is involved in HCO$_3^-$ sensing from cyanobacteria and has been mainly associated with ion/pH regulatory epithelia (Tresguerres et al., 2011). sAC has been demonstrated to be an important regulator of primarily active ion pumps including NKA (Schmitz et al., 2014) and V-type H$^+$-ATPase (Tresguerres et al., 2011). Accordingly, it can be hypothesized that also in molluscs like O. vulgaris sAC has inherited the evolutionary conserved role as a regulator of acid-base homeostasis. Recent advances in understanding branchial pH regulation and excretion in cephalopods have led to first models of proton and NH$_4^+$ secretion pathways. The cephalopod gill is equipped with acid-base transporters including NHE3, VHA, and NKA that are stimulated by a hypercapnia-induced acidosis (Hu et al., 2014b). Furthermore, a Rhesus protein (RhP) that is co-localized with NHE3 in apical membranes has been suggested to be involved in an acid-trapping mechanism of NH$_3$ by protons in the semi-tubular lamellar of the squid gill (Hu et al., 2014b). Similar to the situation in decapodiform cephalopods (e.g., squid and cuttlefish) the octopus gill also expresses acid-base transporters, including NHE3, VHA, NKA, and RhP. Predominant localization of NHE3 and RhP in apical membranes and NKA in basolateral membranes suggest that also in octopus branchial epithelia, NHE3, and RhP operate in concert to promote ammonia excretion. Furthermore, the
Role of Different Excretory Organs in Ammonia Homeostasis

Comparisons of substrate preferences in different tissues of octopus indicated that gill, kidney (renal appendages), and liver tissues preferentially oxidize glutamate, which has been speculated to represent an important source of NH$_4^+$ (Hochachka and Fields, 1982). In vitro determinations of glutamate oxidation rates in gill and kidney tissues of Octopus macropus were 1.1 and 1.3 μmol h$^{-1}$ g$^{-1}$ FM, respectively (Hochachka and Fields, 1982). Furthermore, purine catabolism has been suggested to represent another source of gill ammoniagenesis supported by the evidence of high adenosine deaminase activity in the squid gill (Hoeger et al., 1987). These biochemical data corroborate with our functional results demonstrating that the isolated octopus gill is capable of generating NH$_3$/NH$_4^+$ at rates of 4.55 ± 1.73 μmol h$^{-1}$ g$^{-1}$ FM under simulated in vivo conditions with no NH$_3$ added to the perfusion saline. Here it remains to be investigated through which pathways endogenous ammonia is exported across the basolateral membrane. In the thick ascending limb of the mammalian kidney NH$_4^+$ can exit the basolateral membrane via NHE4 and through a so far uncharacterized, vesicular content is excreted by exocytosis (Weiner and Verlander, 2013). These biochemical data indicate that the octopus gill is capable of mediating NH$_4^+$ excretion and pH regulation including renal- and branchial heart- appendages. It can be hypothesized that coordination of excretion and acid-base regulation between different organs is controlled by endocrine mechanisms. Future studies will aim at improving this gill perfusion technique by using solutions that are closer to those seen by the gill under in vivo conditions using seawater (external medium) and species specific perfusion salines. These studies will include a deeper analysis of blood acid-base parameters, including changes in pH, HCO$_3^-$, and pCO$_2$ during passage of the perfused octopus gill. Furthermore, using this perfusion technique in different cephalopod excretory organs in combination with synthesized hormone peptides (e.g., octopressin) will help to develop an ex vivo model to better understand the regulatory mechanisms of excretion and pH homeostasis in cephalopods.

CONCLUSION

The present work highlighted the importance of a strictly regulated NH$_4^+$ dependent acid-base homeostasis in the cephalopod O. vulgaris. Our results demonstrated that these animals not only excrete ammonia into the surrounding sea water but are capable of maintaining an ammonia concentration of ~300 μM in their blood. In contrast to terrestrial vertebrates that have normal venous plasma values ranging between 5 and 40 μM NH$_3$ (Cooper and Plum, 1987) aquatic species are generally characterized by extracellular NH$_4^+$ concentrations in the range of 100–600 μM (Wood, 1993; Weihrauch et al., 2004; Cruz et al., 2013). In the light of our results that demonstrated a direct link between H$_2$O regulation and NH$_4^+$ excretion it is tempting to speculate that blood [NH$_4^+$] in the range of 200–300 μM are essential to maintain acid-base regulatory capacities in octopus.

AUTHOR CONTRIBUTIONS

MH, PH, YT, and DW designed and conducted experiments of the present work. YG performed molecular cloning of candidate genes from O. vulgaris. PS and JL conducted in vivo pH and NH$_4^+$ measurements and immunohistochemical analyses. MH, DW, and YT wrote the first draft of the manuscript and all authors contributed to the completion of the manuscript and analyses of the data.

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SUPPLEMENTARY MATERIAL

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