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COMMENTARY

Novel and potential physiological roles of vacuolar-type H+-ATPase in marine organisms

Martin Tresguerres*

ABSTRACT

The vacuolar-type H^+-ATPase (VHA) is a multi-subunit enzyme that uses the energy from ATP hydrolysis to transport H^+ across biological membranes. VHA plays a universal role in essential cellular functions, such as the acidification of lysosomes and endosomes. In addition, the VHA-generated H^+-motive force can drive the transport of diverse molecules across cell membranes and epithelia for specialized physiological functions. Here, I discuss diverse physiological functions of VHA in marine animals, focusing on recent discoveries about base secretion in shark gills, potential bone dissolution by *Osedax* bone-eating worms and its participation in a carbon-concentrating mechanism that promotes coral photosynthesis. Because VHA is evolutionarily conserved among eukaryotes, it is likely to play many other essential physiological roles in diverse marine organisms. Elucidating and characterizing basic VHA-dependent mechanisms could help to determine species responses to environmental stress, including (but not limited to) that resulting from climate change.

KEY WORDS: pH, Soluble adenylyl cyclase, Carbonic anhydrase, Carbon-concentrating mechanism, Ocean acidification, *Osedax*

Introduction

The vacuolar-type H^+-ATPase (VHA) is a multi-subunit enzyme that is ubiquitous in eukaryotic cells. VHA uses the energy from ATP hydrolysis to drive H^+ transport against an electrochemical gradient, generating a potential gradient in the process (Mindell, 2012; Wagner et al., 2004). Together with the concomitant transport of a counter ion (typically Cl^-), this process acidifies lysosomes, endosomes, multivesicular bodies and clathrin-coated, Golgi-derived and synaptic vesicles, among other intracellular compartments (Stevens and Forgac, 1997). VHA-mediated acidification in these vesicular compartments is essential for macromolecule digestion and processing, receptor-mediated endocytosis and the coupled transport of small molecules such as nutrients and neurotransmitters (Beyenbach and Wieczorek, 2006; Mindell, 2012; Stevens and Forgac, 1997). VHA subunit structure and function is best known in yeast (Benlekbir et al., 2012; Zhao et al., 2015). VHA has two regions, a V_o domain that spans the lipidic membrane and functions as the H^+ turbine, and a catalytic cytoplasmic V_1 domain (Fig. 1). For detailed information about VHA structure and molecular function in animals, the reader is referred to several excellent recent reviews (Beyenbach and Wieczorek, 2006; Cotter et al., 2015; Drory and Nelson, 2006; Marshansky et al., 2014; Nelson and Harvey, 1999; Stevens and Forgac, 1997; Wagner et al., 2004).

As well as being located in the membranes of vesicular compartments, VHA is also present in the plasma membrane of specialized epithelial cells, where it secretes H^+ for systemic acid/base (A/B) regulation, ion regulation, osmoregulation and extracellular acidification or alkalinization for the purposes of NH_3 excretion, carbonate dissolution and nutrient uptake. The physiological roles of VHA in insects (Baumann and Walz, 2012; Onken and Moffett, 2009; Wieczorek et al., 2009) and mammals (Brown et al., 2009; Jefferies et al., 2008; Saroussi and Nelson, 2009; Wagner et al., 2004) are periodically reviewed. Similarly, recent comprehensive reviews have discussed the involvement of VHA in H^+ secretion and NaCl absorption in freshwater crustaceans, fish and amphibians (Evans et al., 2005; Larsen et al., 2014; Perry and Gilmour, 2006). The purpose of this Commentary is to describe some recent discoveries and hypotheses on the roles of VHA in marine organisms, where it is less well studied, and to place VHA function into the broader context of organismal physiology. Specifically, this Commentary focuses on transepithelial base secretion and acid reabsorption in shark gills, acid secretion by *Osedax* boneworms and symbiosome acidification in corals. The final section presents a discussion of the potential functions of VHA in relation to ocean acidification (OA), an increasingly important topic in marine science.

Acid/base regulation in elasmobranchs: VHA-dependent epithelial HCO_3^- secretion and H^+ reabsorption

Cellular and epithelial HCO_3^- secretion is essential for various physiological functions, such as systemic A/B regulation by the gill and kidney, calcification in bones by osteoblasts (see Glossary) (as well as in the fish intestine, mollusk mantle and, potentially, coral skeleton) and alkaline fluid secretion by the pancreas, intestine and mosquito midgut. The transport of ions against a concentration gradient must be powered by ATPases; however, no HCO_3^- ATPases have yet been molecularly identified in animals. Instead, some cells power HCO_3^- secretion using VHA, which removes H^+ generated from the reaction catalyzed by carbonic anhydrase (CA; $CO_2+H_2O \Leftrightarrow HCO_3^- + H^+$) into the internal compartment (e.g. blood or hemolymph). This 'pulls' the reaction to the right, promoting the generation of HCO_3^-, which is then secreted through anion exchangers or channels into the opposite compartment (the surrounding water or lumen of the organ). Additionally, VHA activity hyperpolarizes the cell membrane and generates an electrical driving force across the membrane of the epithelial cells, which can drive the movement of anions such as Cl^- and HCO_3^- through channels and electrogenic exchangers and cotransporters.

The gill of elasmobranch fishes (sharks, rays and relatives) is an excellent model with which to study the cellular mechanisms of A/B regulation: gills are the only organ responsible for elasmobranch

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List of abbreviations

A/B	acid/base
β-IC	β-intercalated cell
CA	carbonic anhydrase
CCM	carbon-concentrating mechanism
DIC	dissolved inorganic carbon
e	extracellular (subscript)
HKA	H^+/K^+-ATPase
i	intracellular (subscript)
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+-ATPase
OA	ocean acidification
sAC	soluble adenylyl cyclase
VHA	vacuolar-type H^+-ATPase

blood A/B regulation (Heisler, 1988), they only have two A/B regulatory cell types, they do not secrete NaCl for osmoregulation (for which elasmobranchs use the rectal gland; see Glossary), and elasmobranch gills routinely deal with massive A/B fluctuations as a result of feeding, exercise and environmental conditions (reviewed in Tresguerres, 2014; Tresguerres et al., 2014). By contrast, mammals use both lungs and kidneys (reviewed in Boron and Boulpaep, 2012), and their renal cells have multiple A/B regulatory cell types, which are difficult to isolate and cannot withstand large A/B fluctuations *in vitro* (Breton and Brown, 1998; Gong et al., 2010). The gills of teleost fishes also have several cell subtypes, and serve a dual purpose in A/B regulation and NaCl transport for osmoregulation (Evans et al., 2005). Elasmobranch gills contain two types of specialized acid- and base-secreting cells that regulate the blood A/B status (Piermarini and Evans, 2001; Reilly et al., 2011; Roa et al., 2014; Tresguerres et al., 2005). Base-secreting cells are 'VHA-rich', express abundant intracellular CA (Tresguerres et al., 2007b) and apical pendrin-like (see Glossary) anion exchangers (Piermarini et al., 2002; Reilly et al., 2011; Roa et al., 2014), and are mitochondrion-rich (Roa et al., 2014). Normally, fish metabolism is net acidic because of CO_2 release from mitochondria, H^+ derived from anaerobic glycolysis and NH_4^+ resulting from amino acid catabolism. Because base secretion would therefore be counterproductive, gill base-secreting cells are inactive, and VHA is located in cytoplasmic vesicles (Tresguerres et al., 2005). However, sharks need to secrete HCO_3^- in the post-feeding period, because H^+ secretion into the stomach lumen by the H^+/K^+-ATPase (HKA; to aid food digestion) is accompanied by HCO_3^- secretion from the stomach epithelium into the blood, thus inducing a systemic alkalosis that must be counteracted (Wood et al., 2007, 2005, 2009). This process also happens in mammals (Niv and Fraser, 2002), and may be widespread among animals that acidify their stomach for digestion – extreme examples are found in ectothermic vertebrates that eat large, infrequent meals (reviewed in Wang et al., 2001). In fact, transport of H^+ across one side of the epithelium and concomitant transport of HCO_3^- across the other side is a nearly universal phenomenon in physiology.

