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Evolutionary insights into gut acidification: invertebrate-like mechanisms in the basal vertebrate hagfish

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ABSTRACT

Acidification is a key component of digestion throughout metazoans. The gut digestive fluid of many invertebrates is acidified by the vesicular-type H⁺-ATPase (VHA). In contrast, vertebrates generate acidic gut fluids using the gastric H⁺/K⁺-ATPase (HKA), an evolutionary innovation linked with the appearance of a true stomach that greatly improves digestion, absorption and immune function. Hagfishes are the most basal extant vertebrates, and their mechanism of digestive acidification remains unclear. Herein, we report that the stomachless Pacific hagfish (*Eptatretus stoutii*) acidify their gut using the VHA, and searches of *E. stoutii* gut transcriptomes and the genome of a closely related hagfish species (*E. burgerii*) indicate they lack HKA, consistent with its emergence following the 2R whole-genome duplication. Immunostaining revealed prominent VHA presence in the apical membrane of enterocytes and sub-apical expression of both VHA and soluble adenylyl cyclase. Interestingly, akin to vertebrates, VHA was also observed in immature pancreatic-like zymogen granules and was noticeably absent from the mature granules. Furthermore, isolated gut sacs from fed hagfish demonstrate increased VHA-dependent luminal H⁺ secretion that is stimulated by the cAMP pathway. Overall, these results suggest that the hagfish gut shares the trait of VHA-dependent acidification with invertebrates, while simultaneously performing some roles of the pancreas and intestine of gnathostomes.

KEY WORDS: Proton pump, Postprandial, Adenylyl cyclase, Zymogen, Agnathan, Bafilomycin

INTRODUCTION

Acidification plays a ubiquitous role in the mechanisms used to digest food, absorb nutrients and defend from pathogens (Koelz,

1992). Protists and multiple invertebrate metazoans rely upon intracellular digestion, wherein food particles are engulfed via endocytosis to form intracellular vesicles (i.e. phagosomes) that fuse with acidic lysosomes for digestion (He et al., 2018; Steinmetz, 2019). A major evolutionary advancement was the emergence of extracellular digestion, which involves secretion of enzymes into the lumen of a digestive organ, ultimately increasing the size, and therefore variety, of food that is able to be digested (Yonge, 1937). Multiple invertebrate phyla utilize a combination of both intra- and extracellular digestion; however, vertebrates solely rely upon extracellular mechanisms for digestion (Steinmetz, 2019; Yonge, 1937).

It has been posited that extracellular digestion evolved through the co-opting of the intracellular endocytic pathway for the excretion of lysosomal digestive enzymes (Steinmetz, 2019). This hypothesis is supported by the presence of a common mediator in both mechanisms, the vesicular-type ATPase (VHA). This multi-subunit proton pump enzyme is ubiquitously expressed in eukaryotes (Maxson and Grinstein, 2014) and is responsible for the maturation and maintenance of an internal acidic pH as low as 4.5 within lysosomes (Trivedi et al., 2020). Further, VHA plays a role in extracellular digestion in numerous invertebrates. For example, lipophil cells responsible for external digestion in the placozoan *Tricoplax adherens* are enriched in VHA (Romanova et al., 2021), loss of VHA function in the roundworm *Caenorhabditis elegans* prevents gut luminal acidification (Allman et al., 2009; Ji et al., 2006), and VHA energizes mid-gut acidification in the larval fruit fly *Drosophila melanogaster* and adult mosquito *Aedes aegypti* (Nepomuceno et al., 2017; Overend et al., 2016). Whereas many invertebrates utilize VHA to acidify their digestive fluid to ~pH 4–6 (100–1 μmol l⁻¹ [H⁺]), vertebrates with a true stomach (i.e. gnathostomes diverging after the 2R whole-genome duplication that contain gastric glands and the digestive protease pepsinogen; Castro et al., 2014) utilize the H⁺/K⁺-ATPase (HKA; Fig. 1). Compared to VHA, HKA induces a much stronger acidification of digestive fluids to pH as low as 0.8 (160 mmol l⁻¹ [H⁺]; Koelz, 1992). The catalytic subunit of HKA (α-HKA) is closely related to that of the Na⁺/K⁺-ATPase (α-NKA) and thus is purported to have evolved from a common deuterostome α-NKA (Sáez et al., 2009). Genome mining indicates HKA is absent in the most basal extant chordate lineage, the cephalochordates, and also in their closest relatives, the echinoderms (Sáez et al., 2009). Indeed, gut fluids seem to be only moderately acidified in adults of echinoderms (~pH 4–7), as generally determined with indicator dyes under unclear experimental conditions (reviewed by Lawrence, 1982).

Hagfish belong to the infraphylum Agnatha, the sister group of the gnathostomes at the base of the Vertebrata (Miyashita et al., 2019). Alongside lamprey, hagfish are the only extant representatives of the agnathans, and thus provide useful information regarding vertebrate evolution. Although unique aspects of hagfish digestion have been

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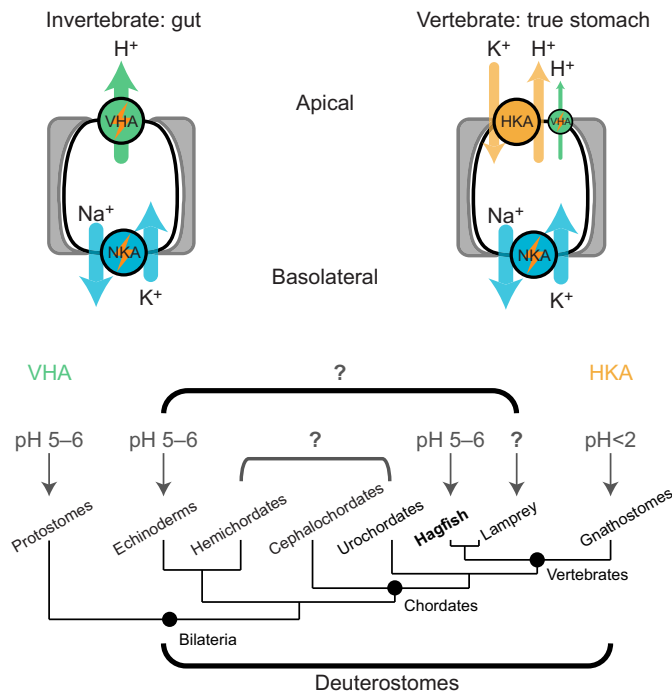


Fig. 1. Mechanisms of extracellular digestive acidification across animal phylogeny. Numerous invertebrates (left) utilize apical vesicular-type ATPase (VHA) in acidifying epithelia to generate a mild acidification of the gut lumen (~pH 5–6), whereas vertebrates with a stomach (i.e. gnathostomes; right) utilize the proton–potassium ATPase (HKA), which is capable of generating a strong acidification (pH <2). VHA appears to colocalize to digestive tissues in gnathostomes and can contribute to mild acidification. In both cases, basolateral sodium–potassium ATPase (NKA) generates electrochemical gradients that assist in ion flux. The mechanisms of digestive acidification in many deuterostomes remain unknown, but mild acidifications (pH 5–6) have been reported for echinoderms and hagfish (bottom); thus, we hypothesize that the invertebrate VHA model is utilized by these animals.

