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Journal

Journal of Experimental Zoology Part A Ecological and Integrative Physiology, 333(6)

ISSN

2471-5638

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Publication Date

2020-07-01

DOI

10.1002/jez.2367

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Evolutionary links between intra- and extracellular acid-base regulation in fish and other aquatic animals

Running title: Acid-base regulatory mechanisms

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Abstract

The acid-base relevant molecules carbon dioxide (CO_2), protons (H^+), and bicarbonate (HCO_3^-) are substrates and end products of some of the most essential physiological functions including aerobic and anaerobic respiration, ATP hydrolysis, photosynthesis, and calcification. The structure and function of many enzymes and other macromolecules are highly sensitive to changes in pH, and thus maintaining acid-base homeostasis in the face of metabolic and environmental disturbances is essential for proper cellular function. On the other hand, CO_2, H^+ and HCO_3^- have regulatory effects on various proteins and processes, both directly through allosteric modulation and indirectly through signal transduction pathways. Life in aquatic environments

presents organisms with distinct acid-base challenges that are not found in terrestrial environments. These include a relatively high CO_2 relative to O_2 solubility that prevents internal CO_2/HCO_3^- accumulation to buffer pH, a lower O_2 content that may favor anaerobic metabolism, and variable environmental CO_2, pH and O_2 levels that require dynamic adjustments in acid-base homeostatic mechanisms. Additionally, some aquatic animals purposely create acidic or alkaline microenvironments that drive specialized physiological functions. For example, acidifying mechanisms can enhance O_2 delivery by red blood cells, lead to ammonia trapping for excretion or buoyancy purposes, or lead to CO_2 accumulation to promote photosynthesis by endosymbiotic algae. On the other hand, alkalinizing mechanisms can serve to promote calcium carbonate skeletal formation. This non-exhaustive review summarizes some of the distinct acid-base homeostatic mechanisms that have evolved in aquatic organisms to meet the particular challenges of this environment.

Keywords: ammonia, coral reef, hemoglobin, hypoxia, ocean acidification, oxygen transport, preferential pHi regulation, proton pump, soluble adenylyl cyclase, symbiosome

Introduction

Proper cellular function depends on a series of tightly regulated, enzymatically catalyzed biochemical reactions, and therefore all organisms have evolved mechanisms to maintain appropriate conditions in their extra- and intracellular compartments. In biological systems, hydrogen ions (H^+) are produced and consumed through various chemical reactions. Due to its extremely large charge to size ratio, H^+ immediately react with the electron cloud of adjacent molecules including the carboxy and amino functional groups of proteins thereby altering their ionization status, conformation, and function. Thus, the ability to regulate intracellular pH (pHi) is essential for life.

The origin of pHi regulatory mechanisms is most likely rooted in the first proto-cells. It has been theorized that an important step in the origin of the first cell was the trapping of ribozymes and substrates within a membrane vesicle bilayer (Koch & Silver, 2005). An ability to maintain protoplasm pH within a range compatible with ribozyme activity would have enabled faster reaction rates and some degree of independence from the surrounding environment, providing a strong selective advantage. Regardless of its evolutionary origin, pHi regulation is essential for all extant organisms because intracellular acid-base homeostasis is continuously challenged by net H^+ production from ATP-consuming reactions. During aerobic conditions, the synthesis of ATP through mitochondrial oxidative phosphorylation acts as a major sink for H^+ that closely matches the H^+ production from ATP hydrolysis (Hochachka & Mommsen, 1983). However, O_2 limitation typically leads to increased H^+ production through anaerobic pathways, which, together with a lag in the mitochondrial H^+ sink, can acidify pHi. Moreover, intracellular acidification can occur even during aerobic conditions if the outwardly directed CO_2

partial pressure (PCO_2) gradient decreases due to an increase in PCO_2 in the extracellular fluids or the environment. In this case, some of the CO_2 that is produced as an end product of cellular respiration will accumulate inside the cell and combine with water to produce H^+ according to the reactions shown in Figure 1A.

These pH-dependent reactions have important implications for acid-base physiology: (1) in the presence of carbonic anhydrase (CA), an enzyme that catalyzes the left most reaction, equilibria are reached virtually instantaneously; (2) an increase in CO_2 leads to increased $[H^+]$ and thus has an acidifying effect; (3) most biological fluids and aquatic environments have a pH between 6-8, resulting in HCO_3^- as the dominant carbon species (Fig. 1B); and (4) active acidification and alkalinization of a compartment can be used to modulate the ratio between the different carbon species, such as to accumulate CO_2, HCO_3^-, or CO_3^-. Also important for acid-base regulation, H^+, HCO_3^-, and CO_3^{2-} (as well as NH_4^+) are charged molecules and therefore require carrier proteins to cross lipid membranes. On the other hand, CO_2 (as well as O_2 and NH_3) as a gas can rapidly diffuse through membranes and the process can be further facilitated by aquaporin and Rhesus (Rh) channel proteins (Musa-Aziz, Chen, Pelletier, & Boron, 2009).

To avoid the adverse effects of intracellular acidification, cells must be able to buffer excess H^+ or actively extrude them at a rate equal to that of production. While all organisms routinely experience acid-base stress, life in aquatic environments poses particular acid-base challenges that are not found in terrestrial environments. In water, acid-base equivalents may be dissolved as ions, such as H^+ and HCO_3^-, whereas in gaseous air, the only acid-base equivalent is CO_2. In addition, the solubility of CO_2 in

88 water is about 30 times higher than that of O_2, and the diffusion ratio between the two
89 gases is higher in water compared to air. Also, the O_2 content in water is much lower
90 than in air. Thus, the ventilatory volume of water required to meet the O_2 demands of
91 water breathers is far in excess of that required to ensure efficient excretion of
92 respiratory CO_2 from blood to the water. Therefore, water breathers cannot elevate
93 blood PCO_2 and [HCO_3^-] to values as high as air-breathers, and thus they have a
94 correspondingly lower internal fluid HCO_3^--buffering capacity (Dejours, 1994). In
95 addition, ventilatory regulation of CO_2 excretion is not an effective strategy for acid-base
96 regulation in water breathers because decreases in ventilation rate to elevate blood
97 PCO_2 would limit O_2 uptake, and blood PCO_2 is already so low that hyperventilation is
98 largely ineffective in reducing blood PCO_2 (Gilmour, 2001). As a result, systemic acid-
99 base regulation in aquatic animals is largely dependent upon active transport of acid-
100 base equivalents in exchange for counter ions between the animal and the environment.
101 The first part of this article will discuss the most common acid-base disturbances in
102 aquatic environments and some intra- and extra-cellular acid-base regulatory strategies
103 that are uniquely used by aquatic animals.

104 In addition to challenging acid-base homeostasis, variations in CO_2, pH and
105 HCO_3^- can be important modulators of physiological processes. The second part of this
106 article will discuss three such examples: (1) the frequent pHi changes experienced by
107 red blood cells (RBCs) as they circulate between the respiratory surfaces and the
108 tissues, and how these pHi changes plays an essential role in the delivery of O_2
109 throughout the body; (2) the effect of pH on ammonia metabolism, and how active
110 acidification of intra- and extracellular compartments can be used to promote ammonia

excretion or accumulation, and (3) the extreme pH microenvironments that are generated by coral cells, and their roles in mediating metabolic communication with their photosynthetic symbionts and in promoting skeletal calcification. Some of the potential evolutionary links between pHi regulation and other physiological processes are emphasized throughout this article.

Common acid-base disturbances in aquatic environments

In many water bodies, photosynthetic activity during the day may outpace respiration and result in elevated environmental O_2 (hyperoxia), reduced PCO_2 (hypocapnia), and elevated pH. However at night, respiration in the absence of photosynthesis can result in hypoxia, elevated PCO_2 (hypercapnia), and decreased pH. Hypercapnia is particularly evident in environments with high densities of organisms and slow water flow such as lakes, swamps, kelp forests, reefs, mangroves, and tide pools (Duarte, Ferreira, Wood, & Val, 2013; Hofmann et al., 2011; Kline et al., 2012; Truchot & Duhamel-Jouve, 1980). Another example of environmental hypercapnia is the gradual elevation of PCO_2 in water bodies due to increased absorption of anthropogenic CO_2 emissions ("ocean acidification" and "freshwater acidification"; Caldeira & Wickett, 2003; Van deWaal, Verschoor, Verspagen, van Donk, E. & Huisman, 2009).