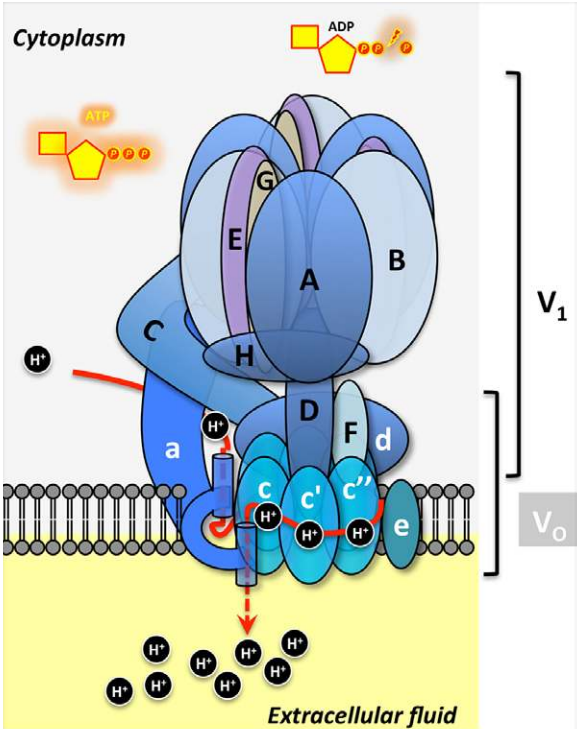

Fig. 1. Vacuolar-type H^+-ATPase (VHA) subunit composition and structure. VHA is composed of cytosolic domain V_1 (subunits A–G in black), and membrane domain V_o (subunits a, c, c′, c″, d and e in white). By convention, subunits in the V_o domain are written in lowercase letters, and subunits in the V_1 domain are written in uppercase letters. A and B are catalytic subunits, D and F are rotor subunits, and E and G are stator subunits. Together, C, H, E and G form the peripheral stalk that connects V_o and V_1 (reviewed in Cotter et al., 2015). In yeast, VHA subunit stoichiometry follows the formula $A_3B_3CDE_3FG_3Hadec_xc'_yc'_z$, where the subscripts indicate the number of each subunit, and x, y, z indicate unknown stoichiometry for subunits c, c′ and c″. The stoichiometry of VHA in other organisms is similar to that of yeast, but can vary in a species- and cell-specific manner. Based on Beyenbach and Wieczorek (2006), and modified from Benlekbir et al. (2012) and Zhao et al. (2015).

Glossary

β-intercalated cell
Kidney cell specialized for HCO_3^- secretion (via pendrin) and H^+ reabsorption (via VHA) in the distal collecting duct of the mammalian nephron.

Ecdysial space
Space that is formed between the hypodermis and the cuticle before the exoskeleton is absorbed as part of the moulting process in crustaceans (and ecdysozoan animals in general).

Etching cells
These cells are believed to secrete acid, thus allowing certain sponges to bore into calcium carbonate substrates.

Osteoblast
Specialized cells in vertebrate animals that synthesize bone.

Pendrin
Electroneutral anion exchanger protein; its main physiological function is epithelial absorption of chloride in exchange for bicarbonate. Encoded in humans by the SLC26A4 gene (Solute carrier family 26, member 4), it owes its name to Pendred syndrome, an autosomal recessive hearing disease caused by mutations in pendrin.

Pillar cells
Gill epithelial cells that connect the two epithelial sheets of gill lamellae. The space delimited between two pillar cells and lamellar cells forms a channel through which blood flows in a counter-current manner compared with the water flowing over the gill.

Rectal gland
A diverticulum of the distal intestine of elasmobranch fishes essential for osmoregulation. The rectal gland epithelium secretes excess NaCl from blood into the cloaca, and has been extensively used as a model system for epithelial NaCl secretion.

Ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo)
This enzyme catalyzes the first major step of carbon fixation in the Calvin–Benson cycle in photosynthetic organisms.

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In air-breathing vertebrates, compensation of blood alkalosis largely results from a hypoventilatory adjustment that retains CO_2, and secretion of HCO_3^- into the small intestine by the pancreas and duodenal glands (reviewed in Wang et al., 2001). However, aquatic animals rely heavily on the active secretion of H^+ or HCO_3^- across the gills to compensate for A/B stress (reviewed in Evans et al., 2005). To secrete excess blood HCO_3^-, elasmobranch base-secreting gill cells are activated through a mechanism that involves CAs and the A/B sensor soluble adenylyl cyclase (sAC) (Fig. 2). First, blood plasma HCO_3^- is dehydrated into CO_2 by an extracellular CA isoform reported to be associated with the cell membrane of pillar cells (see Glossary) and by an intracellular CA isoform in red blood cells (Gilmour et al., 2007, and references therein). CO_2 then diffuses into base-secreting cells and is subsequently rehydrated by an intracellular CA isoform into HCO_3^- and H^+ (Tresguerres et al., 2007b). The elevation in the concentration of intracellular HCO_3^- ($[HCO_3^-]i$) stimulates sAC, which, potentially through the cAMP–PKA pathway, triggers the microtubule-dependent insertion of VHA-containing vesicles from the cytoplasm into the basolateral membrane (Tresguerres et al., 2006c, 2010; Roa and Tresguerres, 2016). Postprandial alkalosis also results in the insertion of pendrin into the apical membrane (Roa et al., 2014), although it is not known whether this is also mediated by sAC. From its basolateral location, VHA 'pumps' H^+ into the blood and energizes HCO_3^- secretion to seawater in exchange for Cl^- through apical pendrin, counteracting blood alkalosis. In the basolateral membrane, Cl^- probably exits the VHA-rich cell into the blood through currently unidentified channels; this step would be essential to maintain a low $[Cl^-]i$, and dissipates the transmembrane electrical force generated by VHA (Stevens and Forgac, 1997). Therefore, HCO_3^- secretion in base-secreting cells is likely to be driven by a combination of increased $[HCO_3^-]i$ and reduced $[Cl^-]i$, both powered by VHA and assisted by CA, which supplies H^+ and HCO_3^- at a sufficiently fast rate.

VHA translocation is gradual and dynamic rather than an 'on/off' process (Tresguerres et al., 2011). This is illustrated in the immunostained gill section in Fig. 2, which shows various stages of VHA translocation in the same gill filament. VHA-containing vesicles are likely to be constantly shuttled between the cytoplasm and the basolateral membrane, thus adjusting the intracellular pH (pHi) of the VHA-rich cells and, consequently, maintaining systemic A/B homeostasis. Therefore, mild and short-lasting alkaline stress can be corrected by the translocation of pre-existing VHA, but the homeostatic response to more extreme alkaline stress additionally involves upregulation of VHA synthesis (Tresguerres et al., 2006c).

The mechanism for sensing and counteracting blood alkalosis in VHA-rich gill cells is likely to be applicable to other epithelial base-secreting cells. For example, β-intercalated cells (β-ICs; see Glossary) in the mammalian nephron also express VHA, pendrin and sAC (Păunescu et al., 2008); however, functional studies with β-ICs are notoriously difficult as a result of the low abundance of this cell type, the lack of cell cultures and their compromised viability upon exposure to stressful conditions (Breton and Brown, 1998; Gong et al., 2010). Sculpin, a species of marine teleost fish, also have a subpopulation of gill ionocytes that express high levels of VHA in cytoplasmic vesicles or possibly in tubular infoldings of the basolateral membrane (Catches et al., 2006). VHA in these cells presumably mediates H^+ reabsorption and energizes apical HCO_3^- secretion as in elasmobranchs; however, this has not yet been confirmed experimentally. Hagfish gills seem to contain a single type of A/B regulatory ionocyte (Tresguerres et al., 2006b), which can perform either acid or base secretion depending on the blood A/B physiological needs. However, hagfish ionocytes have an extensive basolateral tubulovesicular system (Bartels and Welsch, 1986), as found in teleost fish gill ionocytes, which makes it difficult to determine the subcellular location of proteins. Nonetheless, immunohistochemistry and western blots on samples