studied (e.g. extra-intestinal nutrient acquisition; Glover et al., 2011b), mechanisms of digestion within the intestine remain poorly understood. Hagfish lack a true stomach, which is thought to represent an extant primitive condition rather than the derived state arising from stomach loss (Smit, 1968). Instead, the hagfish gut is a straight tube; the anterior foregut houses all the mucous cells and some digestive enzymes, and the hindgut comprises digestive pancreatic-like zymogen granule cells (ZGCs) interspersed amongst absorptive enterocytes (Adam, 1963; Weinrauch et al., 2015, 2019a). Hagfish are carnivorous scavengers but also active predators of small fish (Martini, 1998; Zintzen et al., 2011), and thus the acidification of extracellular digestive fluids is likely. Indeed, the hagfish gut can reach a pH of at least 5.5 during active food digestion (Nilson and Fänge, 1970); however, whether this is the result of active proton secretion or some other method, such as microbial fermentation, remains unknown. Interestingly, hagfish gut extracts demonstrate optimal protease activity at pH 4 (Nilson and Fänge, 1970), which could indicate a higher degree of acidification of digestive fluids, intracellular digestion, or both. Moreover, the blood in the intestinal vein undergoes an alkalization in the postprandial period alongside secretion of base equivalents to the surrounding water (Weinrauch et al., 2018), which indicates an ‘alkaline tide’ resulting from acid secretion into the gut and concomitant base absorption into the blood (Hersey and Sachs, 1995). However, although the combined evidence suggests that hagfish may utilize acidic extracellular digestion, this

process, and the underlying mechanisms, have not been formally established.

Given that hagfish diverged from the gnathostomes prior to the 2R whole-genome duplication (Yu et al., 2024), we hypothesized that a mechanism other than HKA is responsible for extracellular digestive acidification. We hypothesized that VHA would contribute to luminal acidification alongside other constituents that are common to acidifying epithelia (Gleeson, 1992; Pacey and O’Donnell, 2014). For instance, carbonic anhydrase (CA) catalyzes the formation of H^+ and HCO_3^- from metabolic CO_2 and water. In acidifying epithelia, the resultant protons are secreted across the apical membrane (via HKA or VHA), and basolateral NKA recycles Na^+ and K^+ ions across the parietal cell membrane to maintain a gradient for proton secretion (Gleeson, 1992; Hersey and Sachs, 1995; Pacey and O’Donnell, 2014). Further, we hypothesized that the second messenger pathway responsible for activation of these transporters during active food digestion would be conserved with other vertebrates [i.e. the cyclic adenosine monophosphate (cAMP) signaling pathway; Anttila et al., 1976; Choquet et al., 1990; Ruiz et al., 1993; Trischitta and Schettino, 1998; Vucic et al., 2003]. This pathway is conserved across the tree of life; in metazoans it encompasses two enzymatic sources: (1) hormonally stimulated transmembrane adenylyl cyclases (tmACs), and (2) soluble adenylyl cyclase (sAC), which is stimulated directly by HCO_3^- and not by hormones (Levin and Buck, 2015). Stimulation of either AC type catalyzes the conversion of ATP into cAMP, a second messenger molecule that regulates a variety of downstream effects, including the stimulation of gastric acid secretion via HKA (Yao et al., 1996) and the insertion of VHA into target membranes for increased proton secretion (reviewed by Tresguerres et al., 2014). To investigate the mechanisms of digestive acidification in Pacific hagfish (*Eptatretus stoutii*), we first utilized transcriptomics and immunohistochemistry. We then conducted functional characterization of proton secretion mechanisms using pharmacological agents. Specifically, inhibitors of VHA (bafilomycin) or HKA (omeprazole) were applied to hindguts of fed hagfish to determine their role in proton secretion. Next, we examined the effects of a potent tmAC activator, forskolin, on fasting hagfish hindguts to observe whether we could elicit proton secretion similar to vertebrate stomachs (Wilson and Main, 1986), and the sAC inhibitor KH7 on fed hagfish hindguts to observe whether proton secretion could be inhibited, revealing a role for cAMP in acidification. Together, the findings help to resolve our understanding of the transition from invertebrate-like to gnathostome-like roles for VHA in digestion.

MATERIALS AND METHODS

Animal care and husbandry

Pacific hagfish, *Eptatretus stoutii* (Lockington 1878), were collected using baited traps in Trevor Channel, Bamfield, BC, Canada, and immediately transferred to darkened ~200 l holding tanks with continuously flowing seawater (12±3°C) at Bamfield Marine Sciences Centre (BMSC). All experiments were conducted with the approval of the Canadian Council of Animal Care under protocols for the University of Alberta [AUP 0001126 (2017)] and BMSC (RS17-03) and with a collection permit from the Department of Fisheries and Oceans Canada (XR-136-2017). Hagfish were also transported to the University of Alberta and housed in tanks containing recirculating artificial seawater (Instant Ocean: 32 ppt). These hagfish were sampled for RNA extraction and transcriptomics experiments (see below). Hagfish were fasted at least 1 week prior to experimentation or fed squid (*Loligo* sp.) until satiated and then euthanized 8 h following feeding to correspond with previous determinations of altered acid–base status in this species (Weinrauch et al., 2018).

Hagfish gut reference transcriptome

Following euthanasia in artificial seawater containing 1.2 g l⁻¹ NaOH and 4 g l⁻¹ tricaine methanesulfonate (Syndel Laboratories) anterior hindguts from fasted ($N=4$; 41.7±16.5 g; mean±s.d.) or fed ($N=6$; 58.1±31.9 g) hagfish were removed in their entirety and flushed with a 500 mmol l⁻¹ NaCl solution containing 100 mmol l⁻¹ PMSF and 500 mmol l⁻¹ EDTA. A sagittal cut then opened the gut and TRIzol was applied directly to the tissue for 5 min before a glass microscope slide was used to scrape off the luminal epithelial layer directly into a tube containing 1 ml of ice-cold TRIzol. Total RNA was then immediately extracted following the manufacturer's instructions (Invitrogen) and RNA integrity was assessed using a BioAnalyzer (Agilent Technologies) at the Molecular Biology Service Unit (University of Alberta). Total RNA with a RIN value >9 was submitted to Genome Quebec for paired-end next-generation sequencing (100 base pair reads) with an Illumina NovaSeq 6000. Initial quality control of raw sequence reads (65 million reads per library) was conducted using FastQC version 0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Raw reads were then trimmed using Trimmomatic version 0.39 (Bolger et al., 2014), after which trimmed reads were again screened with FastQC. Trimmed reads from all hagfish were combined into a single *de novo* reference transcriptome using Trinity version 2.12.0 (Grabherr et al., 2011). Annotation of the *de novo* transcriptome assembly was performed following the Trinotate pipeline (Bryant et al., 2017) using the following programs: TransDecoder version 5.5.0 (<https://github.com/TransDecoder/TransDecoder/wiki>), blast+ version 2.12.0 (Altschul et al., 1990), HMMER version 3.2.1 (Wheeler and Eddy, 2013), SignalP version 4.1f (Petersen et al., 2011), TMHMM version 2.0c (Krogh et al., 2001) and Trinotate version 3.2.2.