In the majority of naturally occurring cases of environmental hypercapnia, PCO_2 remains lower than that of the internal fluids of the organism. However, the reduced PCO_2 gradient between the internal fluids and the environment limits excretion of endogenously produced CO_2, leading to an elevation in PCO_2 and a higher $[H^+]$ (and $[HCO_3^-]$) by law of mass action (Fig. 1A, Melzner et al., 2009). But in environments with

a very high density of respiring biomass at night (Furch & Junk, 1997), in the proximity of geological CO_2 seeps (Hall-Spencer et al., 2008), and in high-density aquaculture systems (Ellis, Urbina, & Wilson, 2017), environmental PCO_2 can be elevated above the internal PCO_2 of an organism. In these cases, CO_2 will diffuse into the animal down its partial pressure gradient, and induce a much more pronounced acidosis.

A metabolic acidosis in fish is routinely observed after exhaustive exercise (e.g. Milligan & Wood, 1986) and during exposure to environmental hypoxia (e.g. Thomas & Hughes, 1982) due to increased reliance on anaerobic metabolism and the higher H^+ production associated with ATP depletion relative to ATP production. Sessile aquatic invertebrates living in the intertidal zone may likewise experience hypoxia or anoxia during aerial emersion at low tide, as their gas exchange surfaces are ineffective in air, or the animal needs to minimize gas exchange to avoid desiccation, or both (Bayne, Bayne, Carefoot, & Thompson, 1976). A metabolic alkalosis typically develops in the blood of fish upon the consumption of a large meal ("alkaline tide"), which is due to the secretion of H^+ into the stomach and the absorption of HCO_3^- into the blood (e.g. Wood, Kajimura, Mommsen, & Walsh, 2005). Conversely, a blood metabolic acidosis develops after feeding in agastric fish, which is due to the secretion of HCO_3^- into the gastrointestinal tract and the absorption of H^+ into the blood ("acidic tide") (Wood, Bucking, & Grosell, 2010).

Basic concepts of pH regulation

The logarithmic pH scale, where $pH = -\log [H^+]$ (Sørensen, 1909) easily masks relative changes in the actual $[H^+]$ within a fluid, which is the ion that directly interacts

157 with mlecules. As such, at any point of the pH scale, a 1 unit pH change represents a
158 10-fold change in $[H^+]$, a 0.3 pH unit change is a ~2-fold change in $[H^+]$, and a 0.1 pH
159 unit change is a ~25% change in $[H^+]$. Consequently, the range of the pH scale over
160 which changes are observed matters greatly when assessing the magnitude of an acid-
161 base disturbance. For example, a decrease from pH 7.4 to 7.3 reflects a 10 nM increase
162 in $[H^+]$, but a decrease from pH 8.0 to 7.9 reflects only a 2.5 nM increase. Thus, the H^+
163 load associated with a pH change from 7.4 to 7.3 is four-fold larger than the H^+ load that
164 changes pH from 8.0 to 7.9, and will require proportionally more resources in terms of
165 buffering capacity or energy to remove the excess H^+ from a given compartment and
166 prevent adverse effects on cellular function.
167 Intracellular buffering is a first line of defense against fluctuations in pHi. The total
168 intracellular buffering capacity is determined by the sum of the HCO_3^- and non-HCO_3^-
169 buffering systems, which can bind and release H^+ to lessen an acidosis or an alkalosis,
170 respectively. The main component of the non-HCO_3^- system is imidazole of the histidine
171 groups in side chains of amino acids. Their pK_a is in the range of ~6.0-7.0, which is
172 close to the pHi set point of most cells and therefore histidine groups are particularly
173 effective at buffering excess H^+ during physiologically relevant decreases in pHi. For
174 instance, muscle contraction generates a large H^+ load that can induce muscle fatigue
175 and contractile failure (Jarvis, Woodward, Debold, & Walcott, 2018) and accordingly,
176 fish muscle cells contain large amounts of the histidine-rich dipeptides carnosine,
177 anserine, and balenine resulting in a non-HCO_3^- buffering capacity that is ~2-3 times
178 greater than that of other tissues (Walsh & Milligan, 1989). Also, different types of
179 muscle fibers create different acidic conditions that determine the cellular strategy of

180 homeostasis. Fast-twitch white muscle relies largely on anaerobic metabolism that
181 produces H^+ (Kieffer, 2000), whereas slow-twitch, red muscle relies on aerobic
182 metabolism and predominantly produces CO_2. Thus, to compensate for a more rapid
183 and pronounced metabolic acidosis, white muscle contains more histidine-rich
184 dipeptides that elevate the intracellular buffering capacity over that of red muscle (Dolan
185 et al., 2019).

186 As a rapid, reversible, and passive strategy buffers are critical to maintaining pHi
187 homeostasis; however, their capacity is finite and when overwhelmed, pHi regulation
188 hinges on active mechanisms (Fig. 2). In most animal cells, Na^+/K^+-ATPase (NKA)
189 activity drives H^+ excretion by secondarily active transporters such as the ubiquitous
190 Na^+/H^+ exchanger isoform 1 (NHE1). NKA activity can also drive HCO_3^- uptake *via*
191 Na^+/HCO_3^- cotransporters (NBCs) and Na^+-dependent Cl^-/HCO_3^- exchangers
192 (NDCBEs); an increase in intracellular [HCO_3^-] reacts with and decreases [H^+], thus is
193 equivalent to active H^+ extrusion (reviewed in Casey, Grinstein, & Orlowski, 2010). In
194 cancer cells growing in acidic microenvironments, the V-type H^+-ATPase (VHA) is
195 another active H^+ extruding mechanism that helps counteract intracellular acidification
196 (reviewed in Torigoe et al, 2002). Notably, H^+ excretion by VHA does not depend on
197 NKA activity; however, VHA-dependent H^+ excretion must occur in concert with the net
198 transport of a counter ion (typically Cl^- excretion or Na^+ absorption) (Tresguerres, 2016).

199 Cells that produce lactate as the end product of fermentation typically excrete H^+
200 together with lactate through monocarboxylate-H^+ cotransporters (MCTs). However,
201 several important differences exist between aquatic animals and mammals. For
202 example, white muscle in fish expresses MCTs at a relatively low abundance and

retains a significant proportion of the lactate that is produced during exhaustive exercise, which allows for the localized replenishment of glycogen stores *in situ* from lactate (reviewed in Weber, Choi, Gonzalez, & Omlin, 2016). Likewise, aquatic invertebrates that produce imino acids ("opines") in the final step of fermentation may retain these end products intracellularly for later oxidization when aerobic conditions return, or reconvert them into the original pyruvate and amino acid substrates (Ellington, 1983). In some cases, the accumulation of fermentative metabolites may be associated with a pronounced intracellular acidification that can inhibit glycogenic and gluconeogenic metabolism through pH effects on enzyme activity and substrate or end-product inhibition (Walsh & Milligan, 1989).

Cases of intracellular alkalinization are less common than those of acidification. Nonetheless, when an alkaline load is experienced, some cells use anion exchangers (AEs) that excrete HCO_3^- in exchange for Cl^-, thereby acting as "acid-loading" transporters that help counteract an alkaline load. And some cells experiencing elevated intracellular Ca^{2+} levels use Plasma Membrane Ca^{2+}-ATPases (PMCAs) to extrude Ca^{2+} in exchange for extracellular H^+ thereby acidifying the cytosol (reviewed in Casey et al., 2010).