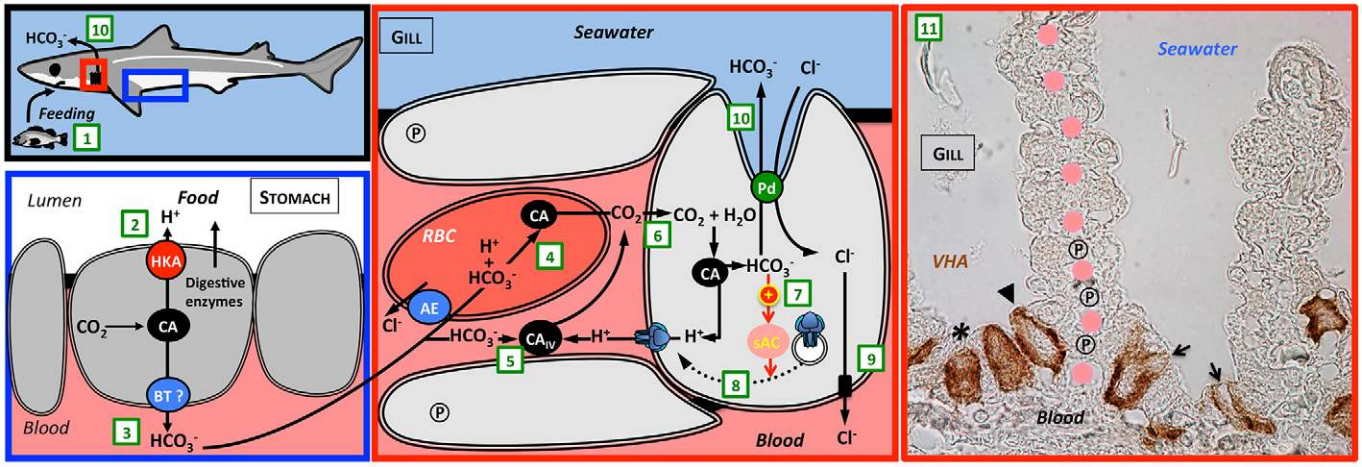

Fig. 2. VHA translocation in shark gill base-secreting cells. (1) Sharks feed opportunistically on a variety of fish and invertebrate prey. (2) H^+/K^+-ATPase (HKA, do not confuse with VHA) is involved in the secretion of HCl into the stomach lumen to aid in food digestion. (3) H^+ secretion is accompanied by HCO_3^- secretion into the blood, inducing alkalosis. BT, bicarbonate transporter. (4) HCO_3^- travels in blood plasma and in red blood cells (RBC) to the gills. Inside RBCs, intracellular carbonic anhydrase (CA) hydrates HCO_3^- into CO_2. (5) In addition, extracellular CA located in the cell membrane of pillar cells (Ⓟ) hydrates plasma HCO_3^- into CO_2. (6) CO_2 from both sources diffuses into VHA-rich base-secreting cells, where intracellular CA rehydrates it into HCO_3^- and H^+. (7) Intracellular HCO_3^- stimulates soluble adenylyl cyclase (sAC), which (8) triggers the translocation of cytoplasmic vesicles containing VHA (blue icon) to the cell basolateral membrane. VHA then secretes H^+ into the blood. (9) A putative basolateral channel brings Cl^- from VHA-rich cells into the blood. (10) Intracellular HCO_3^- is secreted to seawater in exchange for Cl^- via apical pendrin (Pd)-like anion exchangers. The combined action of H^+ reabsorption by VHA and HCO_3^- secretion by pendrin corrects blood alkalosis. (11) Immunostaining of the VHA A subunit (brown) in gills of Pacific dogfish *Squalus acanthias* in early stages of blood alkalosis. VHA-rich cells display various stages of VHA translocation from fully cytoplasmic (asterisk) to fully basolateral (arrowhead). Arrows indicate the apical opening of two VHA-rich cells. Some lamellar blood spaces are labeled with pink circles, and Ⓟ indicate some of the pillar cells. Based on Gilmour et al. (2007), Roa et al. (2014) and Tresguerres et al. (2005, 2006c, 2007b, 2010).

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enriched in gill cell membranes from control and base-infused hagfish suggest that VHA is present both in cytoplasmic vesicles and in the basolateral membrane, and that – similar to results from sharks – VHA inserts into the basolateral membrane and corrects blood alkalosis (Tresguerres et al., 2007a). Cells with basolateral VHA are also found in gills from squid (Hu et al., 2014), crab (Tresguerres et al., 2008) and bone-eating *Osedax* worms (Tresguerres et al., 2013) (see below). However, it is not known whether VHA energizes HCO_3^- secretion in any of these cells.

Apical pendrin and basolateral Cl^- transport across channels results in Cl^- loading in the blood of sharks. For example, sharks fed a meal corresponding to ∼5% of their body mass excrete HCO_3^- at an average rate of ∼230 µmol kg^{-1} h^{-1} (with peak rates of >600 µmol kg^{-1} h^{-1} in some cases), with a total of ∼10.5 mmol kg^{-1} HCO_3^- excreted to seawater during the 2–48 h postprandial period (Wood et al., 2007). Although this is a significant amount of HCO_3^- from an A/B perspective, the equivalent Cl^- loading is negligible when considering that there is a constant concentration gradient that favours Cl^- diffusion from seawater (∼500 mmol l^{-1}) into the blood (∼270 mmol l^{-1}). Furthermore, continuous infusion of $NaHCO_3$ at a rate of 1 mmol kg^{-1} h^{-1} for 24 h did not significantly affect plasma [Cl^-] (Tresguerres et al., 2005), demonstrating that the Cl^- load associated with HCO_3^- secretion is efficiently handled by rectal gland NaCl secretion. However, the Cl^- uptake that results from HCO_3^- secretion has implications for ionoregulation in freshwater fishes. For example, the rates of Cl^- uptake in larval (Bayaa et al., 2009) and adult zebrafish (Boisen et al., 2003), catfish (Goss et al., 1992) and rainbow trout (reviewed in Goss et al., 1994) have been reported as 200–700 µmol kg^{-1} h^{-1}, and are therefore comparable to the rates of HCO_3^- secretion (and thus Cl^- uptake) in post-fed dogfish sharks. Additionally, basolateral VHA has been reported in gill ionocytes from freshwater stingray (Piermarini and Evans, 2001; Piermarini et al., 2002), bull shark (Reilly et al., 2011), killifish (Katoh et al., 2003) and rainbow trout (Tresguerres et al., 2006a), as well as in skin ionocytes from medaka embryos (Lin et al., 2012). An early study found evidence for Cl^-/HCO_3^- exchange in the osmoconforming hagfish, and proposed that the driving force for the evolution of ion uptake in freshwater fish was A/B regulation (Evans, 1984). Here, I propose that one of the cellular mechanisms that allowed the ancestors of modern-day freshwater fish to invade freshwater was the translocation of VHA to the basolateral membrane, possibly as a result of food abundance that caused a prolonged (or continuous) alkaline tide. Over evolutionary time, mutations in genes encoding components of the sensing or translocation mechanisms could have resulted in VHA being permanently located in the basolateral membrane and acting primarily for Cl^- uptake rather than HCO_3^- secretion (but with the two processes remaining linked). Unfortunately, this idea cannot be experimentally tested.

Potential role of VHA in bone dissolution by *Osedax* worms

Osedax is a genus of mouthless and gutless marine worms that live on the seafloor and feed on the bones of dead vertebrates (Rouse et al., 2004). The posterior end of *Osedax* branches out and penetrates deep into the bone, resembling the roots of a tree, and these roots host symbiotic heterotrophic bacteria. *Osedax* worms derive their nutrition from collagen and lipids trapped in the bones (Goffredi et al., 2005; Rouse et al., 2004), which they release by secreting acid using VHA that is present at high levels in the apical membrane of root epithelial cells (Tresguerres et al., 2013). The proposed *Osedax* feeding mechanism is a multistep process that starts with VHA-mediated secretion of H^+ onto the bone, which dissolves the calcium phosphate matrix and releases collagen and lipids (Fig. 3). In order to produce H^+, CO_2 is hydrated to H^+ and HCO_3^- by CA. The CO_2 is generated by aerobic metabolism – *Osedax* has a robust circulatory system that ensures sufficient O_2 delivery to the root epithelium (Huusgaard et al., 2012), and

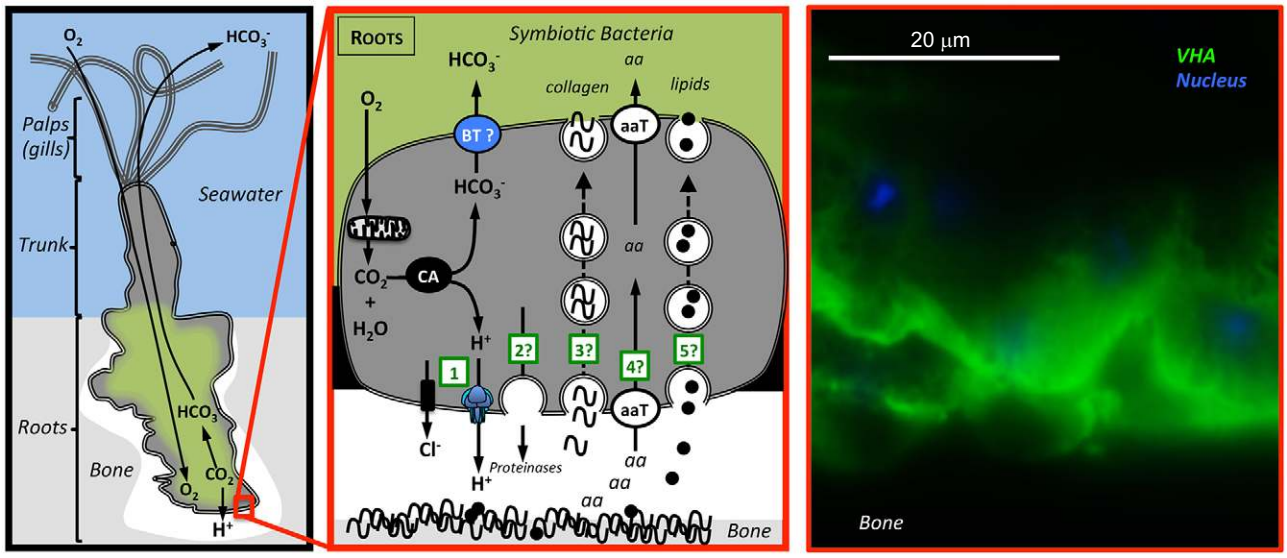

Fig. 3. Acid secretion by VHA in *Osedax* boneworm roots and putative mechanisms for nutrient absorption. Left: The posterior end of the *Osedax* body is modified into 'roots' that penetrate into bone. Symbiotic bacteria are indicated in green. Center: The question marks indicate aspects of the mechanism that are speculative. (1) H^+ secretion by VHA dissolves the bone inorganic carbon matrix. The equimolar HCO_3^- produced from the carbonic anhydrase (CA)-catalyzed CO_2 hydration is likely to be secreted by putative bicarbonate transporters (BT?) in the basolateral cell membrane. (2?) Secreted proteinases break down bone protein matrix. (3?) Collagen-derived peptides are taken up by transcytosis. (4?) Amino acids (aa) are taken up via amino acid transporters (aaT). (5?) Lipids are absorbed by transcytosis. Right: VHA immunostaining (green) in the apical membrane of cells in the *Osedax* root epithelium, facing the bone. Based on Goffredi et al. (2005), Rouse et al. (2004) and Tresguerres et al. (2013).

epithelial cells that are rich in mitochondria and CA (Tresguerres et al., 2013). This implies that as H^+ is secreted onto the bone, an equimolar amount of HCO_3^- is secreted into the worm's tissues. This excess HCO_3^- load is likely transported in the blood to the gills and then secreted to the surrounding seawater. Because a subset of gill epithelial cells has immunostaining that is consistent with basolateral VHA localization (Tresguerres et al., 2013), HCO_3^- might be secreted following the cellular mechanism described above for shark gills.