Immunohistochemical analysis of hagfish gut

Antibodies

NKA was immunodetected with a mouse monoclonal α5 antibody, which was purchased from the Developmental Studies Hybridoma Bank (DSHB, The University of Iowa, Iowa City, IA, USA, cat. no. a5). VHA was immunodetected using custom-made rabbit polyclonal antibodies against a peptide in the B subunit (epitope: AREEVPGRRGFPY; GenScript). sAC was immunodetected using custom-made rabbit polyclonal antibodies against a peptide in the second catalytic domain of dogfish (*Squalus acanthias*) sAC (epitope: INNEFRNYQGRINKC; Roa and Tresguerres, 2016). Antisera against either peptide was generated in rabbits and affinity-purified (GenScript). HKA was immunodetected with the rabbit polyclonal antibody against the α subunit of human HKA (119101, MilliporeSigma) (epitope: GVRCCPGSWWDQELYY). Secondary antibodies used for western blots were goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP conjugate (Bio-Rad, cat. no. 1706516 and 1706515, respectively) and for immunohistochemistry goat anti-mouse Alexa Fluor 546 or goat anti-rabbit Alexa Fluor 555 (Invitrogen, cat. no. A-11030 and A-21428, respectively).

Immunohistochemistry

Gut segments of fasted and fed hagfish were fixed in 4% paraformaldehyde in 2× PBS (pH 7.4; Electron Microscopy Sciences) overnight at 4°C, then serially dehydrated in a graded ethanol series (Kwan et al., 2020). Following paraffin embedding, samples were sectioned (7 μm) and placed on Superfrost Plus microscope slides (Thermo Fisher Scientific). Slides were deparaffinized by incubating for 10 min in each of the following: SafeClear (×3), 100% ethanol, 95% ethanol, 70% ethanol, and PBS-T (1× PBS and 0.2% Tween 20). Citrate unmasking buffer (10 mmol l⁻¹

citric acid, Tween 20, pH 6; five incubations at 95°C, 3 min each) and sodium borohydride [1 mg ml⁻¹ in PBS; five incubations at room temperature (RT), 5 min each] were used for antigen retrieval and to reduce autofluorescence, respectively. After 1 h incubation in blocking buffer (PBS-T with 2% normal goat serum, 0.02% keyhole limpet hemocyanin, pH 7.7), slides were incubated overnight in primary antibody [1.5 μg ml⁻¹ anti-dogfish sAC (Roa and Tresguerres, 2016), 1.5 μg ml⁻¹ anti-NKA or 12 μg ml⁻¹ anti-VHA]. Slides were washed in PBS-T (three 5 min washes) and incubated in the appropriate secondary antibody (1:500) for 1 h, before a 10 min incubation in Hoechst 33342 nuclear stain (1:1000 in blocking buffer; Invitrogen). The slides were washed in PBS-T (three 5 min washes) and mounted with FluoroGel (EMS) for visualization on a Zeiss Axio Observer ZA inverted microscope. Zeiss AxioVision software and Adobe Photoshop were used to adjust contrast and brightness only. Peptide pre-absorption controls did not show any visible immunoreactivity (Fig. S1). Antibodies were validated using western blots, which showed antibody reactivities with proteins at their expected, respective molecular weights (Fig. S2; see Supplementary Materials and Methods).

Functional examination of mechanisms of digestive acidification

Hagfish were euthanized in seawater containing 1.2 g l⁻¹ NaOH and 4 g l⁻¹ tricaine methanesulfonate (Syndel Laboratories) and dissected bilaterally on the ventral side to remove the entire gut. When present, gut fluid was collected, and pH was measured using a thermo-jacketed pH electrode (Fisher Scientific Accumet glass body combination electrode). Net fluxes of protons and ions in fasted ($N=6$; 118.8±57.5 g) and fed ($N=6$; 74.5±33.0 g) hagfish were determined as follows. Upon excision of the gut, a sac was tied as previously described (Glover et al., 2011a). Briefly, the gut was sutured at both ends with a sampling port of PE50 inserted in one end. Sacs were filled with hagfish saline (in mmol l⁻¹: NaCl 450, KCl 8, CaCl₂·2H₂O 5, MgSO₄·7H₂O 4, MgCl₂ 11, NaHCO₃ 1, glucose 1; all chemicals from MilliporeSigma), weighed to the nearest gram, and placed in aerated solution of the same composition. Gut proton secretion (J_{H^+}) into the gut lumen was calculated using a double end-point titration method as described previously (Hills, 1973). Briefly, the pH of an aliquot of luminal sample at the start and end of the flux period was recorded while under continuous aeration and temperature (10°C). HCl (0.02 N) was added to each sample while continuously mixing the sample with a stir bar. pH was measured using a thermo-jacketed pH electrode until a stable reading was obtained (drift <0.002 pH units 10 s⁻¹ for 1 min). NaOH (0.02 N) was then added until the pH returned to that of the original sample and stabilized (as above). The titration data (μ equivalents H⁺ required for change in pH unit) was plotted and the slope of a linear regression indicated buffering capacity (β; μmol pH⁻¹). J_{H^+} was calculated as adapted from Wood et al. (2010):

$$J_{H^+} = \frac{\Delta pH \times \beta}{SA \times t}, \quad (1)$$

where ΔpH is the difference between the lowest and highest recorded pH, β is the buffering capacity of the solution (μmol pH⁻¹), SA is the gut sac surface area (cm²) and *t* is flux time (h).

To identify cellular mechanisms involved in acidification, gut sacs from fed hagfish were exposed to various pharmacological inhibitors. Preliminary trials measured J_{H^+} ($N=5$; 61.8±23.7 g) over a 1 h control flux period followed by a second 1 h flux where 0.1% DMSO was added and which had no statistical effect (data not shown; $t=-0.11$, d.f.=4, $P=0.92$). Experimental trials consisted of a 1 h control flux (0.1% DMSO), followed by a second 1 h flux where the luminal saline

was then replaced with new solution containing 0.1% DMSO and either the HKA inhibitor omeprazole (MilliporeSigma 50 $\mu\text{mol l}^{-1}$; $N=5$; 109.1 ± 49.7 g), the VHA inhibitor bafilomycin (MilliporeSigma 1.6 $\mu\text{mol l}^{-1}$; $N=4$; 93.3 ± 3.7 g) or the sAC inhibitor KH7 (Tocris Bioscience, Minneapolis, MN, USA; 10 $\mu\text{mol l}^{-1}$; $N=10$; 74.4 ± 24.6 g), and J_{H^+} was again calculated (as above).

To investigate whether proton secretion was stimulated by tmAC-derived cAMP, forskolin (MilliporeSigma) was applied to the gut vasculature of gut sacs from a subset of fasted animals ($N=5$; 84.5 ± 9.7 g). Specifically, the entire gut was removed and filled with hagfish saline as above. Then, the vena supaintestinalis was cannulated with PE 20 tubing and hagfish saline containing forskolin (10 $\mu\text{mol l}^{-1}$) was perfused through the vasculature at a rate of 4 ml h^{-1} for 15 min (Buckling et al., 2011) to stimulate tmACs from the serosal side. The cannula was then removed from the vasculature for the remainder of the experiment. The gut sac was emptied and refilled with fresh hagfish saline, transferred to a bath of aerating saline, and net proton secretion (J_{H^+}) determined as above.

Additional ion transport parameters were measured and compared between the fasted and fed animals. Ion concentrations were determined using atomic absorption spectrophotometry (iCE 3000 Series, AA Spectrophotometer; Thermo Fisher Scientific) with the appropriate matrix modifier, or, in the case of chloride, using a digital chloridometer as per the manufacturer's specifications (Labconco). Ion absorption rates ($\mu\text{mol cm}^{-2} \text{h}^{-1}$) were calculated as follows (based on Wood et al., 2010):

$$\text{Ion absorption rate} = \frac{(V_i - C_i) - (V_f - C_f)}{\text{SA} \times t}, \quad (2)$$

where V_i and V_f represent the initial and final volumes in the gut sac (ml), C_i and C_f represent the initial and final measured concentration of the respective ion ($\mu\text{mol ml}^{-1}$), SA is the gut sac surface area (cm^2) and t is the duration of the flux (h).