Active pHi regulation requires the ability to sense disturbances from a set point and to trigger compensatory responses. One such mechanism relies on pH-dependent amino acid conformational changes that render acid-secreting proteins such as NHE1 inactive when pH increases, and acid-loading proteins such as AE3 inactive when pH decreases (reviewed in Casey et al., 2010). Other molecular acid-base sensors are coupled to signal transduction pathways (Tresguerres, Buck, & Levin, 2010), of which

226 the soluble adenylyl cyclase (sAC) is arguably the best characterized in aquatic animals
227 (Tresguerres, Barott, Barron, & Roa, 2014). This evolutionarily conserved enzyme is
228 directly stimulated by HCO_3^- to produce the ubiquitous second messenger cyclic
229 adenosine monophosphate (cAMP) (Chen et al., 2000) that regulates the activity of
230 effector proteins *via* PKA-dependent phosphorylation, Exchange Protein Activated by
231 cAMP (EPAC), and cAMP gating of membrane channels (Fig. 3). In many systems, sAC
232 activity is directly stimulated by HCO_3^-; however, in the presence of CA, changes in
233 $[HCO_3^-]$ almost instantaneously reflect changes in $[CO_2]$ and $[H^+]$, enabling sAC to
234 indirectly sense extracellular and intracellular acid-base disturbances of any origin
235 (Tresguerres, Levin, & Buck, 2011). Indeed, sensing cytosolic $[HCO_3^-]$ may be more
236 rapid and reliable for pHi regulation than sensing $[H^+]$ because the repeated association
237 and dissociation with cytosolic macromolecules slows down H^+ diffusion, which may
238 confound the detection of a H^+ load (Chang & Oude Elferink, 2014). To our knowledge,
239 a role of sAC in pHi regulation has only been established in corals (Barott, Barron, &
240 Tresguerres, 2017). However, given that corals are phylogenetically deeply rooted
241 metazoans, the role of sAC in pHi regulation most likely extends to most other animal
242 Phyla. In addition, the presence of sAC in the nucleus of mammalian (Zippin et al.,
243 2004) and shark (Roa & Tresguerres, 2017) cells suggests a conserved role in
244 regulating gene expression in response to changing acid-base conditions (Fig. 3).
245 Aquatic animals have specialized cells (“acid-base ionocytes”) on the gills and
246 skin epithelia that actively maintain blood pH by exchanging acid-base equivalents with
247 the environment; this centralized strategy of pHe homeostasis lessens the need for pHi
248 regulation by every individual cell (reviewed in Larsen et al. 2014). The identity and

kinetics of the ion transporting proteins involved in pHe regulation varies greatly between species and environments; however, these proteins are all derived from those involved in pHi regulation (i.e. CAs, NKA, NHEs, NBCs, NDBCEs, VHA, AEs, sAC). The differential placement of transport proteins in the ionocyte's apical or basolateral membrane allows for the vectorial transport of H^+ and HCO_3^- between the internal fluids and the external environment for the purposes of pHe regulation. Similarly, many of the ion-transporting proteins and regulatory pathways involved in pHi regulation take on novel physiological functions when regulating the pH of other internal compartments to promote systemic O_2 transport, ammonia excretion, biomineralization, and CO_2 delivery to photosymbionts.

Coupled pH regulation and preferential pHi regulation

A severe acid-base challenge that overwhelms the capacity for pHe regulation will result in a disturbance to both pHe and pHi. However, some animals are able to tightly regulate pHi even when the pHe defenses have been breached. This capacity gives them an unusual resilience to environmental hypercapnia, and possibly during other acid-base challenges as discussed below.

Environmental hypercapnia results in a sustained elevation in blood PCO_2 and potentially a large acid-base disturbance, and permits investigating the relative contributions of pHi and pHe regulation. Exposure to severe hypercapnia induces a rapid and large reduction in pHe that is often followed by a less pronounced reduction in pHi. During continuous exposure to CO_2, complete pHi recovery is associated with significant (>50-100%) pHe recovery, and therefore this acid-base regulatory pattern

has been termed “coupled pH regulation” (Shartau, Baker, Crossley & Brauner, 2016). Coupled pH regulation during exposure to a respiratory acidosis has been observed in most amphibians, reptiles, and mammals investigated to date, and thus coupled pH regulation appears to be widespread amongst vertebrates (Shartau, Baker, et al., 2016). This pattern has also been observed in the few invertebrates where simultaneous measurements of tissue pHe and pHi have been made during exposure to hypercapnia, and include the land snail (*Otala lacteal*; Barnhart & McMahon, 1988), a deep-sea bivalve (*Acesta excavate*; Hammer, Kristiansen, & Zachariassen, 2011), and the peanut worm (*Sipunculus nudus*; Pörtner, Reipschläger, & Heisler, 1998).

However, some aquatic vertebrates display a different pattern of acid-base regulation, where tissue pHi is completely regulated despite large reductions in pHe during the first few hours of exposure to environmental hypercapnia, and in some cases pHi even increases relative to control values despite a large reduction in pHe (Baker et al., 2009). This pattern of rapid and tight pHi regulation during a transient reduction in pHe during acute CO_2 exposure has been termed “preferential pHi regulation”. Importantly, it does not imply the absence of pHe regulation; just that pHi regulation may be virtually instantaneous and more robust than pHe regulation.

In white sturgeon (*Acipenser transmontanus*) exposed to a PCO_2 of 6 kPa, pHe was reduced by 0.7 pH units within 15 minutes (Baker et al., 2009). Despite the severe blood acidosis, heart pHi increased by 0.05 pH units and was maintained over the subsequent 90 min of hypercapnia (Baker, 2010). Similarly, when sturgeon were exposed to 3 and 6 kPa PCO_2, the pHi of brain, liver and white muscle was tightly regulated. At that time, blood pH was reduced below the blood buffer line indicating a

net acid excretion from the cells to the blood, and this reflects the preferential regulation of the intra- over the extracellular compartment (Baker et al., 2009). Therefore in hypercapnic sturgeon pHi regulation occurs more rapidly than pHe regulation, resulting in a H^+ transfer from the cells to the blood that is faster than their excretion to the environment at the gills.

In addition to sturgeon, preferential pHi regulation has been observed in a number of other fishes including the armored catfish (*Pterygoplichthys pardalis*), the marbled swamp eel (*Synbranchus marmoratus*), the striped catfish (*Pangasianodon hypophthalmus*), and three species of gar (*Lepisosteus oculatus*, *L. osseus*, and *Atractosteus spatula*) (reviewed in Shartau et al., 2020). Preferential pHi regulation was also observed in the late stage developing embryos of the common snapping turtle (*Chelydra serpentine*; Shartau, Crossley, Kohl, & Brauner, 2016) and American alligator (*Alligator mississippiensis*; Shartau, Crossley, Kohl, Elsey, & Brauner, 2018). Thus, it has been proposed that preferential pHi regulation may be a general trait in vertebrate embryos prior to the complete development of the extracellular compartments and structures for acid-base regulation (Shartau, Baker, et al, 2016). This trait is then either retained or lost during development depending on the animal's life history and/or the environment. For example, the greater siren (*Siren lacertian*) is the only tetrapod known to retain preferential pHi regulation into adulthood (Heisler, Forcht, Ultsch, & Anderson, 1982), and this eel-like amphibian inhabits stagnant wetlands where the water is routinely hypercapnic and hypoxic, or even anoxic (Ultsch, 1973, Ultsch & Anthony 1973). To our knowledge, there is no evidence for preferential pHi regulation in