As the bone calcium phosphate matrix is dissolved, lipids and collagen are released and digested, with digestion of the latter likely aided by proteolytic enzymes (cf. Goffredi et al., 2005). The released nutrients must then be absorbed across the root epithelium and transported to the symbiotic bacteria hosted in *Osedax* roots, which then metabolize these nutrients into diverse compounds. Finally, these metabolites must be transferred back to the worm or possibly the worm digests the bacteria, or both (Katz et al., 2011). Further research is needed to definitively characterize the *Osedax* feeding mechanism; however, this task is not trivial because *Osedax* typically live at depths of between 300 and 3000 m [although some *Osedax* species may be found at depths as shallow as 30 m off the coast of Sweden (Huusgaard et al., 2012)].

Acid secretion by VHA is a characteristic likely shared by other marine invertebrates that bore into the substrate. For example, VHA is highly abundant in the brush border of cells in the accessory boring organ from the snail *Nucella lamellosa* (Clelland and Saleuddin, 2000), which drills through the calcium carbonate shells of its prey. And, although VHA has not yet been described in boring sponges, the etching cells (see Glossary) of these organisms have abundant vacuoles and CA activity (Pomponi, 1980), consistent with the presence of VHA. Furthermore, the proposed mechanism by which *Osedax* might dissolve bone and absorb nutrients has striking similarities to the mechanism of bone resorption by vertebrate osteoclasts, which involves acid secretion by apical VHA (Blair et al., 1989; Li et al., 1999), the formation of a 'resorptive pit', the degradation of the bone organic matrix by proteolytic enzymes (Gowen et al., 1999), the absorption of bone-derived substances and the removal of HCO_3^- (reviewed in Väänänen et al., 2000). If confirmed, this would represent a remarkable example of convergent evolution between an epithelium specialized for feeding (*Osedax* roots) and cells specialized for bone remodelling (osteoclasts).

VHA and a carbon-concentrating mechanism in the coral symbiosome

Reef-building corals are cnidarian animals with endosymbiotic *Symbiodinium* dinoflagellate algae living inside their gastrodermal cells. Photosynthesis by *Symbiodinium* produces abundant carbohydrates, most of which are transferred to the animal host to be used as fuel to sustain metabolism (Falkowski et al., 1984; Muscatine et al., 1984). Because the ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo; see Glossary) enzyme of dinoflagellates has a low affinity for its CO_2 substrate (Rowan et al., 1996), CO_2 must be present at high concentrations in order for *Symbiodinium* to photosynthesize. Thus, a carbon-concentrating mechanism (CCM) is necessary to sustain photosynthesis and prevent photorespiration (Reinfelder, 2011). However, this presents a physiological challenge, as there are no known molecular mechanisms to actively transport gases such as CO_2 and, at the pH of seawater and coral cell cytoplasm, the most abundant dissolved inorganic carbon (DIC) species is, by far, HCO_3^-. Recently, VHA in the host-derived symbiosome membrane has been shown to promote *Symbiodinium* photosynthesis (Barott et al., 2015). The proposed mechanism (Fig. 4) involves acidification of the symbiosome space by VHA to pH~4, which presumably energizes the parallel transport of HCO_3^- and favours the chemical speciation of DIC into CO_2. The evidence in support of this mechanism includes a high abundance of VHA in symbiosome membranes of gastrodermal cells, alkalinization of the symbiosome space from pH~4 to pH~5 in response to bafilomycin (a specific

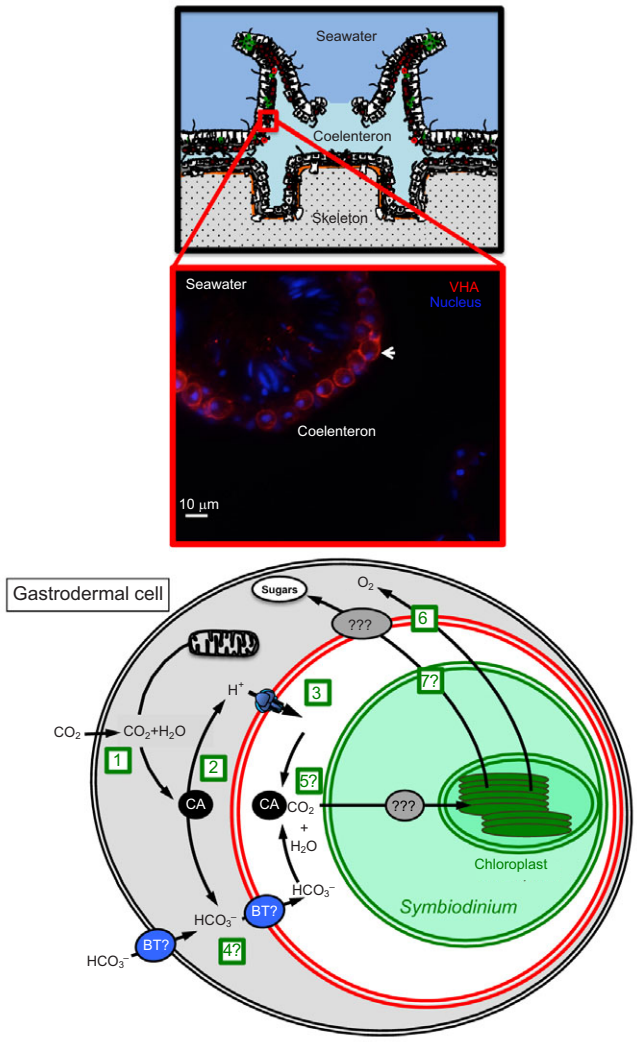

Fig. 4. VHA-dependent carbon-concentrating mechanism (CCM) in coral. Top: Diagram of coral tissues. The red box indicates the location of gastrodermal cells hosting *Symbiodinium*. Middle: VHA immunostaining (red) in the symbiosome membrane of coral gastrodermal cells (arrow). Bottom: Proposed coral CCM. The question marks indicate aspects of the mechanism that are speculative. (1) CO_2 (potentially derived from coelenteron and adjacent cells, or from metabolic processes) enters the gastrodermal cell. (2) Intracellular carbonic anhydrase (CA) catalyzes the hydration into H^+ and HCO_3^-. (3) VHA (blue) in the symbiosome membrane 'pumps' the H^+ into the symbiosome space, acidifying it to pH~4. (4?) Putative bicarbonate transporters (BT?) in the cell membrane could import HCO_3^- into the cytoplasm, and a different subset of BTs in the symbiosome membrane could import HCO_3^- into the symbiosome space. (5?) H^+ and HCO_3^- combine to form CO_2 in the symbiosome space, which first diffuses inside *Symbiodinium* and eventually reaches the site of RuBisCo through unknown mechanisms (???). (6) *Symbiodinium* photosynthesis produces oxygen, as well as carbohydrates that are translocated to the coral host cell through (7?) unknown mechanisms. Based on Barott et al. (2015) and Tresguerres et al. (in press).

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inhibitor of VHA) in isolated cell experiments, and a significant decline in O_2 production in coral branches exposed to bafilomycin (Barott et al., 2015). This mechanism has been reported for two coral species belonging to the two different coral clades, robust and complex (Barott et al., 2015), so it is likely to apply to most, if not all, reef-building corals. Furthermore, given that most marine photosynthesizing organisms require a CCM (Reinfelder, 2011), and that VHA is evolutionarily conserved in eukaryotic cells, the mechanism described for carbon concentrating in corals may be widespread. For example, VHA mRNA abundance was upregulated in coccolithophores (unicellular eukaryotic phytoplankton with external calcium carbonate plates) exposed to low CO_2 levels (Bach et al., 2013); however, the functional relevance of this finding is not yet known.