Statistical analysis

R (<https://www.r-project.org/>; RStudio v.1.4.1106) was used for statistical analyses and graphical outputs with a 0.05 significance threshold used throughout. All data was assessed for normality using a Shapiro–Wilk test and were transformed when necessary to meet test assumptions. Specifically, data for fasted versus fed fluxes (Fig. 1A), KH7 application (Fig. 1E) and sodium fluxes (Table 1) were log transformed to meet test assumptions. Data were analyzed using paired t -tests to examine within sac differences (i.e. drug applications) as they were repeated measures on the same gut sac and a Welch two-sample t -test was used to identify differences in J_{H^+} between fasted and fed individuals to compare two populations with unequal variance. Datasets are presented as means $\pm$ s.e.m. with individual data points shown.

RESULTS

Identification of VHA in the hagfish gut transcriptome

The sequences from both fed and fasted anterior hagfish hindgut sections were combined into a single reference transcriptome to determine whether the proton transporters of interest (i.e. HKA and

VHA) were localized to this tissue. The final reference transcriptome contained 893,682 transcripts representing 568,201 genes. VHA subunits were identified in the annotated transcriptome and there was a 100% sequence identity of hagfish VHA B subunit with the epitope used for immunohistochemistry (epitope: AREEVPGRRGFPGY). Conversely, the annotated transcriptome did not contain any transcripts with sequences analogous to gastric (*atp4*) or non-gastric (*atp12*) HKA in the hagfish despite the occurrence of other P-Type ATPases [e.g. NKA (*atp1a*), sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA; *atp2a*)]. Likewise, a BLAST search of the inshore hagfish *E. burgerii* genome (Yu et al., 2024) did not yield conserved sequences for either HKA. To verify that the absence of *atp4* was not an artifact of the annotation procedure, we further examined the transcriptome using a compiled query sequence as described previously (Weinrauch et al., 2022). In brief, using the standard BLASTp function on NCBI protein sequences were curated separately for the catalytic α and non-catalytic β subunits of gastric HKA from multiple vertebrates. These sequences were aligned using MUSCLE (Edgar, 2004). This consensus sequence was used to search the reference transcriptome for conserved and possibly unannotated sequences using HMMER v.3.2.1. Again, no positive hits were identified for either subunit of *atp4*. Only the closely related NKA and SERCA were identified (and subsequently verified as such on NCBI) as related sequences.

Immunohistochemical localization of VHA, sAC and NKA in hagfish gut

With VHA confirmed within the hagfish gut transcriptome, its localization within the tissue was explored alongside that of its putative regulator sAC as well as NKA, a prominent transporter involved in ion cycling and the generation of cellular electrochemical potential. Antibodies were first verified and showed no staining in the peptide pre-absorption controls (Fig. S1). Further, western blots verified that antibody reactivity occurred at the expected molecular weight for all investigated proteins (Fig. S2). VHA and sAC were both expressed throughout the sub-apical region of enterocytes (Fig. 2A–D). Abundant VHA expression was localized to the apical membrane of enterocytes, in both fasted and fed conditions (Fig. 2C,D), whereas NKA was localized basolaterally as expected. However, NKA appeared to be more tightly localized to the basolateral membrane in fed hagfish than in fasted hagfish, in which diffuse NKA staining was observed basolaterally and extended laterally (Fig. 2E,F). In addition, sAC appeared to localize within the ZGCs (Fig. 2A,B), and VHA showed differential ZGC staining patterns between fed and fasted hagfish (Fig. 2C,D). A more detailed examination of the ZGCs revealed the absence of VHA in ‘mature’ ZGCs in fasted hagfish with full granule stores in anticipation of the next meal (Fig. 3A,C). However, VHA was abundant within ‘immature’ ZGCs in fed hagfish that had discharged their contents during food digestion (Fig. 3B,D).

Functional examination of the mechanisms of digestive acidification

With apical VHA confirmed in the hagfish gut, we next explored its functional role in gut acidification by directly measuring proton flux

Table 1. Intestinal ion absorption rates in fasted and fed *Eptatretus stoutii*

	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}
Fasted	0.0009 ± 0.002^a	3.56 ± 0.56^a	0.25 ± 0.04^a	0.17 ± 0.05^a	0.36 ± 0.06^a
Fed	-0.002 ± 0.003^a	8.73 ± 2.12^b	0.49 ± 0.06^b	0.40 ± 0.05^b	0.55 ± 0.07^a

Data are means $\pm$ s.e.m. of $N=6$ fed and $N=6$ fasted *E. stoutii*. Positive values indicate absorption and negative values indicate ion secretion in $\mu\text{mol cm}^{-2} \text{h}^{-1}$. Different letters indicate significant differences ($P<0.05$) between the fasted and fed groups as determined using unpaired t -tests.