317 invertebrates; however, very few studies have simultaneously measured pHe and pHi in
318 invertebrates during the early stages of exposure to severe hypercapnia.
319 While exposure to elevated PCO_2 has been used as a tool to induce a large
320 acidosis to investigate the presence or absence of preferential pHi regulation, a more
321 common acid-base disturbance may result from anaerobic metabolism due to an O_2
322 limitation. Armored catfish can tolerate long periods of severe hypoxia or even anoxia
323 (Armbruster, 1998), where they preferentially regulate pHi of brain, heart, liver and white
324 muscle despite a severe blood acidosis (Harter et al., 2014). Also white sturgeon are
325 considered hypoxia-tolerant (Cech & Crocker, 2002, however not to the extent of the
326 air-breathing armored catfish. During a hypoxic challenge induced by air exposure,
327 sturgeon demonstrated some capacity for preferential pHi regulation in heart and brain;
328 however, the pHi of liver and white muscle decreased during this challenge (Shartau,
329 Baker, & Brauner, 2017). These findings on armored catfish and white sturgeon may
330 point towards a link between preferential pHi regulation and the ability to survive in O_2
331 limited environments; however, this hypothesis must be tested with further comparative
332 studies. In addition questions remain regarding the molecular and cellular mechanisms
333 underlying preferential pHi regulation that provide exciting avenues to further investigate
334 the evolution and prevalence of preferential pHi regulation.
335
336 **The role of red blood cell pHi on systemic gas transport**
337 RBCs in the vertebrate circulatory system come in close contact with every other
338 cell type and carry high concentrations of hemoglobin (Hb) and CA that enhance O_2
339 delivery and CO_2 removal in all tissues. Cardiovascular gas transport is modulated by

340 the RBC microenvironment and by the fluctuations in pH that occur between arterial and
341 venous blood, with every pass through the circulatory system. The diffusion of CO_2 into
342 the blood at the tissue capillaries causes a decrease in blood pH, whereas the systemic
343 excretion of CO_2 at the gas exchange surfaces causes an increase in pH (Fig. 4A).
344 These cyclical changes in pH between the arterial and venous systems are dampened
345 by the buffer capacity of the blood that, in water breathers, is largely provided by non-
346 bicarbonate buffers. In fishes, these buffers are proteins in the plasma and Hb and
347 organic phosphates within the RBCs and, while most species rely on both intra- and
348 extracellular buffers, their individual contributions may vary greatly among the major fish
349 lineages. On one end of the spectrum are the Antarctic icefishes that have lost RBCs
350 and Hb from the circulation, and where the only non-bicarbonate buffers in the blood are
351 histidine-rich plasma proteins (Feller, Poncin, Aittaleb, Schyns, & Gerday, 1994). On the
352 other hand, lamprey rely almost entirely on buffers within the RBC, which prevent pHi
353 fluctuations but lead to large arterial-venous changes in blood pHe (Tufts & Boutilier,
354 1989). In most vertebrates, Hb is the principal blood buffer and some H^+ binding sites
355 on Hb are modulated by oxygen. This Haldane effect links the transport of O_2 and CO_2
356 in the blood and is particularly important in teleost fishes (Harter & Brauner, 2017).
357 Due to their charge, extracellular H^+ have no direct access to RBC intracellular
358 buffers, such as Hb. However, the Jacobs-Stewart cycle (Jacobs & Stewart, 1942) links
359 the activities of H^+ to the transmembrane fluxes of CO_2 and HCO_3^- by the reversible
360 hydration and dehydration reactions in the plasma and within the RBC (Fig. 4B). CO_2
361 crosses the RBC membrane by diffusion (Wagner, 1977), a process that may be
362 facilitated by aquaporin 1 and RhAG (Muza-Asis et al., 2009), while HCO_3^- is

363 transported by the abundant RBC AE, Band 3 (Romano and Passow 1984). Within the
364 RBC the equilibration between CO_2, HCO_3^- and H^+ is catalyzed by CA (Itada & Forster,
365 1977), whereas the blood plasma of many vertebrates lacks CA (Henry & Swenson,
366 2000) and often contains CA inhibitors that ensure an absence of CA activity against a
367 background of constant RBC lysis that releases soluble CA (Henry, Gilmour, Wood, &
368 Perry, 1997). Without CA activity, the uncatalyzed CO_2 hydration and dehydration
369 reactions in the plasma are slow and typically the rate-limiting step in the Jacobs-
370 Stewart cycle (Motais, Fievet, Garcia-Romeu, & Thomas, 1989). However, at the tissue
371 capillaries, membrane-bound, plasma-accessible CA (paCA) isoforms that are
372 unaffected by plasma CA inhibitors (Gervais & Tufts, 1998; Heming et al., 1993), will
373 accelerate the Jacobs-Stewart cycle and effectively link pHe and RBC pHi.
374 In a theoretical steady state, H^+ are passively distributed across the RBC
375 membrane in a Donnan-like equilibrium; however, the negative charge of Hb and
376 organic phosphates favors a higher [H^+] inside the RBC, resulting in a lower pHi relative
377 to pHe (Jensen, 2004). This pH gradient across the RBC membrane is of physiological
378 significance as it renders the blood an effective sink for CO_2 that removes the gas from
379 the tissues. Due to the higher plasma pHe (typically 7.8-8 in fishes) and the relatively
380 low pK_a of the CO_2-HCO_3^- equilibrium [~6.1; (Boutilier et al., 1984)] more than 90% of
381 CO_2 can be transported as HCO_3^- in the plasma. This increases the capacitance of
382 blood for CO_2 far beyond the physical solubility of the gas in plasma and severely
383 reduces the convection requirements for CO_2 excretion (Tufts & Perry, 1998).
384 The active regulation of RBC pHi is largely driven by the Na^+ and K^+ gradients
385 that are generated by RBC NKA activity (Fig. 4C; Thomas & Egée, 1998). A decrease in

386 RBC pHi is typically due to a net K^+ efflux *via* a K^+-$2Cl^-$-cotransporter (KCC) and the
387 loss of Cl^- displaces H^+ from equilibrium *via* the Jacobs-Stewart cycle (Cossins &
388 Gibson, 1997). Whereas an increase in RBC pHi is driven by a net Na^+ influx, either
389 through Na^+-K^+-Cl^--cotransporters (NKCC) or NHEs (Nikinmaa 2003). The β-
390 adrenergically activated NHEs (β-NHE) of teleosts are particularly effective regulators of
391 RBC pHi and presumably have evolved to protect O_2 transport by pH-sensitive Hb
392 during a systemic acidosis (Berenbrink et al., 2005). The binding of H^+ to Hb decreases
393 its affinity for O_2 and this Bohr effect (Bohr, Hasselbalch, & Krogh, 1904) describes the
394 prominent role of pH in fine-tuning cardiovascular O_2 transport in nearly all vertebrates
395 (Fig. 4A). Teleost fishes have exceptionally pH-sensitive Hbs with large Bohr
396 coefficients and in addition have a Root effect where H^+ binding prevents a complete O_2
397 saturation of Hb even at very high PO_2 (see Berenbrik et al 2005). Based on this high
398 pH-sensitivity of Hb, several physiological mechanisms have evolved in teleosts that
399 actively acidify the blood to increase O_2 unloading to specialized tissues.
400 Perhaps the best-known examples are the teleost *retia mirabilia*, vascular
401 counter-current exchangers that are coupled to acidifying tissues that trigger the Root
402 effect. This mechanism can produce PO_2 values of several hundred atmospheres
403 allowing teleosts with gas-filled bladders to regulate buoyancy at depth (Nielsen &
404 Munk, 1964; Pelster 1997) and to drive O_2 across large diffusion distances to their
405 avascular retinas (Wittenberg & Wittenberg, 1962). Similarly, the intestine of marine
406 teleosts, which secretes large amounts of HCO_3^- into the lumen, may acidify the blood
407 sufficiently to enhance Hb-O_2 unloading and thereby meet its high metabolically
408 demand for O_2 (Cooper, Regan, Brauner, De Bastos, & Wilson, 2014).