Many questions about the potential coral CCM remain unanswered, including those about the source(s) of HCO_3^- and H^+, the existence and identities of HCO_3^-- and additional H^+-transporting proteins in the various biological membranes of coral host cells and *Symbiodinium*, and whether VHA–symbiosome acidification actually promotes carbon fixation (and not just O_2 production). The role of the P-type H^+-ATPase [the plasma membrane H^+-ATPase typical of plants and fungi that is expressed by symbiotic but not free-living *Symbiodinium* (Bertucci et al., 2010)], is also unknown. Furthermore, the symbiosome space is equally acidic during both light and dark periods, at least under the isolated cell conditions that have been tested thus far (Barott et al., 2015). This raises the possibility that symbiosome acidification is not only required under light conditions, when photosynthesis is occurring, but is also involved in additional metabolic exchanges across the symbiosome membrane, such as the transport of NH_4^+ and photosynthates, or the regulation of the *Symbiodinium* cell cycle.

Role of VHA in calcification

Although the mechanisms for making calcium carbonate skeletons and the chemical composition of such structures may vary greatly from organism to organism, some basic unifying principles seem to apply to all: Ca^{2+} and DIC must be transported into a confined space (intra- or extra-cellular), nucleating compounds (macromolecules or other ions) must be present and H^+ must be removed to create a high pH environment. Conceivably, VHA could be involved in removing H^+ from the site of calcification and providing the driving force for DIC (and maybe Ca^{2+}) transport. Evidence for the role of VHA in calcification was provided by studies on sternal epithelial cells from the terrestrial isopod *Porcellio scaber*: VHA exhibits a switch in epithelial polarity during the moulting period, from the apical membrane during skeleton reabsorption to the basolateral membrane during skeleton deposition (Ziegler et al., 2004). The switch in VHA polarity coincided with a reversal of the H^+ gradient between the ecdysial space (see Glossary) and the hemolymph. Although the latter maintains a constant pH of ∼7.6, the former has an acidic pH of ∼6.9 during skeleton resorption and an alkaline pH of ∼8.2 during skeleton deposition (Ziegler, 2008).

Basolateral VHA is also present in marine teleost intestinal epithelial cells (Guffey et al., 2011), which secrete HCO_3^- into the intestinal lumen to promote $CaCO_3$ precipitation and water absorption (Wilson et al., 2002), and it secretes an equimolar amount of H^+ into the blood (Grosell and Genz, 2006). Other calcifying cells from invertebrate animals that express VHA include those in molluscan mantle (Barron et al., 2012; Li et al., 2016; Mann and Jackson, 2014) and hemocytes (Barron et al., 2012). However, it is currently unknown whether VHA is involved in promoting calcification in any of those cells. Coral calcifying cells have been proposed to also use VHA for intracellular pH regulation and for removing H^+ from the site of calcification (Allemand et al., 2004); however, expression of VHA in those cells has not yet been determined.

There is also indirect evidence for the involvement of VHA in calcification in coccolithophores, as VHA was found on the coccolith vesicle membrane and is hypothesized to alkalinize the coccolith vesicle to promote coccolith formation (Corstjens et al., 2001). However, this mechanism would require pumping of H^+ from an endomembrane into the cytosol, which has never been reported in any system to date (Mackinder et al., 2010), or the action of as yet unidentified electrogenic exchangers such as $Na^+/2H^+$, taking advantage of the voltage set by VHA across the coccolith vesicle membrane to remove H^+ and alkalinize the vesicle. This would be similar to the alkalinization mechanism described in the insect midgut (reviewed in Wieczorek et al., 1999).

VHA in relation to ocean acidification

The partial pressures of gases in the atmosphere and in the ocean are in equilibrium. As atmospheric CO_2 levels rise as a result of anthropogenic activity, CO_2 in the ocean also increases, which is predicted to decrease the surface ocean pH at a rate faster than the most rapid events in the geological past (Caldeira and Wickett, 2003). Understandably, studies on the responses of marine organisms to OA have recently greatly increased in number (at the time of this writing, a search for 'ocean acidification' returned 285 peer-reviewed papers in PubMed and over 7200 results in Google Scholar for publications since 2015). However, a solid understanding about the basic physiological mechanisms of these responses is in most cases lacking, preventing proper interpretation of results. *A priori*, VHA could be involved in at least three processes directly related to OA: (1) systemic and intracellular A/B regulation, (2) CCM and (3) calcification. The potential roles of VHA in each of these processes are discussed in more detail below.

A/B regulation

Because VHA secretes H^+, a popular assumption is that VHA activity may be necessary to counteract acidosis in systemic fluids under conditions of OA (e.g. Deigweiher et al., 2010; Harms et al., 2014; Hu et al., 2011; Kaniewska et al., 2012; Tseng et al., 2013). However, thermodynamic and energetic considerations of intra- and extra-cellular pH and $[Na^+]$ favour H^+ secretion via Na^+/H^+ exchangers (NHEs) instead of VHA in marine environments. Briefly, the $[Na^+]$ in seawater (∼500 mmol l^{-1}) is several-fold higher than that inside cells (10–100 mmol l^{-1}) (Willmer et al., 2004) because of the action of Na^+/K^+-ATPase (NKA), which actively transports Na^+ out of cells while pumping K^+ in. Conversely, the H^+ concentration is much lower in seawater (pH∼8.0, 10 nmol l^{-1}) than in cells (pH∼7.4, 40 nmol l^{-1}) or in internal extracellular fluids such as blood (pH∼7.70, 20 nmol l^{-1}). Even during unrealistically extreme OA scenarios, the Na^+ gradient would be great enough to drive H^+ secretion via NHEs. In fact, H^+ secretion based on NKA and NHEs is favoured in any aquatic environment with $[Na^+]$ equal to or higher than 5 mmol l^{-1} (Parks et al., 2008). A few studies have reported downregulation of VHA mRNA in gills from fish exposed to OA (e.g. Esbaugh et al., 2012; Tseng et al., 2013), which, as pointed out in a recent review (Heuer and Grosell, 2014), was most likely due to downregulation of branchial HCO_3^- secretion to seawater and H^+ reabsorption into the blood.

CCM

As explained above, VHA in the symbiosome of reef-building corals' gastrodermal cells is important for promoting O_2 production by symbiotic *Symbiodinium*, probably as part of a CCM (Barott et al., 2015). As an elevated level of CO_2 diminishes the need for an energy-consuming CCM, the abundance of symbiosomal VHA would potentially be reduced during OA. Consistent with this hypothesis, reduced VHA mRNA abundance has been reported in two previous studies on corals (Kaniewska et al., 2012, 2015); however, based on the transcriptomics approach used, it was not possible to determine in which cell type(s) these changes took place. Thus, the hypothesized reduction in the level of symbiosomal VHA in response to OA still requires experimental validation.

Calcification

OA may affect biological calcification in at least three, not mutually exclusive, ways: (1) by reducing the seawater concentration of CO_3^{-} [which some people believe is the 'building block' of skeletons (e.g. Cohen and Holcomb, 2009; Kleypas and Langdon, 2006), although others (Bach, 2015; Cyronak et al., 2015; Tresguerres et al., in press) disagree], (2) by increasing dissolution of already deposited calcium carbonate skeletons (Andersson et al., 2009; Eyre et al., 2014) and (3) by limiting the rate of H^+ removal from the calcification site ('proton flux hypothesis'; Jokiel, 2011). As described above, VHA could conceivably be relevant for the proton pump hypothesis. However, detailed descriptions of the potential effect of OA on VHA-dependent calcification in presumably vulnerable organisms such as corals, pteropods and mollusks is not possible at this point, because of the lack of knowledge about the cellular and molecular mechanisms responsible for the calcification process. In fact, the OA research field often uses the term 'proton pumping' rather loosely to refer to any mechanism that may, actively or otherwise, transport H^+. Such mechanisms include VHA, HKA, NKA, P-type H^+-ATPase and Ca^{2+}-ATPase, and in many cases no specific ATPase is even mentioned (e.g. Cai et al., 2016; Cohen and Holcomb, 2009; Hohn and Merico, 2015; Nakamura et al., 2013; Ries, 2011; Waldbusser et al., 2013). Regardless of the ATPases involved, transporting H^+ against the increased seawater $[H^+]$ during OA would result in increased energy expenditure. However, the exact mechanism will determine the amount of additional energy that would be required (Ries, 2011), and the proportion of the total energy budget it would represent. Clearly, basic information about calcifying mechanisms, including the role of VHA (or lack thereof), is urgently needed in order to understand and predict effects of OA on calcifying organisms. Below, I discuss some energetic considerations about potential effects of OA on VHA-dependent physiological functions.

Energetic considerations

Depending on the coupling efficiency between ATPase activity and H^+ transport, the action of modulator proteins, the dissipation of membrane potential, the H^+ permeability and other unidentified factors (reviewed in Stevens and Forgac, 1997), VHA can transport H^+ against concentration gradients (ΔpH) of over 1000-fold (Davies et al., 1994; Kettner et al., 2003; Mindell, 2012; Müller et al., 1996). Logically, the ATP demand for H^+ pumping increases proportionally to ΔpH, which might suggest that a drop in ocean pH associated with OA would result in an increased energy requirement for VHA activity. The ATP requirement of VHA at different values of ΔpH has been experimentally calculated in isolated yeast vesicles (Kettner et al., 2003) and plant vacuoles (Davies et al., 1994), and the ratio of $0.7H^+$/ATP per ΔpH unit is very consistent in those two distantly related organisms. For example, at ΔpH=4, yeast VHA can pump ∼2 H^+ for every ATP that is hydrolyzed ($2H^+$/ATP), and at ΔpH=0 the ratio is over $4H^+$/ATP (Kettner et al., 2003). Based on these values, OA is unlikely to significantly affect the VHA-dependent energy budget of an organism. In a scenario where ΔpH=0.3 (equivalent to a drop in ocean pH from 8.0 to 7.70), the VHA would use 1 ATP to pump ∼3.8 H^+ instead of ∼4, which represents a reduction in efficiency of only 5%. However, as mentioned above, VHA is unlikely to pump H^+ to seawater for the purposes of systemic or pHi regulation; if anything, it is involved in CCM or calcification. To calculate the change in VHA energetic demand, it is necessary to first establish the cellular and subcellular localization of VHA, the concentration of relevant ions in the different compartments and how (or whether) these parameters change in response to OA.