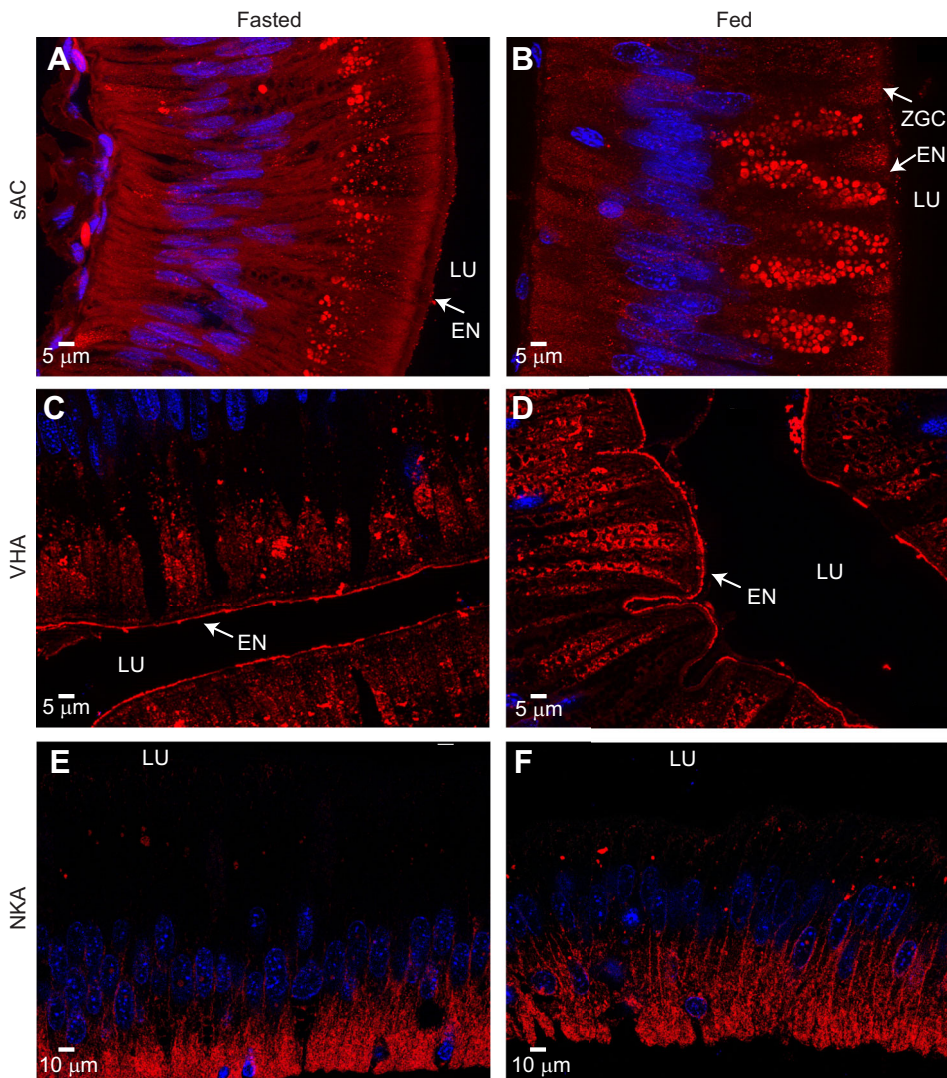


Fig. 2. Immunolocalization of VHA, sAC and NKA in fasted and fed *Eptatretus stoutii* hindguts. (A,B) sAC (red) was localized ubiquitously throughout the enterocyte and within the zymogen granule cells (ZGC) of fasted (A) and fed (B) hindguts. (C,D) VHA (red) was localized to the apical membrane of the enterocyte (EN) near the luminal (LU) surface and sub-apically of both fasted (C) and fed (D) hagfish hindguts. (E,F) NKA (red) was localized to the basal surface of the enterocyte in both fasted (E) and fed (F) hindguts. Nuclei are stained blue in all panels with Hoechst 33342.

in combination with specific pharmacological stimulants or inhibitors. A $\sim 130\%$ increase in J_{H^+} was observed following feeding from 0.049 ± 0.009 to $0.114 \pm 0.024 \mu\text{mol cm}^{-2} \text{h}^{-1}$ (Fig. 4A; $t = -2.67$, d.f.=9.45, $P = 0.02$). After feeding, the stimulated J_{H^+} was significantly reduced with the application of the HKA inhibitor omeprazole (Fig. 4B; $t = 3.12$, d.f.=4, $P = 0.04$), and the VHA inhibitor bafilomycin (Fig. 4C; $t = 4.67$, d.f.=3, $P = 0.02$). In the fasting condition, J_{H^+} was significantly increased by $\sim 90\%$ following application of the tmAC stimulator forskolin (Fig. 4D; $t = -3.50$, d.f.=4, $P = 0.02$), whereas application of the sAC inhibitor KH7 (Fig. 4E; $t = 5.54$, d.f.=8, $P < 0.001$) significantly reduced J_{H^+} after feeding.

Feeding did not change the rate of intestinal chloride transport ($t = 0.84$, d.f.=9.17, $P = 0.42$), but it did alternate from net absorption to net secretion following feeding albeit with large variation across individuals and near net zero (Table 1). Significant increases in absorption rate were observed after feeding for sodium ($t = -3.06$, d.f.=9.18, $P = 0.01$), potassium ($t = -3.29$, d.f.=8.72, $P < 0.001$) and magnesium ($t = -2.91$, d.f.=8.66, $P = 0.01$), but not for calcium ($t = -2.17$, d.f.=9.87, $P = 0.06$; Table 1).

DISCUSSION

The present study is the first examination of the mechanisms of digestive acidification in a hagfish species. Our results indicate that

apical VHA is responsible for digestive acidification in hagfishes, akin to the mechanism present in a number of invertebrate species that results in a mild acidification ($\sim \text{pH } 5\text{--}6$). Additionally, VHA is found within the digestive zymogen granules, much like observations in the mammalian pancreas, demonstrating the earliest known phylogenetic appearance of this conserved function. Further, we provide evidence of cAMP-mediated activation of digestive acidification and potential for sAC to regulate the expression of VHA on its target membranes.

Given that hagfish diverged prior to the 2R whole-genome duplication, when HKA is thought to have arisen (Castro et al., 2014), yet are themselves vertebrates, we considered the possibility that either VHA- or HKA-mediated acidification could occur, with the hypothesis that VHA would be responsible for the previously characterized mild acidification to $\sim \text{pH } 5\text{--}6$ (Nilson and Fänge, 1970). We first verified that feeding elicits a significant acidification of the gut lumen. The guts of fasted hagfish had very little to no fluid, which precluded accurate measures of gut pH. However, digesta in fed guts had a pH between 5.3 and 6.0, a range similar to that measured during the titrations. Although this pH matches the few published reports available in the literature (Glover et al., 2011a; Nilson and Fänge, 1970), it does not indicate proton secretion per se, as it could be the result of microbial fermentation. Thus, J_{H^+} was measured in freshly

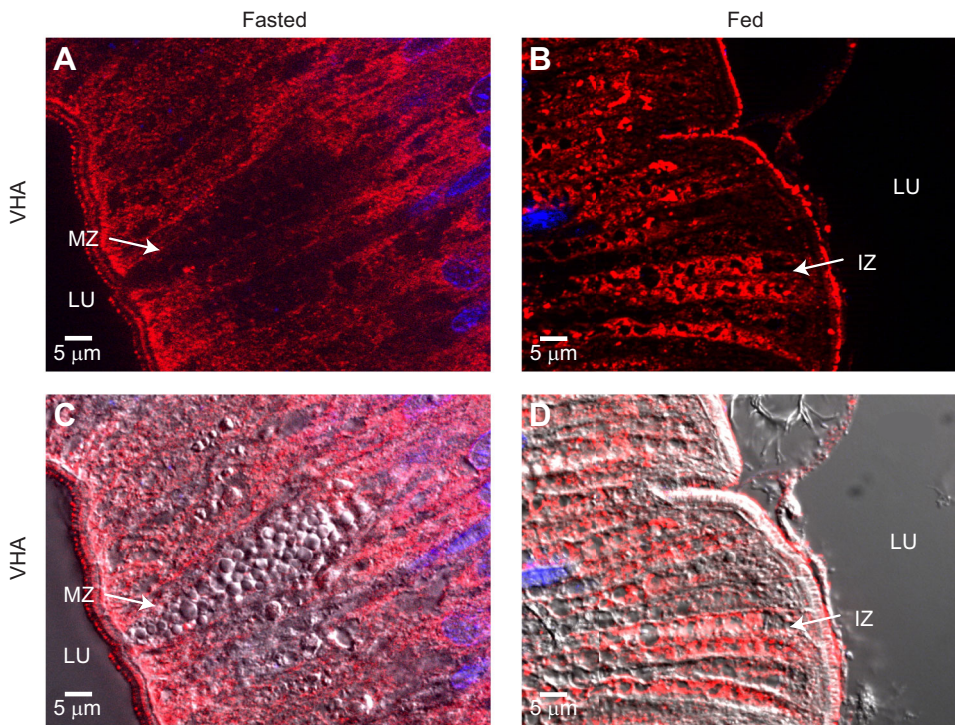


Fig. 3. Immunolocalization of VHA in *Eptatretus stoutii* ZGCs of the hindgut demonstrates differences in expression with maturation. (A,B) VHA (red) is absent from mature zymogen (MZ) cells found in fasted animals (A), but is localized to the cytoplasm of immature zymogen (IZ) cells found in fed animals (B). Nuclei are stained with Hoechst 33342 in all panels. (C,D) Differential interference contrast images of the images shown in A and B to highlight the changing abundance of granules. LU, lumen.

dissected guts. Net proton secretion was continuous and increased significantly following feeding, as has been broadly documented across vertebrates (e.g. from elasmobranchs to mammals; Papastamatiou and Lowe, 2004; Sachs, 1994). With acidification established, the cellular mechanisms responsible were investigated.