More recently, *in vivo* studies have shown that rainbow trout may actively modulate RBC pHi to enable higher tissue PO_2 compared to those in mammals (Rummer, McKenzie, Innocenti, Supuran, & Brauner, 2013) and that can be maintained even in the face of exercise or hypoxia (McKenzie et al., 2004). When the RBC β-NHEs are activated by catecholamines, the extrusion of H^+ exceeds the rate of re-equilibration *via* the Jacobs-Stewart cycle due to the absence of CA activity in teleost plasma. However, when RBCs reach the tissue capillaries the Jacobs-Stewart cycle accelerates in the presence of paCA and the sudden linkage between pHe and pHi effectively "short-circuits" β-NHE activity. The result is a rapid transfer of H^+ into the RBC that enhances O_2 unloading to the tissue *via* the Bohr effect. When the RBCs leave the capillaries and the site of CA, β-NHE activity recovers pHi and Hb-O_2 affinity during venous transit, securing the renewed oxygenation of Hb at the gills (Harter, May, Federspiel, Supuran, & Brauner, 2018). This mechanism of β-NHE short-circuiting is not tied to morphological structures, such as the *retes* and therefore, it may be generally available to enhance Hb-O_2 unloading to all tissues in teleosts (Randall, Rummer, Wilson, Wang, & Brauner, 2014). In fact, in swimming Atlantic salmon β-NHE short-circuiting allows for a reduction in cardiac output by nearly a third, which may enable the athletic performance of this migratory species (Harter, Zanuzzo, Supuran, Gamperl, & Brauner, 2019). Many other teleost species, besides salmonids, also have RBC β-NHE that may be short-circuited to enhance O_2 unloading (Berenbrink et al., 2005; Harter & Brauner, 2017). Whether other transporters that create H^+ gradients across the RBC membrane (i.e. NHE, KCC, NKCC) can also be short-circuited in the presence of CA

remains unexplored, and if substantiated may extend the relevance of this mechanism to species that lack β-NHE, such as other fishes, birds and mammals.

Furthermore, many invertebrate species also have Hbs or hemocyanins, some of which display pH-sensitive O_2 binding characteristics that resemble the Bohr effect of vertebrate Hbs (van Holde & Miller, 1995). Invertebrate respiratory pigments that are dissolved in the plasma lack the cellular mechanism that fine-tune gas transport in vertebrates by modulating the RBC microenvironment. However, as shown in cephalopods, the changes in hemolymph pH during circulatory transit may be sufficient to alter the O_2 binding properties of their hemocyanins, and thus, to modulate cardiovascular O_2 transport and facilitate pH homeostasis, much like in vertebrates (Brix, Lykkeboe, & Johansen, 1981). This remarkable example of convergent evolution illustrates the powerful regulatory effects of pH on physiological systems and its ubiquity across animal taxa.

Links between pH and ammonia metabolism

In solution, NH_3 and NH_4^+ follow the pH-dependent equilibrium shown in Figure 5A. Since the pKa of this reaction is ~9.3, over 95% of ammonia will be present as NH_4^+ at the pH values found in most biological fluids and the external environment (Fig. 5B). Furthermore, NH_3 is a gas and thus crosses cellular membranes much faster than NH_4^+ ions (Boron, 2010). Thus, small but physiologically relevant pH changes result in relatively large changes in the partial pressure of NH_3 (PNH_3), and the resulting difference in partial pressure can drive the diffusion of the gas across a cellular membrane. Additionally, NH_4^+ has nearly identical hydration shell sizes, ionic

conductance, and water mobility rates compared to those of K^+, which allows NH_4^+ to "hijack" K^+ carrier proteins such as NKA, NKCC, and K^+ channels (Wiener & Verlander, 2017). In combination, these physico-chemical characteristics permit the unregulated entry of ammonia into cells and subcellular compartments, where it can have toxic effects through disruptions in pH, membrane potential, the inner mitochondrial H^+ gradient, cell volume, and the Krebs cycle (see Ip & Chew 2010 for review).

The main source of endogenous ammonia production (ammoniagenesis) in animals is as a by-product of the transdeamination reactions during amino acid catabolism within the mitochondrial matrix. These reactions result in the equimolar production of NH_4^+ and HCO_3^-, and predominantly take place in the kidney in mammals (Weiner & Verlander, 2017) and in the liver in fish (Ip & Chew 2010). Additionally, the intestine of carnivorous fishes can catabolize amino acids and produce significant ammonia load following a meal (Karlsson, Eliason, Mydland, Farrell, & Kiessling, 2006). The deamination of serine by serine-dehydratase is another important ammoniagenic pathway, especially in mollusks. The purine nucleotide cycle is a third ammoniagenic pathway, and is prominent during pHi acidification induced by anaerobic metabolism in both fish white muscle (Mommsen and Hochachka, 1988) and intertidal invertebrates (Campbell, 1991).

In mammals, the most common causes of ammonia build up are due to diseases that alter ammonia metabolism and excretion (Wiener & Verlander, 2017). However, aquatic animals may be exposed to high environmental ammonia levels resulting from organic matter degradation, during hypoxic conditions that impair nitrification, in overcrowded and confined environments, and from agricultural, sewage, and industrial

run-offs (Alabaster & Lloyd, 1980). These conditions can impair the excretion of endogenous ammonia and in extreme cases result in ammonia influx leading to internal ammonia accumulation in internal fluids. In general, the toxicity of a given environmental ammonia concentration increases as environmental pH increases due to the resulting increase in the proportion of ammonia present as NH_3, which more readily diffuses into the animal (Randall and Tsui, 2002).

In aquatic animals, waste ammonia is typically excreted to the surrounding water across the gills and the skin (Weihrauch & Allen, 2018). The transport of ammonia across cellular membranes is facilitated by ammonium transporters (AMTs) and Rh glycoproteins (Rhs) (Fig. 6). AMTs are broadly present in bacteria, algae and invertebrates (Huang & Peng, 2005) and can electrogenically excrete NH_4^+ into the external medium (Wacker, Garcia-Celma, Lewe, & Andrade, 2014). In the anal papillae of mosquito larvae, AMT1 was found in the basolateral membrane of epithelial cells (Chasiotis et al., 2016). Although AMTs have also been proposed to be present in the apical membrane of gill epithelial cells of marine polychaetes (Thiel et al., 2017), this putative cellular localization has not been confirmed. In addition to AMTs, invertebrates have the Rh isoform Rhp1, which is expressed in the apical membrane of ammonia excreting epithelial cells (Hu et al., 2014, 2017, Thomsen et al., 2016). On the other hand, vertebrates lack Amts and express several Rh isoforms. The most comprehensive analysis of Rh localization in fish has been performed in pufferfish (*Takifugu rubripes*), which express Rhcg1 and Rhcg2 in the apical membrane of ionocytes and pavement cells, and Rhbg in the basolateral membrane of pavement cells (Nakada, Westhoff, Kato, & Hirose, 2007). Additionally, Rhag is expressed in

RBCs, and in some cases is present in the apical and basolateral membranes of fish epithelial cells (reviewed in Wright & Wood, 2009). Although non-tetrapod vertebrates have an Rhp2 gene, its expression has only been shown in sharks (Nakada et al., 2010). Rhp2 mRNA is highly expressed in shark kidney, and the protein is present in the basolateral membrane of renal tubule cells (Nakada et al., 2010). Lower levels of Rhp2 mRNA were also present in shark blood, gill, brain, intestine, liver, rectal gland and stomach (Nawata, Walsh, & Wood, 2015), however, cellular localization in these tissues has not been explored.

The substrate specificity of the various Rh channels has large implications for the reciprocal relationship between pH and ammonia transport. When NH_3 is transported into a compartment its protonation to NH_4^+ consumes H^+ and thus has an alkalinizing effect, a response that is stimulated by a greater H^+ availability in compartments that have a lower pH (the opposite is the case for NH_4^+ transport). Unfortunately, substrate specificity studies are not trivial due to the interrelationship between pHi, pHe, NH_3/NH_4^+ ratios, and the greater molecular mass and higher pKa of the radiolabeled NH_4^+ analogue, ^{14}C-methyl-ammonium, compared to NH_4^+ (~10.6 vs. ~9.3). Heterologous expression in *Xenopus* oocytes suggests that mammalian Rhag and Rhbg can transport both NH_3 and NH_4^+, and that Rhcg exclusively transports NH_3 (Caner et al., 2015); however, this remains a highly debated subject (Weiner & Verlander, 2017). Knowledge about the substrates transported by the Rhs from aquatic species is even more limited: the substrate for Rhp1 is unknown, Rhp2 seems to preferentially transport NH_3 (Nakada et al., 2010), and detailed substrate specificity studies for other Rhs from aquatic species are lacking. In addition, some Rhs may facilitate CO_2 transport (Musa-Aziz et

al., 2009), and therefore caution should be used when inferring potential Rh functions in aquatic organisms.