Experimental considerations

The potential simultaneous VHA localization in multiple cell types and organelles, together with its potential involvement in various cellular processes, greatly complicates studying responses to environmental stress using common proteomics and transcriptomics techniques. For example, under OA conditions, an organism could plausibly downregulate VHA in one location for CCM, but upregulate it in a separate compartment to allow calcification [such a situation is plausible in coccolithophores (Bach et al., 2013) and coral, among others]. However, analyses of VHA mRNA or protein abundance in whole tissues/organisms would not discriminate between the various pools of VHA, and therefore could lead to erroneous interpretation of the organism's response to OA. Furthermore, as shown in the base-secreting shark gill cells, VHA (and most other proteins) can be regulated by post-translational means such as insertion or removal from the cell membrane, or phosphorylation, which are not detectable by transcriptomic and regular proteomic techniques. To understand the responses of VHA (and any other protein) to OA (and to any other source of stress), the localization of VHA in specific cells and subcellular compartments should be first determined, for example, using immunohistochemistry. After this basic information is obtained, systematic experiments should determine its physiological role. Only after the basic mechanism has been characterized can -omics techniques be confidently used.

Conclusions

Although VHA always pumps H^+ across a biological membrane, its ultimate physiological role is determined by its subcellular localization in apical, basolateral or intracellular membranes, and its colocalization and interaction with other proteins. In conjunction with regulatory signalling cascades, this allows the VHA to mediate diverse functions such as A/B regulation, bone dissolution and carbon concentrating for photosynthesis, among others. Given the great biodiversity found in the oceans, future studies are likely to unveil novel VHA functions in marine organisms. Basic information about VHA presence, localization, function and regulation is essential for us to be able to understand potential responses to environmental challenges.

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Competing interests
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References

Allemand, D., Ferrier-Pagès, C., Furla, P., Houlbrèque, F., Puverel, S., Reynaud, S., Tambutté, E., Tambutté, S. and Zoccola, D. (2004). Biomineralisation in reef-building corals: from molecular mechanisms to environmental control. *C. R. Palevol.* **3**, 453-467.

Andersson, A. J., Kuffner, I. B. and Mackenzie, F. T. (2009). Net loss of $CaCO_3$ from a subtropical calcifying community due to seawater acidification: mesocosm-scale experimental evidence. *Biogeosciences* **6**, 1811-1823.

Bach, L. T. (2015). Reconsidering the role of carbonate ion concentration in calcification by marine organisms. *Biogeosciences* **12**, 4939-4951.

Bach, L. T., Mackinder, L. C. M., Schulz, K. G., Wheeler, G., Schroeder, D. C., Brownlee, C. and Riebesell, U. (2013). Dissecting the impact of CO_2 and pH on the mechanisms of photosynthesis and calcification in the coccolithophore *Emiliania huxleyi*. *New Phytol.* **199**, 121-134.

Barott, K. L., Venn, A. A., Perez, S. O., Tambutté, S. and Tresguerres, M. (2015). Coral host cells acidify symbiotic algal microenvironment to promote photosynthesis. *Proc. Natl. Acad. Sci. USA* **112**, 607-612.

Barron, M. E., Roa, J. N. B. and Tresguerres, M. (2012). Pacific oyster mantle, gill and hemocytes express the bicarbonate-sensing enzyme soluble adenylyl cyclase. *FASEB J.* **26**, 1070.2.

Bartels, H. and Welsch, U. (1986). Mitochondria-rich cells in the gill epithelium of cyclostomes. A thin section and freeze fracture study. In *Indo-Pacific Fish Biology Proceedings of the Second International Conference on Indo-Pacific Fishes* (ed. T. Uyeno, T. Taniuchi and K. Matsuura). pp. 58-72. Tokyo: Ichthyological Society of Japan.

Baumann, O. and Walz, B. (2012). The blowfly salivary gland – a model system for analyzing the regulation of plasma membrane V-ATPase. *J. Insect Physiol.* **58**, 450-458.

Bayaa, M., Vulesevic, B., Esbaugh, A., Braun, M., Ekker, M. E., Grosell, M. and Perry, S. F. (2009). The involvement of SLC26 anion transporters in chloride uptake in zebrafish (*Danio rerio*) larvae. *J. Exp. Biol.* **212**, 3283-3295.

Benlekbir, S., Bueler, S. A. and Rubinstein, J. L. (2012). Structure of the vacuolar-type ATPase from *Saccharomyces cerevisiae* at 11-Å resolution. *Nat. Struct. Mol. Biol.* **19**, 1356-1362.

Bertucci, A., Tambutté, E., Tambutté, S., Allemand, D. and Zoccola, D. (2010). Symbiosis-dependent gene expression in coral–dinoflagellate association: cloning and characterization of a P-type H^+-ATPase gene. *Proc. R. Soc. B Biol. Sci.* **277**, 87-95.

Beyenbach, K. W. and Wieczorek, H. (2006). The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* **209**, 577-589.

Blair, H. C., Teitelbaum, S. L., Ghiselli, R. and Gluck, S. (1989). Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **245**, 855-857.

Boisen, A. M. Z., Amstrup, J., Novak, I. and Grosell, M. (2003). Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochim. Biophys. Acta* **1618**, 207-218.

Boron, W. F. and Boulpaep, E. L. (2012). *Medical Physiology*. Philadelphia, PA: Saunders.

Breton, S. and Brown, D. (1998). Cold-induced microtubule disruption and relocalization of membrane proteins in kidney epithelial cells. *J. Am. Soc. Nephrol.* **9**, 155-166.

Brown, D., Păunescu, T. G., Breton, S. and Marshansky, V. (2009). Regulation of the V-ATPase in kidney epithelial cells: dual role in acid-base homeostasis and vesicle trafficking. *J. Exp. Biol.* **212**, 1762-1772.

Cai, W.-J., Ma, Y., Hopkinson, B. M., Grottoli, A. G., Warner, M. E., Ding, Q., Hu, X., Yuan, X., Schoepf, V., Xu, H. et al. (2016). Microelectrode characterization of coral daytime interior pH and carbonate chemistry. *Nat. Commun.* **7**, 11144.

Caldeira, K. and Wickett, M. E. (2003). Oceanography: anthropogenic carbon and ocean pH. *Nature* **425**, 365-365.

Catches, J. S., Burns, J. M., Edwards, S. L. and Claiborne, J. B. (2006). Na^+/H^+ antiporter, V-H^+-ATPase and Na^+/K^+-ATPase immunolocalization in a marine teleost (*Myoxocephalus octodecemspinosus*). *J. Exp. Biol.* **209**, 3440-3447.

Clelland, E. S. and Saleuddin, A. S. M. (2000). Vacuolar-type ATPase in the accessory boring organ of *Nucella lamellosa* (Gmelin) (Mollusca: Gastropoda): role in shell penetration. *Biol. Bull.* **198**, 272-283.

Cohen, A. and Holcomb, M. (2009). Why corals care about ocean acidification: uncovering the mechanism. *Oceanography* **22**, 118-127.

Corstjens, P. L. A. M., Araki, Y. and González, E. L. (2001). A coccolithophorid calcifying vesicle with a vacuolar-type ATPase proton pump: cloning and immunolocalization of the V_0 subunit *c*. *J. Phycol.* **37**, 71-78.

Cotter, K., Stransky, L., McGuire, C. and Forgac, M. (2015). Recent insights into the structure, regulation, and function of the V-ATPases. *Trends Biochem. Sci.* **40**, 611-622.

Cyronak, T., Schulz, K. G. and Jokiel, P. L. (2015). The Omega myth: what really drives lower calcification rates in an acidifying ocean. *ICES J. Mar. Sci.* **73**, fsv075-fsv562.

Davies, J. M., Hunt, I. and Sanders, D. (1994). Vacuolar H^+-pumping ATPase variable transport coupling ratio controlled by pH. *Proc. Natl. Acad. Sci. USA* **91**, 8547-8551.

Deigweiher, K., Hirse, T., Bock, C., Lucassen, M. and Pörtner, H. O. (2010). Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. *J. Comp. Physiol. B* **180**, 347-359.

Drory, O. and Nelson, N. (2006). The emerging structure of vacuolar ATPases. *Physiology* **21**, 317-325.

Esbaugh, A. J., Heuer, R. and Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B* **182**, 921-934.