Analyses of the inshore hagfish *E. burgerii* genome (Yu et al., 2024) and of *E. stoutii* gut transcriptomes did not find evidence for subunits of either the gastric (*atp4*) or non-gastric (*atp12*) HKA. These observations indicate that hagfish lack HKA, supporting the hypothesis that this gastric proton pump evolved with the gnathostomes (Castro et al., 2014). Despite this, application of the HKA inhibitor omeprazole resulted in a significant reduction in proton secretion of fed guts. This reduction likely indicates off-target effects despite using a dose ($50 \mu\text{mol l}^{-1}$) that falls within the range of reported doses used across vertebrate species ($10\text{--}200 \mu\text{mol l}^{-1}$; Kitay et al., 2018; Kopic et al., 2012; Mattsson et al., 1991; Paradiso et al., 1989; Seidler et al., 1992; Wood et al., 2009). Given that omeprazole binds to the α subunit of HKA, a subunit that is highly conserved with NKA (S  ez et al., 2009), this could indicate inhibition of this other P-type ATPase, or even VHA. Further, at concentrations from 0.01 to $100 \mu\text{mol l}^{-1}$, omeprazole can inhibit CA activity in a tissue-specific manner in humans (Puscas et al., 1999). If CA was inhibited, proton availability might be reduced and lead to the observed reduction of luminal proton secretion. Although one report suggests that CA activity is lacking in hagfish gut (Esbaugh et al., 2009), this study investigated membrane-bound isoforms only. Cytosolic isoforms are likely present within this tissue and remain unstudied.

Within the hagfish transcriptomes and the *E. burgerii* genome, VHA subunits were readily apparent, and VHA_B protein was abundant within the apical membrane of the hagfish gut as evidenced by immunohistochemistry. Further, functional evidence for the involvement of VHA in digestive acidification was observed with a $\sim 60\%$ reduction of J_{H^+} by bafilomycin to fed guts. Bafilomycin sensitivity and apical VHA localization is typical of

multiple acid-secreting digestive epithelia of invertebrates, including the midgut of the adult mosquito *A. aegypti* (Nepomuceno et al., 2017; Patrick et al., 2006), the intestine of the round worm *C. elegans* (Allman et al., 2009; Ji et al., 2006) and the midgut of larval *Drosophila* (Overend et al., 2016). Despite HKA being the dominant proton-secreting transporter of most gnathostomes, VHA has also been noted within the gastric tissue of mammals. Specifically, when HKA is pharmacologically inhibited by omeprazole in rat parietal cells, proton secretion is accomplished, although it is unclear whether this is a result of intracellular proton buffering or apical proton secretion by VHA (Kitay et al., 2018; Kopic et al., 2012; Paradiso et al., 1989). Perhaps this highlights an evolutionary retention of VHA function from the invertebrate to vertebrate lineage despite the acquisition of the evolutionarily advantageous HKA (Koelz, 1992).

Beyond the gastric tissue, VHA is also expressed apically within the intestine of vertebrates. In mammals, VHA is colocalized with the cystic fibrosis transmembrane conductance regulator (CFTR; a chloride transporter) where it is hypothesized to be important for proton extrusion (Collaco et al., 2013a,b; Jakab et al., 2013). Yet knowledge regarding intestinal VHA function in mammals is limited. In marine fishes, intestinal VHA is likewise linked to chloride transport where it plays an important role in osmoregulation (Grosell et al., 2009a,b; Tresguerres et al., 2010). Specifically, apical VHA (Grosell et al., 2009a) works in concert with an anion exchanger to secrete chloride and bicarbonate, ultimately resulting in water absorption (Grosell et al., 2009a,b). The osmoregulatory role of VHA is clearly demonstrated during osmotic challenges [e.g. hypersalinity (60 ppt)] where VHA expression and activity increase in the posterior intestine of the toadfish *Opsanus beta* (Guffey et al., 2011). An osmoregulatory role for VHA in the iso-osmotic hagfish (Currie and Edwards, 2010) is unlikely. However, divalent ions including magnesium and calcium are physiologically regulated at the level of the plasma in hagfish (Sardella et al., 2009; Giacomini et al., 2019), and here we