Acid-trapping of ammonia

Acidification of a given compartment favors ammonia speciation into NH_4^+ and reduces PNH_3, thus facilitating NH_3 diffusion into the compartment and trapping it as NH_4^+. This mechanism is known as “acid-trapping of ammonia”, and is an effective means for excreting ammonia or for accumulating it into subcellular compartments. The acidification can be generated by the hydration of excreted CO_2 at the external surfaces and by H^+ excretion *via* VHA and NHEs, while the diffusion of NH_3 across cellular membranes is facilitated by Rh channels (Figure 6).

Acid-trapping is a generalized strategy to excrete ammonia by freshwater fishes (Ip & Chew, 2010) and invertebrates (Weihrauch & O’Donnell, 2017). In theory, acid-trapping of ammonia is less advantageous for marine animals due to the challenge of secreting sufficient H^+ to acidify highly buffered seawater (Wilkie, 2002). Indeed, the marine polychaetae (*Eurythoe complanate*) directly excretes NH_4^+ across its gills, possibly through AMTs (Thiel et al., 2017). However, many marine invertebrates use acid-trapping to excrete ammonia into a stagnant or enclosed fluid. For example, the gills of cephalopods have intricate infoldings that create a semi-tubular luminal space that limits ventilation volume, permitting apical excretion of H^+ *via* NHE to acidify the seawater and acid trap NH_3 that diffuses through apical Rhp (Hu et al., 2014). Likewise, excretion of CO_2 and H^+ into the palial cavity of bivalves results in a typical pH of ~7.5 (and as low as pH 6.5 during aerial exposure) (Littlewood & Young, 1994). The

546 ammonia that is trapped in this acidified fluid can be released into the bulk seawater
547 when the animal opens its valves while submerged, or can potentially be volatilized into
548 air while emerged. Similarly, marine animals may utilize acid-trapping into internal fluids
549 such as that in the renal lumen, a mechanism that has been proposed to contribute to
550 the production of ammonia-rich urine in octopuses (Hu et al., 2017).

551 A variation of acid-trapping of ammonia occurs in the gills of marine carbs and is
552 known as vesicular acid-trapping (Figure 6). Here, acidification of intracellular vesicles
553 by VHA promotes NH_3 diffusion and subsequent trapping as NH_4^+. The vesicles are
554 thereafter trafficked *via* microtubules to the apical membrane, and NH_4^+ is excreted into
555 the environment *via* exocytosis (Weihrauch, Ziegler, Siebers, & Towle, 2002). Vesicular
556 acid-trapping of ammonia has also been proposed in goldfish kidney (Fehsenfeld,
557 Kolosov, Wood, & O'Donnell, 2019).

558 Since NH_4^+ is lighter than seawater, acid-trapping may also be used to
559 accumulate ammonia in coelomic cavities or in vacuoles within body tissues for the
560 purpose of buoyancy (Voight, Pörtner, & O'Dor, 1995). Indeed, the tissues of some
561 deep-sea cephalopods can reach $[NH_4^+]$ upwards of 500 mmol L^{-1} (Seibel, Goffredi,
562 Thuesen, Childress, & Robison, 2004), with ~50-60% of their total body mass being
563 comprised of NH_4^+-rich fluid (Voight et al., 1995). The protein-rich diet of cephalopods
564 enables this buoyancy strategy by providing the necessary source of ammonia thorough
565 amino acid catabolism. These squids maintain their pHe at ~7.2 while the pH of
566 sequestration sites can reach values as low as 5 (Voight et al., 1995). Together, these
567 pH set-points provide the necessary transmembrane pH and $P\mathrm{NH_3}$ gradients to permit
568 acid-trapping of ammonia, first within the hemolymph and thereafter within the

sequestration sites. Similar NH_4^+ sequestration may provide buoyancy in tunicate embryos (Lambert & Lambert, 1978) and pelagic crustaceans (Sanders & Childress, 1988). Although the molecular mechanisms underlying NH_4^+ sequestration have yet to be elucidated, they likely involve Rhp and VHA.

Extreme pH microenvironments in coral cells

Reef-building corals that host photosymbiotic algae experience some of the most extreme acid-base disturbances found among animals. While a carbon concentrating mechanism (CCM) promotes photosynthesis by acidifying the algal microenvironment to pH values as low as 4, skeleton calcification is promoted by creating an alkaline microenvironment where pH values can be greater than 9 (reviewed in Tresguerres et al, 2017). Remarkably, this 100,000-fold difference in $[H^+]$ exists over just a few hundred microns that separate the cells that host symbiotic algae from those that build the skeleton (Fig. 7A). Since corals lack specialized organs, acid-base homeostasis relies on regulatory mechanisms within each individual cell.

The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) catalyzes the first major step in photosynthetic CO_2 fixation (Cooper, Filmer, Wishnick, & Lane, 1969). However, rubisco's relatively low affinity for CO_2 compared to contemporary environmental PCO_2 levels and to its significant affinity for O_2 may lead to photorespiration and diminished carbon fixation efficiency (Tamiya & Huzisige, 1948). In response, many phytoplankton species have developed CCMs that elevate PCO_2 at the site of rubisco (Reinfelder, 2011). Likewise, a CCM is essential for sustaining the photosynthetic activity of coral's symbiotic algae (Yellowlees, Rees, & Leggat, 2008).

592 However, these algae reside inside an intracellular compartment of coral gastrodermal
593 cells called the symbiosome (Fig. 7), which can be modulated by the coral host cell.
594 Recently, a novel host-controlled CCM has been identified whereby VHA that is
595 abundantly expressed in the symbiosome membrane acidifies the lumen down to pH~4
596 (Barott, Venn, Perez, Tambutté, & Tresguerres, 2015) (Fig. 7B,C). Together with HCO_3^-
597 transport through yet unidentified mechanisms, this VHA-dependent acidification is
598 thought to drive CO_2 flux into the symbiosome lumen and thereby enhance the delivery
599 of CO_2 to the site of fixation. VHA activity in the coral symbiosome membrane has been
600 proposed to additionally slow down symbiotic alga cell division, as well as to drive the
601 transport of phosphates, amino acids, sugars, and of ammonia by acid-trapping (Tang,
602 2015; Tresguerres et al., 2017, Fig. 7). The presence of an analogous VHA-driven CCM
603 in giant clams that host symbiotic algae in their gut (Armstrong, Roa, Stillman, &
604 Tresguerres, 2018) suggests that this mechanism has evolved convergently in different
605 species.
606 While coral photosynthesis requires an acidified microenvironment, it alkalinizes
607 the rest of the coral because it consumes CO_2 and H^+ and it generates OH^- (Allemand,
608 Furla, & Bénazet-Tambutté, 1998). At the onset of photosynthesis, the pHi of the coral
609 host cells immediately increases from ~7.0 to ~7.4 (Barott et al., 2017; Laurent,
610 Tambutte, Tambutte, Allemand, & Venn, 2013). The rate of photosynthesis increases
611 linearly with light irradiance, and so does the initial alkalinization of the host cell
612 cytoplasm. However, pHi plateaus after ~20 min despite continuous photosynthetic
613 activity, indicating the activation of pHi regulatory mechanisms (Laurent et al., 2013). At
614 this time, cytosolic H^+ are being replenished at the same rate as they are being