Evans, D. H. (1984). Gill Na^+/H^+ and Cl^-/HCO_3^- exchange systems evolved before the vertebrates entered fresh water. *J. Exp. Biol.* **113**, 465-469.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Eyre, B. D., Andersson, A. J. and Cyronak, T. (2014). Benthic coral reef calcium carbonate dissolution in an acidifying ocean. *Nat. Clim. Change* **4**, 969-976.

Falkowski, P. G., Dubinsky, Z., Muscatine, L. and Porter, J. W. (1984). Light and the bioenergetics of a symbiotic coral. *Bioscience* **34**, 705-709.

Gilmour, K. M., Bayaa, M., Kenney, L., McNeill, B. and Perry, S. F. (2007). Type IV carbonic anhydrase is present in the gills of spiny dogfish (*Squalus acanthias*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R556-R567.

Goffredi, S. K., Orphan, V. J., Rouse, G. W., Jahnke, L., Embaye, T., Turk, K., Lee, R. and Vrijenhoek, R. C. (2005). Evolutionary innovation: a bone-eating marine symbiosis. *Environ. Microbiol.* **7**, 1369-1378.

Gong, F., Alzamora, R., Smolak, C., Li, H., Naveed, S., Neumann, D., Hallows, K. R. and Pastor-Soler, N. M. (2010). Vacuolar H^+-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase. *Am. J. Physiol. Renal Physiol.* **298**, F1162-F1169.

Goss, G. G., Laurent, P. and Perry, S. F. (1992). Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). *Cell Tissue Res.* **268**, 539-552.

Goss, G. G., Wood, C. M., Laurent, P. and Perry, S. F. (1994). Morphological responses of the rainbow trout (*Oncorhynchus mykiss*) gill to hyperoxia, base ($NaHCO_3$) and acid (HCl) infusions. *Fish Physiol. Biochem.* **12**, 465-477.

Gowen, M., Lazner, F., Dodds, R., Kapadia, R., Feild, J., Tavaria, M., Bertoncello, I., Drake, F., Zavarselk, S., Tellis, I. et al. (1999). Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. *J. Bone Miner. Res.* **14**, 1654-1663.

Grosell, M. and Genz, J. (2006). Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291**, R1145-R1156.

Guffey, S., Esbaugh, A. and Grosell, M. (2011). Regulation of apical H^+-ATPase activity and intestinal HCO_3^- secretion in marine fish osmoregulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R1682-R1691.

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F., Storch, D., Held, C., Pörtner, H.-O. and Lucassen, M. (2014). Gene expression profiling in gills of the great spider crab Hyas araneus in response to ocean acidification and warming. *BMC Genomics* **15**, 789.

Heisler, N. (1988). Acid-base regulation. In *Physiology of Elasmobranch Fishes* (ed. T. J. Shuttleworth), pp. 215-252. Berlin: Springer-Verlag.

Heuer, R. M. and Grosell, M. (2014). Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R1061-R1084.

Hohn, S. and Merico, A. (2015). Quantifying the relative importance of transcellular and paracellular ion transports to coral polyp calcification. *Front. Earth Sci.* **2**, 37.

Hu, M. Y., Tseng, Y.-C., Stumpp, M., Gutowska, M. A., Kiko, R., Lucassen, M. and Melzner, F. (2011). Elevated seawater PCO_2 differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R1100-R1114.

Hu, M. Y., Guh, Y.-J., Stumpp, M., Lee, J.-R., Chen, R.-D., Sung, P.-H., Chen, Y.-C., Hwang, P.-P. and Tseng, Y.-C. (2014). Branchial NH_4^+-dependent acid–base transport mechanisms and energy metabolism of squid (*Sepioteuthis lessoniana*) affected by seawater acidification. *Front. Zool.* **11**, 55.

Huusgaard, R. S., Vismann, B., Kühl, M., Macnaugton, M., Colmander, V., Rouse, G. W., Glover, A. G., Dahlgren, T. and Worsaae, K. (2012). The potent