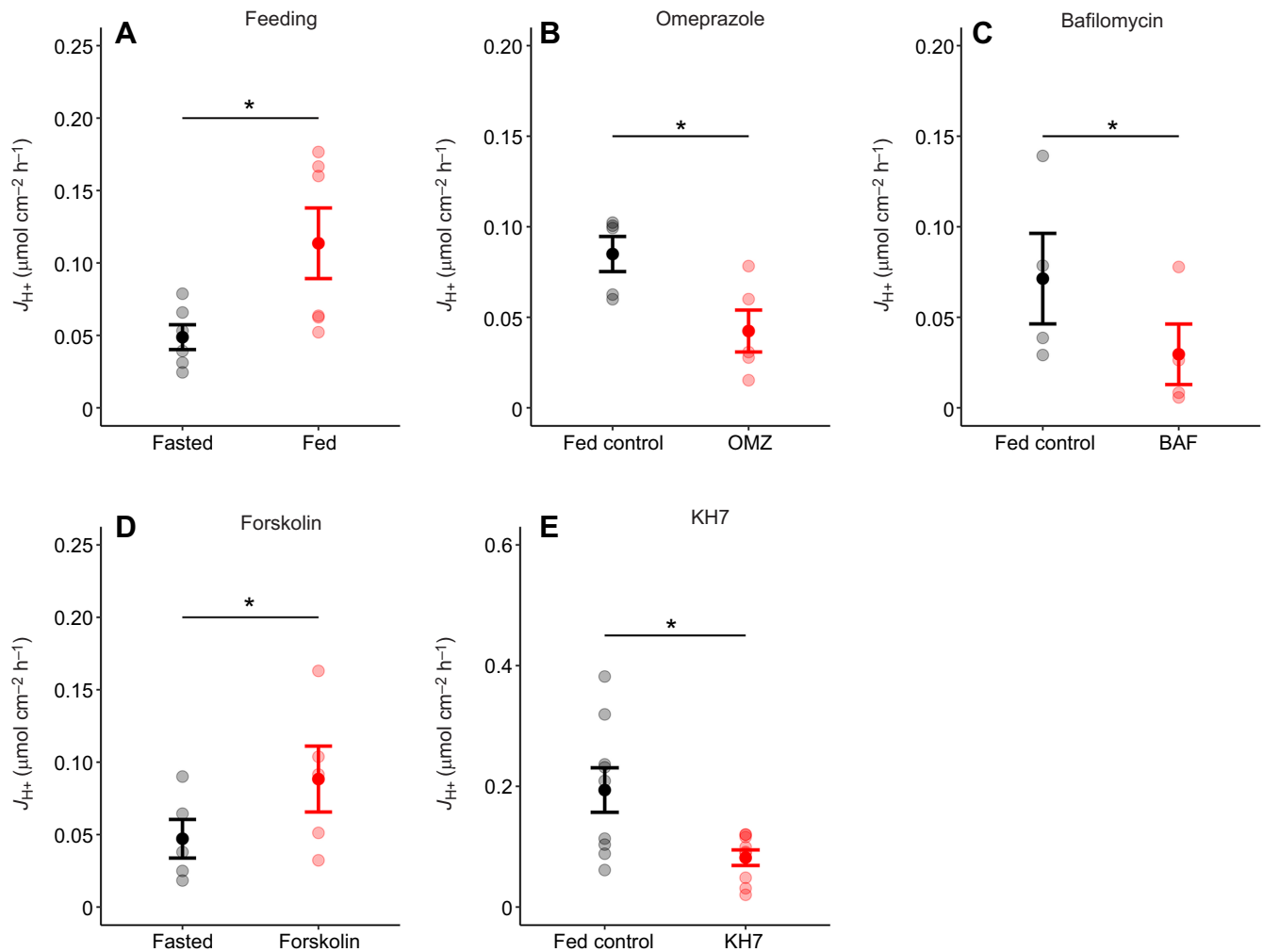


Fig. 4. Functional characterization of *Eptatretus stoutii* gut proton secretion. (A) Stimulation of gut proton secretion (J_{H^+}) with feeding ($N=6$). (B,C) Inhibition of J_{H^+} with omeprazole (OMZ; $N=5$) or bafilomycin (BAF; $N=4$) in fed animals. (D) Effect of forskolin ($N=5$) on fasted gut J_{H^+} secretion. (E) Effect of KH7 ($N=9$) on fed gut J_{H^+} secretion. For all panels, control values are shown in black and treated values in red. For A and D, fasted control animals are shown; for B,C,E, control values were obtained from fed animals. Data are presented as means $\pm$ s.e.m. with individual data points shown. * $P < 0.05$, as determined using unpaired (A) or paired (B-E) t -tests.

demonstrate significant increase of magnesium absorption with feeding. Thus, the hagfish gut may be one tissue where this cation regulation occurs. Further alterations in ion transport were noted for chloride where a switch from net absorption to net secretion occurred (perhaps indicative of HCl formation in the gut lumen), as well as increased absorption of sodium and potassium. These cations are important in establishing electrochemical potential, often through basolateral NKA. Accordingly, immunohistochemistry revealed a basolateral localization of NKA that appears to be more tightly localized to the membrane in fed guts compared with the guts of fasted animals. This may be indicative of upregulated membrane expression to increase transport rates for numerous ions. The sodium electrochemical gradient is of great import not only for ion transport, but other solutes characterized within the hagfish gut, including glucose (Weinrauch et al., 2019b, 2022), amino acids (Glover et al., 2011a) and dipeptides (Weinrauch et al., 2019b), highlighting the need for additional NKA in a digesting gut. Like NKA, VHA is also implicated in the co-transport of nutrients. Specifically, peptide acquisition relies upon a proton gradient, believed to be energized by VHA in freshwater fish (Con et al.,

2017). This remains to be specifically determined in hagfish. However, the apical localization of VHA throughout the intestine suggests that it could play a role in nutrient acquisition as well as acidic digestion.

In addition to acidification, extracellular digestion encompasses the release of digestive enzymes from exocrine cells in the midgut or associated glands, such as the pancreas in some later-diverging vertebrates (Steinmetz, 2019). Pacific hagfish and other basal chordates do not have a discrete pancreas; instead, morphologically and functionally similar ZGCs are found scattered throughout the hindgut enterocytes (Ostberg et al., 1976). Our results show a distinct pattern of VHA ZGC staining between fed and fasted hagfish. Much like mammalian pancreatic acinar cells (Orsi et al., 1987; Thévenod, 2015), VHA is conspicuously absent from 'mature' ZGCs found in fasted hagfish that have replenished their granule stores but is abundant within 'immature' ZGCs in fed animals that have discharged their granules during food activation. Zymogen activation into the proteolytic enzyme is achieved in acidic environments (Gorelick and Otani, 1999); in mammals, VHA may be responsible for said activation or the fusion/exocytosis of the zymogen granules from the cell (Roussa et al., 2001; Waterford

et al., 2005). Therefore, VHA is removed from the granule as it matures to prevent premature activation or release, which may be an evolutionarily conserved trait as it is observed here in the hagfish. Although invertebrates are known to have zymogen granules (LaDouceur, 2021), we cannot find any literature investigating the presence of VHA within these invertebrate cells, so it remains unknown whether this is a vertebrate-specific trait.

With VHA established as a contributor to digestive acidification, we next aimed to characterize the subcellular mechanisms leading to acidification. Similar to feeding, gut proton secretion was stimulated in fasted guts following application of forskolin, a pharmacological agent known to stimulate production of cAMP via tmACs. To verify whether cAMP was involved in the stimulation of luminal acidification, a drug known to limit cAMP production in hagfish tissues (KH7; Wilson et al., 2016) was applied to fed guts. Here, a significant ~60% reduction in the rate of proton secretion was observed, supporting a role for cAMP in the stimulation of proton secretion in the hagfish gut. cAMP is known to stimulate proton secretion through both HKA (Wilson and Main, 1986) and VHA (Dames et al., 2006), with actions at the level of transporter activity, holoenzyme assembly and trafficking to apical membranes (Dames et al., 2006; Sachs, 1994; Wilson and Main, 1986).

To enact its function, VHA must be properly translocated to a targeted membrane. VHA trafficking is known to be induced by cAMP (Collaco et al., 2013a,b) and has been specifically linked to sAC activation in multiple tissues (reviewed by Tresguerres et al., 2011). Given this background and our functional evidence suggesting a role for sAC in mediating proton secretion, we sought to determine sAC localization within the hagfish gut. Like VHA, sAC was expressed in subapical vesicles throughout the hagfish gut where it likely facilitates apical trafficking of transporters, as has been proposed for sub-apical VHA in the intestine of the Atlantic cod *Gadus morhua* (Hu et al., 2016). Indeed, the inhibition of sAC by KH7 may have prevented additional apical translocation of VHA into the membrane, limiting the capacity for proton translocation and resulting in reduced net proton secretion into the gut lumen. sAC was also found within the ZGCs, supporting the hypothesis that sAC could facilitate VHA trafficking within the maturing granules, as is posited to occur in mammals (Koloddecik et al., 2012).

The findings of this study provide evidence that the hagfish gut is a multifunctional organ that can perform the roles of the vertebrate stomach, intestine and pancreas. VHA appears to be the predominant transporter involved in each of these functions and we propose that the apical VHA in the hagfish gut represents an intermediate step between the invertebrate-type acidification machinery and the gnathostome-like intestinal expression, where over evolutionary time VHA progressively loses its physiological importance in luminal acidification and acquires new functional roles in ion regulation and nutrient acquisition. That VHA can contribute to proton secretion when HKA is inhibited in mammals perhaps hints at the retained functions of early-diverging vertebrates in which VHA was the primary source of acidification. The moderate acidification produced by VHA in hagfish gut may have downstream consequences on multiple physiological processes. For example, pathogenic bacteria may be able to more readily colonize the hagfish gut, which could be an important consideration for an animal that lacks essential components of the adaptive immune system (Raison and dos Remedios, 1998). Additionally, the likelihood of encountering pathogenic micro-organisms may be heightened in the scavenging hagfishes, which sometimes feed on dead and decaying organisms that could contain harmful micro-

organisms or viruses (Martini, 1998). Further, hagfish are also known to digest for ~5 days (Eom et al., 2022), yet it is unknown whether luminal pH remains acidic throughout this period or if oscillations in pH occur (as observed in the *C. elegans* gut; Allman et al., 2009). The relative acidity of the lumen may affect not only the efficiency of digestive enzymes, many of which perform optimally at alkaline pH (Nilson and Fänge, 1970), but also dictate which micro-organisms colonize the gut. Finally, given that hagfish are the only vertebrate known to acquire nutrients across their gills and integument (Glover et al., 2011b; Schultz et al., 2014), it would be interesting to investigate the presence and potential digestive function of VHA in these tissues.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.M.W., M.T., G.G.G.; Data curation: A.M.W.; Formal analysis: A.M.W., G.T.K., I.A.B.; Funding acquisition: A.M.W., M.T., G.G.G.; Investigation: A.M.W., G.T.K., M.G.; Methodology: A.M.W., G.T.K.; Project administration: M.T., G.G.G.; Resources: M.T., G.G.G.; Supervision: M.T., G.G.G.; Validation: A.M.W., M.G.; Visualization: A.M.W., G.T.K.; Writing – original draft: A.M.W., M.T.; Writing – review & editing: A.M.W., G.T.K., M.G., I.A.B., M.T., G.G.G.