615 consumed by photosynthesis and a new steady state is reached. The molecular
616 mechanisms involved in this response are unknown. Although certain AEs are a
617 common mechanism used to counteract an intracellular alkalosis (Fig. 2), they extrude
618 HCO_3^- from the cell and this would conflict with the need for dissolved inorganic carbon
619 transport for photosynthesis. Alternatively, transport of HCO_3^- into the symbiosome as
620 proposed in figure 4B would fulfill the need for both pHi regulation and CCM.
621 Intracellular buffering is also important to help cope with the immediate alkalinizing
622 effect of algal photosynthesis, and this is reflected in symbiont-containing cells having
623 ~25% higher buffering capacity compared to symbiont-free cells (Laurent et al., 2014).
624 Indeed, their buffering capacity is higher than that of mussel retractor muscle (Zange,
625 Grieshaber, & Jans, 1990) and squid mantle (Pörtner, Boutilier, Tang, & Toews, 1990),
626 which may imply that the magnitude of the alkaline challenge induced by symbiont
627 photosynthesis is greater than the acidic challenge resulting from muscle contraction.
628 On the other hand, coral calcification takes place in an actively alkalinized
629 environment and represents a source of acidic stress for the rest of the coral. The cells
630 responsible for coral skeleton formation are called calicoblastic cells, and form an
631 epithelium that is situated directly above the extracellular calcifying fluid (ECF) that
632 separates it from the skeleton. The calicoblastic cells express an abundance of SLC4
633 transporters that presumably help deliver HCO_3^- to the ECF (Barott, Perez, Linsmayer,
634 & Tresguerres, 2015; Tresguerres et al., 2017; Zoccola et al., 2015). These cells also
635 express Na^+/Ca^{2+} exchangers (Barron et al, 2018) and plasma membrane Ca^{2+}-
636 ATPases that help deliver the required Ca^{2+} (Zoccola et al. 2004; Barott, Perez, et al
637 2015); the latter might additionally mediate H^+ removal from the ECF. The combined

activities of these transporters generate an elevated aragonite saturation state in the ECF that promotes skeleton calcification and counteracts its dissolution. Some of these transporters are likely under regulatory control by sAC, which is expressed in calicoblastic cells and mediates the alkalinization of the ECF and the growth of skeletal $CaCO_3$ crystals (Barott et al., 2020). A similar role of sAC in regulating $CaCO_3$ precipitation has been demonstrated or proposed in the intestine of marine teleosts (Tresguerres, Levin, Buck, & Grosell, 2010) and in the teleost inner ear (Kwan, Smith, & Tresguerres, in review). Thus, an evolutionary patter is emerging whereby sAC-regulated transepithelial acid-base relevant ion-transport generates alkaline conditions that promote calcification in an extracellular space.

Other components of the coral calcification mechanism include acidic proteins that promote Ca^{2+} precipitation (Mass et al., 2013), and abundant vesicles in the calicoblastic cells that are formed by macropinocytosis from the ECF (Ganot et al, 2020) and potentially also by transcytosis from the oral tissues (Mass et al., 2017). Interestingly, calcifying foraminifera produce their chambered shells by endocytosis of seawater into vesicles, which they subsequently alkalinize to a pH >9.0 thus promoting $CaCO_3$ precipitation (Bentov, Brownlee, & Erez, 2009; de Nooijer, Toyofuku, & Kitazato, 2009). Incubation with the VHA inhibitor bafilomycin A1 significantly decreased H^+ efflux from the newly forming chambers and resulted in weakly calcified chamber walls, indicating the involvement of VHA in calcification (Toyofuku et al., 2017). A similar role for VHA in calcification was proposed in the calcifying vesicle of coccolitophorids, which are another eukryotic phytoplankton with an external $CaCO_3$ shell (Corstjens, Araki, & González, 2001; Mackinder et al., 2011). It is worth investigating whether VHA is also

present in the vesicles within coral calicoblastic cells and whether it plays a role in coral skeleton formation.

The H^+ that are removed from the ECF during calcification eventually reach the coral gut cavity, where they combine with HCO_3^- to form CO_2 that may be used by photosynthesis within the symbiotic algae (Fig. 7B). Thus, coral calcification and photosynthesis are linked to each other through the complementary and synergistic production and consumption of CO_2, H^+ and HCO_3^-. This is one of the mechanisms by which the photosynthetic activity of the symbiotic algae stimulates coral skeletal growth, a phenomenon known as "light enhanced calcification" (Kawaguti & Sakumoto, 1948).

Summary

As substrates and products of many biochemical reactions CO_2, H^+, HCO_3^- molecules are intrinsically linked to aerobic and anaerobic metabolism, O_2 transport, ammonia homeostasis, metabolic communication between symbiotic partners, and calcification. Future comparative studies at all levels of organization will undoubtedly continue to reveal novel aspects about the evolutionary links between intra- and extracellular acid-base regulation and their effects on multiple aspects of organismal physiology. From a practical perspective, understanding the effects of metabolic and environmental acid-base disturbances on homeostatic processes may help predict the resilience and vulnerability of species to environmental disturbances, both natural and anthropogenic in origin, as well as to artificial environments such as those experienced in intensive aquaculture.

Acknowledgements

Supported by National Science Foundation (NSF) IOS #1754994 to M.T., SIO Postdoctoral Scholar fellowship to A.M.C., NSF Graduate Research Fellowship to A.B.T., NSF Postdoctoral Fellowship to J.N.R., and Natural Sciences and Engineering Research Council (NSERC) Discovery grant (2018-04172) to C.J.B.

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1177

Figure Legends

Figure 1. pH-dependent chemical equilibria between CO_2, HCO_3^-, and CO_3^{2-}. (A) Equation describing the hydration of CO_2 and its subsequent equilibrium reactions with the other dissolved inorganic carbon (DIC) species. The pKAs shown under each reaction are the negative logarithm of the respective dissociation constant, and indicate the pH at which the relevant DIC species are found in equimolar amounts. The hydration of CO_2 into H_2CO_3 is relatively slow; however, in the presence of carbonic anhydrase (CA) enzymes this reaction takes place virtually instantaneously. (**B)** pH-dependent relative proportion of DIC species. Notice how a more acidic pH favors CO_2/H_2CO_3, a relatively neutral pH in the biological range favors HCO_3^-, and a more alkaline pH favors CO_3^{2-}.

Figure 2. Summary of pHi regulatory mechanisms. Schematic of a generic eukaryotic cell depicting the most common pHi regulatory mechanisms. **(1)** The mitochondrial H^+ sink maintains a close balance between H^+ production and consumption during resting aerobic metabolism. However, an increase in **(2)** anaerobic metabolism or **(3)** PCO_2 can result in an intracellular H^+ load that acidifies pHi. **(4)** The activity of carbonic anhydrase (CA) mediates the nearly instantaneous reversible equilibration between CO_2 with HCO_3^- and H^+. **(5)** Buffering is the first line of defense against pHi fluctuations. **(6)** When an acidic load breaches the buffering capacity, cells actively excrete H^+ through Na^+/H^+ exchanger (NHE), V-type ATPase (VHA) and monocarboxylate transporter (MCTs), or take up HCO_3^- through Na^+/HCO_3^-

cotransporter (NBCs), and/or Na^+-dependent Cl^-/HCO_3^- exchangers (NDCBEs). **(7)** Active intracellular acidification can be mediated by anion exchanger (AE) proteins and by Plasma Membrane Ca^{2+}-ATPase (PMCA) (which links intracellular pH with Ca^{2+} homeostasis). **(8)** The driving force for most of these transporters is provided by the inward directed Na^+ electrochemical gradient and the inside negative membrane potential established by the Na^+/K^+-ATPase (NKA). However, VHA and PMCA directly hydrolyze ATP and their activities are not dependent on NKA. The lighting bolts indicate ATP hydrolysis. **(9)** The end products of anaerobic fermentation can have different fates. The lactate that is produced by lactate dehydrogenase (LDH) is typically excreted from cells together with H^+ *via* MCTs; however, some cells can retain the lactate and use it to replenish their glycogen stores. Similarly, the "opines" produced by opine dehydrogenases (OpDH) during fermentation in invertebrates are retained intracellularly and reconverted into the original substrates upon the return of aerobic conditions.