respiratory system of *Osedax mucofloris* (Siboglinidae, Annelida) – a prerequisite for the origin of bone-eating *Osedax*? *PLoS ONE* **7**, e35975.
Jefferies, K. C., Cipriano, D. J. and Forgac, M. (2008). Function, structure and regulation of the vacuolar H^+-ATPases. *Arch. Biochem. Biophys.* **476**, 33-42.
Jokiel, P. L. (2011). Ocean acidification and control of reef coral calcification by boundary layer limitation of proton flux. *Bull. Mar. Sci.* **87**, 639-657.
Kaniewska, P., Campbell, P. R., Kline, D. I., Rodriguez-Lanetty, M., Miller, D. J., Dove, S. and Hoegh-Guldberg, O. (2012). Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE* **7**, e34659.
Kaniewska, P., Chan, C.-K. K., Kline, D., Ling, E. Y. S., Rosic, N., Edwards, D., Hoegh-Guldberg, O. and Dove, S. (2015). Transcriptomic changes in coral holobionts provide insights into physiological challenges of future climate and ocean change. *PLoS ONE* **10**, e0139223.
Katoh, F., Hyodo, S. and Kaneko, T. (2003). Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. *J. Exp. Biol.* **206**, 793-803.
Katz, S., Klepal, W. and Bright, M. (2011). The Osedax trophosome: organization and ultrastructure. *Biol. Bull.* **220**, 128-139.
Kettner, C., Bertl, A., Obermeyer, G., Slayman, C. and Bihler, H. (2003). Electrophysiological analysis of the yeast V-type proton pump: variable coupling ratio and proton shunt. *Biophys. J.* **85**, 3730-3738.
Kleypas, J. and Langdon, C. (2006). Coral reefs and changing seawater carbonate chemistry. In *Coral Reefs and Climate Change: Science and Management* (ed. J. T. Phinney, O. Hoegh-Guldberg, J. Kleypas, W. Skirving and A. Strong), pp. 73-110. Washington, DC: American Geophysical Union.
Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H. and Weihrauch, D. (2014). Osmoregulation and excretion. *Compr. Physiol.* **4**, 405-573.
Li, Y.-P., Chen, W., Liang, Y., Li, E. and Stashenko, P. (1999). Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. *Nat. Genet.* **23**, 447-451.
Li, S., Huang, J., Liu, C., Liu, Y., Zheng, G., Xie, L. and Zhang, R. (2016). Interactive effects of seawater acidification and elevated temperature on the Transcriptome and Biomineralization in the pearl oyster *Pinctada fucata*. *Environ. Sci. Technol.* **50**, 1157-1165.
Lin, C.-C., Lin, L.-Y., Hsu, H.-H., Thermes, V., Prunet, P., Horng, J.-L. and Hwang, P.-P. (2012). Acid secretion by mitochondrion-rich cells of medaka (*Oryzias latipes*) acclimated to acidic freshwater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, R283-R291.
Mackinder, L., Wheeler, G., Schroeder, D., Riebesell, U. and Brownlee, C. (2010). Molecular mechanisms underlying calcification in coccolithophores. *Geomicrobiol. J.* **27**, 585-595.
Mann, K. and Jackson, D. J. (2014). Characterization of the pigmented shell-forming proteome of the common grove snail *Cepaea nemoralis*. *BMC Genomics* **15**, 249.
Marshansky, V., Rubinstein, J. L. and Grüber, G. (2014). Eukaryotic V-ATPase: novel structural findings and functional insights. *Biochim. Biophys. Acta* **1837**, 857-879.
Mindell, J. A. (2012). Lysosomal acidification mechanisms. *Annu. Rev. Physiol.* **74**, 69-86.
Müller, M., Irkens-Kiesecker, U., Rubinstein, B. and Taiz, L. (1996). On the mechanism of hyperacidification in lemon. Comparison of the vacuolar H^+-ATPase activities of fruits and epicotyls. *J. Biol. Chem.* **271**, 1916-1924.
Muscatine, L., Falkowski, P. G., Porter, J. W. and Dubinsky, Z. (1984). Fate of photosynthetic fixed carbon in light- and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. *Proc. R. Soc. B Biol. Sci.* **222**, 181-202.
Nakamura, T., Nadaoka, K. and Watanabe, A. (2013). A coral polyp model of photosynthesis, respiration and calcification incorporating a transcellular ion transport mechanism. *Coral Reefs* **32**, 779-794.
Nelson, N. and Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol. Rev.* **79**, 361-385.
Niv, Y. and Fraser, G. M. (2002). The alkaline tide phenomenon. *J. Clin. Gastroenterol.* **35**, 5-8.
Onken, H. and Moffett, D. F. (2009). Revisiting the cellular mechanisms of strong luminal alkalinization in the anterior midgut of larval mosquitoes. *J. Exp. Biol.* **212**, 373-377.
Parks, S. K., Tresguerres, M. and Goss, G. G. (2008). Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **148**, 411-418.
Păunescu, T. G., Da Silva, N., Russo, L. M., McKee, M., Lu, H. A. J., Breton, S. and Brown, D. (2008). Association of soluble adenylyl cyclase with the V-ATPase in renal epithelial cells. *Am. J. Physiol. Renal Physiol.* **294**, F130-F138.
Perry, S. F. and Gilmour, K. M. (2006). Acid–base balance and CO_2 excretion in fish: unanswered questions and emerging models. *Respir. Physiol. Neurobiol.* **154**, 199-215.
Piermarini, P. M. and Evans, D. H. (2001). Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (Dasyatis sabina): effects of salinity and relation to Na^+/K^+-ATPase. *J. Exp. Biol.* **204**, 3251-3259.
Piermarini, P. M., Verlander, J. W., Royaux, I. E. and Evans, D. H. (2002). Pendrin immunoreactivity in the gill epithelium of a euryhaline elasmobranch. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R983-R992.
Pomponi, S. A. (1980). Cytological mechanisms of calcium carbonate excavation by boring sponges. *Int. Rev. Cytol.* **65**, 301-319.
Reilly, B. D., Cramp, R. L., Wilson, J. M., Campbell, H. A. and Franklin, C. E. (2011). Branchial osmoregulation in the euryhaline bull shark, *Carcharhinus leucas*: a molecular analysis of ion transporters. *J. Exp. Biol.* **214**, 2883-2895.
Reinfelder, J. R. (2011). Carbon concentrating mechanisms in eukaryotic marine phytoplankton. *Annu. Rev. Mar. Sci.* **3**, 291-315.
Ries, J. B. (2011). A physicochemical framework for interpreting the biological calcification response to CO_2-induced ocean acidification. *Geochim. Cosmochim. Acta* **75**, 4053-4064.
Roa, J. N. and Tresguerres, M. (2016). Soluble adenylyl cyclase is an acid/base sensor in epithelial base-secreting cells. *Am. J. Physiol. Cell Physiol.* doi:10.1152/ajpcell.00089.2016.
Roa, J. N., Munévar, C. L. and Tresguerres, M. (2014). Feeding induces translocation of vacuolar proton ATPase and pendrin to the membrane of leopard shark (*Triakis semifasciata*) mitochondrion-rich gill cells. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **174**, 29-37.
Rouse, G. W., Goffredi, S. K. and Vrijenhoek, R. C. (2004). *Osedax*: bone-eating marine worms with dwarf males. *Science* **305**, 668-671.
Rowan, R., Whitney, S. M., Fowler, A. and Yellowlees, D. (1996). Rubisco in marine symbiotic dinoflagellates: form II enzymes in eukaryotic oxygenic phototrophs encoded by a nuclear multigene family. *Plant Cell* **8**, 539-553.
Saroussi, S. and Nelson, N. (2009). Vacuolar H^+-ATPase—an enzyme for all seasons. *Pflügers Arch.* **457**, 581-587.
Stevens, T. H. and Forgac, M. (1997). Structure, function and regulation of the vacuolar H^+-ATPase. *Annu. Rev. Cell Dev. Biol.* **13**, 779-808.
Tresguerres, M. (2014). sAC from aquatic organisms as a model to study the evolution of acid/base sensing. *Biochim. Biophys. Acta* **1842**, 2629-2635.
Tresguerres, M., Katoh, F., Fenton, H., Jasinska, E. and Goss, G. G. (2005). Regulation of branchial V-H^+-ATPase, Na^+/K^+-ATPase and NHE2 in response to acid and base infusions in the Pacific spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* **208**, 345-354.
Tresguerres, M., Katoh, F., Orr, E., Parks, S. K. and Goss, G. G. (2006a). Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? *Physiol. Biochem. Zool.* **79**, 981-996.
Tresguerres, M., Parks, S. K. and Goss, G. G. (2006b). V-H^+-ATPase, Na^+/K^+-ATPase and NHE2 immunoreactivity in the gill epithelium of the Pacific hagfish (*Epatretus stoutii*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **145**, 312-321.
Tresguerres, M., Parks, S. K., Katoh, F. and Goss, G. G. (2006c). Microtubule-dependent relocation of branchial V-H^+-ATPase to the basolateral membrane in the Pacific spiny dogfish (*Squalus acanthias*): a role in base secretion. *J. Exp. Biol.* **209**, 599-609.
Tresguerres, M., Parks, S. K. and Goss, G. G. (2007a). Recovery from blood alkalosis in the Pacific hagfish (*Eptatretus stoutii*): Involvement of gill V-H^+-ATPase and Na^+/K^+-ATPase. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 133-141.
Tresguerres, M., Parks, S. K., Wood, C. M. and Goss, G. G. (2007b). V-H^+-ATPase translocation during blood alkalosis in dogfish gills: interaction with carbonic anhydrase and involvement in the postfeeding alkaline tide. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R2012-R2019.
Tresguerres, M., Parks, S. K., Sabatini, S. E., Goss, G. G. and Luquet, C. M. (2008). Regulation of ion transport by pH and $[HCO_3^-]$ in isolated gills of the crab *Neohelice* (*Chasmagnathus*) *granulata*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1033-R1043.
Tresguerres, M., Parks, S. K., Salazar, E., Levin, L. R., Goss, G. G. and Buck, J. (2010). Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis. *Proc. Natl. Acad. Sci. USA* **107**, 442-447.
Tresguerres, M., Levin, L. R. and Buck, J. (2011). Intracellular cAMP signaling by soluble adenylyl cyclase. *Kidney Int.* **79**, 1277-1288.
Tresguerres, M., Katz, S. and Rouse, G. W. (2013). How to get into bones: proton pump and carbonic anhydrase in *Osedax* boneworms. *Proc. R. Soc. B Biol. Sci.* **280**, 20130625.
Tresguerres, M., Barott, K. L., Barron, M. E. and Roa, J. N. (2014). Established and potential physiological roles of bicarbonate-sensing soluble adenylyl cyclase (sAC) in aquatic animals. *J. Exp. Biol.* **217**, 663-672.
Tresguerres, M., Barott, K. L., Barron, M. E., Deheyn, D. D., Kline, D. I. and Linsmayer, L. B., **Cell biology of reef-building corals** (in press). Ion transport, acid/base regulation, and energy metabolism. In *Acid-Base Balance and Nitrogen Excretion in Invertebrates* (ed. D. Weihrauch and M. O'Donnell). Berlin: Springer.
Tseng, Y.-C., Hu, M. Y., Stumpp, M., Lin, L.-Y., Melzner, F. and Hwang, P.-P. (2013). CO_2-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (*Oryzias latipes*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **165**, 119-130.
Väänänen, H. K., Zhao, H., Mulari, M. and Halleen, J. M. (2000). The cell biology of osteoclast function. *J. Cell Sci.* **113**, 377-381.

Journal of Experimental Biology

Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D. and Geibel, J. P. (2004). Renal vacuolar H^+-ATPase. *Physiol. Rev.* **84**, 1263-1314.
Waldbusser, G. G., Brunner, E. L., Haley, B. A., Hales, B., Langdon, C. J. and Prahl, F. G. (2013). A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. *Geophys. Res. Lett.* **40**, 2171-2176.
Wang, T., Busk, M. and Overgaard, J. (2001). The respiratory consequences of feeding in amphibians and reptiles. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **128**, 535-549.
Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J. and Harvey, W. R. (1999). Animal plasma membrane energization by proton-motive V-ATPases. *Bioessays* **21**, 637-648.
Wieczorek, H., Beyenbach, K. W., Huss, M. and Vitavska, O. (2009). Vacuolar-type proton pumps in insect epithelia. *J. Exp. Biol.* **212**, 1611-1619.
Willmer, P., Stone, G. and Johnston, I. (2004). *Environmental Physiology of Animals*. New York: Wiley-Blackwell.
Wilson, R. W., Wilson, J. M. and Grosell, M. (2002). Intestinal bicarbonate secretion by marine teleost fish—why and how? *Biochim. Biophys. Acta* **1566**, 182-193.
Wood, C. M., Kajimura, M., Mommsen, T. P. and Walsh, P. J. (2005). Alkaline tide and nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*). *J. Exp. Biol.* **208**, 2693-2705.
Wood, C. M., Bucking, C., Fitzpatrick, J. and Nadella, S. (2007). The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias*. *Respir. Physiol. Neurobiol.* **159**, 163-170.
Wood, C. M., Schultz, A. G., Munger, R. S. and Walsh, P. J. (2009). Using omeprazole to link the components of the post-prandial alkaline tide in the spiny dogfish, *Squalus acanthias*. *J. Exp. Biol.* **212**, 684-692.
Zhao, J., Benlekbir, S. and Rubinstein, J. L. (2015). Electron cryomicroscopy observation of rotational states in a eukaryotic V-ATPase. *Nature* **521**, 241-245.
Ziegler, A. (2008). The cationic composition and pH in the moulting fluid of *Porcellio scaber* (Crustacea, Isopoda) during calcium carbonate deposit formation and resorption. *J. Comp. Physiol. B* **178**, 67-76.
Ziegler, A., Weihrauch, D., Hagedorn, M., Towle, D. W. and Bleher, R. (2004). Expression and polarity reversal of V-type H^+-ATPase during the mineralization–demineralization cycle in *Porcellio scaber* sternal epithelial cells. *J. Exp. Biol.* **207**, 1749-1756.