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Data availability

Data are publicly available at Mendeley Data: <https://doi.org/10.17632/558jx98f.1>

Special Issue

This article is part of the special issue 'The Integrative Biology of the Gut', guest edited by Carol Bucking, Matt Regan and John Terblanche. See related articles at <https://journals.biologists.com/jeb/issue/228/14>.

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Supplemental Materials and Methods

Western Blotting

Enterocytes were gently scraped from hagfish guts using a glass slide. Scrapings were flash frozen in liquid N₂ and stored at -80°C. Samples were pulverized under liquid N₂ using a porcelain mortar and pestle prior to homogenization in ice-cold protease-inhibitor buffer (in mM: sucrose 250, EDTA 1, Tris 30, benzamidine hydrochloride hydrate 10, phenylmethanesulfonyl fluoride 200, dithiothreitol 1; pH 7.5) in a glass homogenizer. Following centrifugation (500 g for 10 min, 4°C), the supernatant was collected and centrifuged (21,130 g for 30 min, 4°C). The resultant supernatant and pellet were saved as the “cytoplasmic fraction” and the “plasma membrane” fraction, respectively. The samples were then passed through a 27G syringe (3X) and sonicated (3 min). Samples were then combined with 2X Laemmli buffer (90%), β-mercaptaethanol (10%), and urea (2M; VHA and sAC, 4M; NKA, 8M; HKA). After heating (95°C, 10 min), proteins were separated on a 7.5% polyacrylamide mini gel (60 V 15 min, 200 V 45 min). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a wet transfer cell (90 mAmps, 8 h; Bio-Rad, Hercules, CA, USA). Following transfer, the PVDF membrane was incubated 1 h in blocking buffer (Tris-buffered saline, 1% tween, 10% skim milk, RT) and incubated with the target antibody (10.5 ng/mL NKA; 1.5 µg/mL sAC and VHA; 0.5 µg/mL HKA) overnight (4°C). On the following day, the PVDF membrane was washed 10 min (3X) in Tris-buffered saline + 1% tween (TBS-T), incubated (1 h, RT) in HRP-linked secondary antibody (1:5,000, Bio-Rad), and washed 10 min (3X) in TBS-T. Preabsorption controls for blots were run after pre-absorbing the sAC, VHA and HKA antibodies with 10-fold excess amount of the corresponding antigen peptide, and did not show any bands. Protein bands were visualized with ClarityTM Western ECL Substrate (Bio-Rad), and imaged and analyzed in a Bio-Rad Universal III Hood using ImageQuant software (Bio-Rad; Supplemental Figure 2).

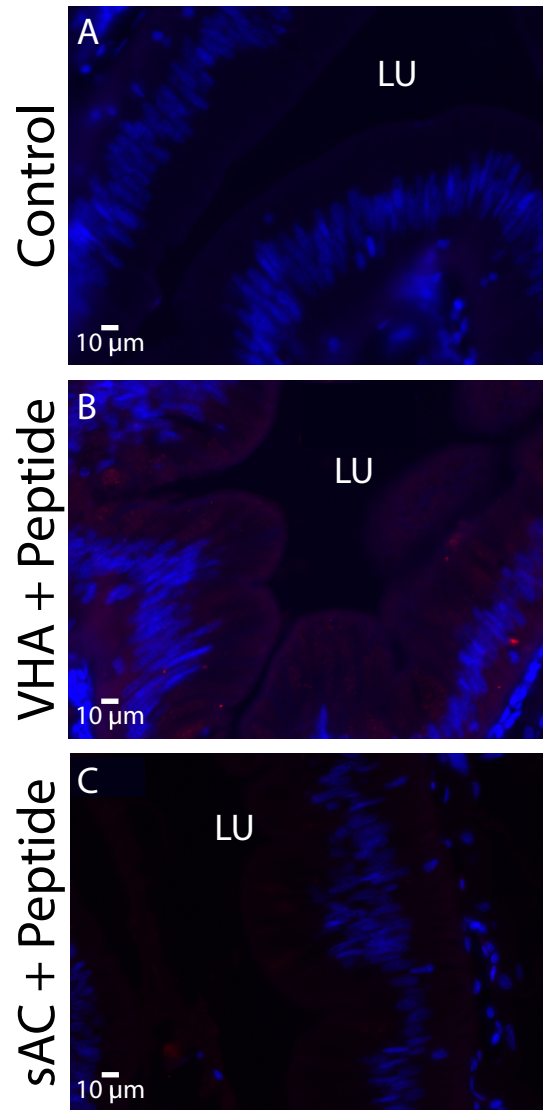


Fig. S1. Immunolocalization controls. Pre-absorption controls (A; red) and peptide absorption controls for VHA (B; red) and sAC (C; red). Nuclei stained blue with Hoescht 33342. LU: lumen.

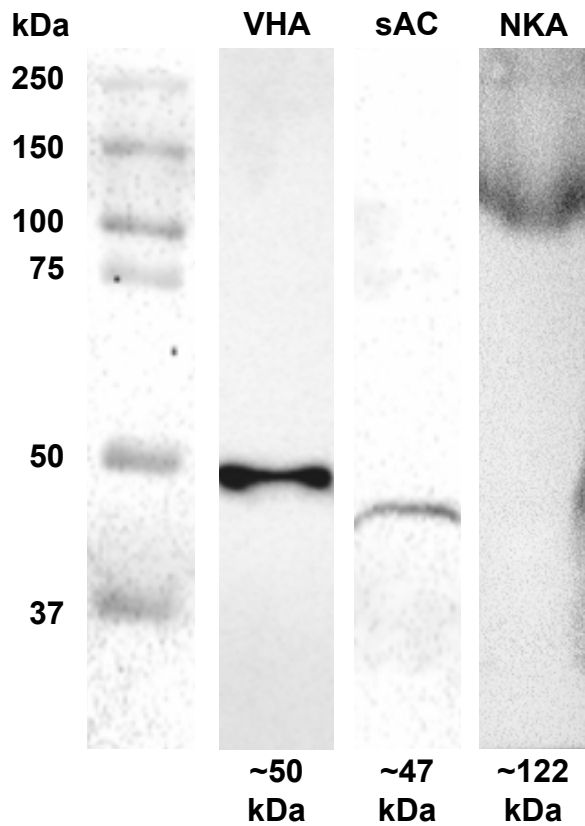


Fig. S2. Verification of antibodies with Western blotting. VHA, sAC, and NKA antibodies specifically recognized a single band at ~50, 47, and 122 kDa, respectively.