Figure 3. Acid-base sensing by sAC. Separate pools of sAC in **(1)** the cytoplasm and **(2)** the nucleus can be stimulated by HCO_3^- from the following sources: **(a)** Carbonic anhydrase (CA)-dependent hydration of external CO_2; **(b)** CA-dependent hydration of CO_2 generated through mitochondrial respiration; **(d)** entry through bicarbonate transporters (BT) such as the ones shown in Figure 2 or through channels such as cystic transmembrane conductance regulator (CFTR). **(e)** The activities of H^+ extruding transporters (HE) (Figure 2) remove H^+ from the cell and may prevent slowing down of the CO_2 hydration reaction. The cyclic adenosine monophosphate (cAMP) that is generated by sAC can regulate the activities of multiple and diverse target proteins *via*

Protein Kinase A (PKA)-dependent phosphorylation, Exchange Protein Activated by cAMP (EPAC) signaling, and gating of membrane channels. In the nucleus, the sAC-cAMP-PKA pathway can regulate the activity of the gene transcription factor, cAMP responsive element binding (CREB). Modified from Tresguerres et al. (2014).

Figure 4. The role of red blood cell (RBC) pHi on systemic O_2 transport in fish. (**A)** At the capillaries CO_2 from the tissues diffuses into the blood and into the RBCs. In the presence of carbonic anhydrase (CA) within the RBC CO_2 is rapidly converted into H^+ and HCO_3^-. The binding of H^+ to Hb decreases its affinity for O_2 (Bohr effect; a right shift in the oxygen equilibrium curve; OEC), which is then released to the tissues. The binding of H^+ to Hb also buffers arterial-venous pH differences promoting pH homeostasis. Within RBCs, the produced HCO_3^- is exported into the plasma by the anion exchanger (AE). At the gills the process is reversed: when CO_2 diffuses out of the blood and into the water, HCO_3^- is taken up into the RBC and converted into CO_2, which maintains the diffusion gradient for excretion. At the same time, the binding of O_2 to Hb decreases its affinity for H^+ (Haldane effect), which are released into the RBC and are used by CA to dehydrate HCO_3^-. In the absence of H^+ binding, Hb increases its affinity for O_2, which promotes oxygenation of the blood (left shift of the OEC). **(B)** The Jacobs-Stewart cycle describes the transfer of H^+ across the RBC membrane. H^+ are charged ions and do not readily diffuse across lipid membranes. In the plasma H^+ react with HCO_3^- to form CO_2 which rapidly diffuses across the membrane and this is often facilitated by channel proteins such as aquaporin 1 (AQP1) and RhAG. Within the RBC CO_2 will dissociate into H^+, that bind to intracellular buffers, and HCO_3^- that is exported

into the plasma by AE. **(C)** Summary of transporters that regulate RBC pHi in fish. The Na^+/K^+-ATPase (NKA) creates trans-membrane gradients for Na^+ and K^+ that are used by secondary active transporters to drive ion transport. Alkalinizing transporters: Na^+-H^+ exchangers (NHE) use the Na^+ gradient to extrude H^+; β-adrenergically activated NHEs (β-NHE) are activated by catecholamine binding to a receptor on the RBC membrane; Na^+-K^+-$2Cl^-$-cotransporter (NKCC) uses the Na^+ gradient to drive net Cl^- uptake. Because the activities of Cl^- are linked to those of H^+ (via the Jacobs-Stewart cycle), NKCC activity will increase RBC pHi. On the other hand the K^+-$2Cl^-$-cotransporter (KCC) will lead to a decrease in RBC pHi due to a net excretion of Cl^-. See text for further details and references.

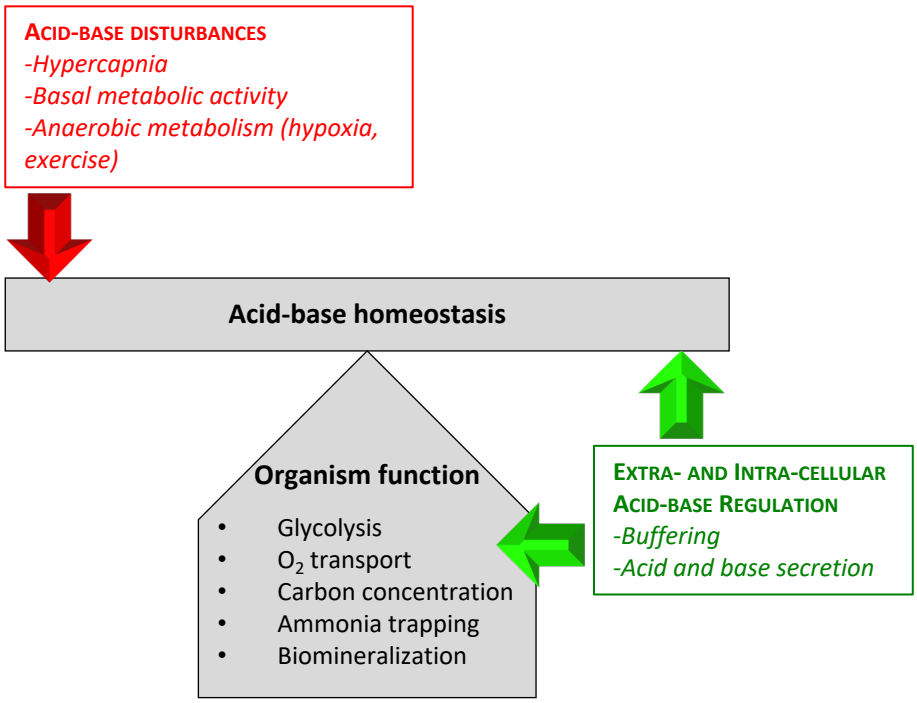
Figure 5. pH-dependent chemical equilibrium of ammonia. (A) Equation describing the hydration of NH_4^+ and the subsequent equilibrium reaction with NH_3. The pKA shown under the reaction is the negative logarithm of the dissociation constant, and indicates the pH at which NH_3 and NH^+ are found in equimolar amounts. **(B)** pH-dependent relative proportion of NH_3 and NH_4^+. Notice that at physiological pH, NH_4^+ is by far the dominant species, that acidification further favors NH_4^+, and that alkalinization favors NH_3.

Figure 6. Acid-trapping of ammonia. (1) Intracellular ammonia (the sum of NH_3 and NH_4^+) is predominantly derived from amino acid catabolism in mitochondria, from facilitated diffusion through ammonium transporters (AMTs) and Rhesus channel glycoptroteins (Rhs), and through import by K^+-transporting proteins (K^+T) such as

Na^+/K^+-ATPase, Na^+-K^+-$2Cl^-$-cotransporter, K^+-$2Cl^-$-cotransporter, and K^+-channels. **(2)** At a typical intracellular pH (pHi), most ammonia is present as NH_4^+ (see Fig. 5). However, acidification of **(3)** the external boundary layer or **(4)** intracellular vesicles acts as a siphon for NH_3, producing NH_4^+ that gets trapped outside of the cell or in the vesicle, while sustaining a favorable NH_3 partial pressure gradient (ΔPNH_3) that promotes further ammonia transport and trapping. The acidification can take place through H^+ transport by Na^+ H^+ Exchanger (NHE) or V-type-H^+-ATPase (VHA), and NH_3 diffusion is facilitated by Rh. In addition, NH_3 may diffuse across cellular membranes or paracellularly (not shown). **(5)** The vesicles can be trafficked to the apical membrane in microtubule-dependent manner, and the trapped NH_4^+ excreted *via* exocytosis, as reported in the gills of some marine crabs (note that the cuticle at the apical side has been omitted for clarity). In addition, similar acidic and NH_4^+-rich vesicles can be stored within the body tissues of deep-sea cephalopods and other marine invertebrates for the purpose of achieving buoyancy (however, the pathways for NH_3 and H^+ transport remain unknown).

Figure 7. Extreme pH microenvironments in corals. (A) Simplified coral histology diagram showing the movements of acid-base relevant molecules between seawater, host cells with algal symbionts, and the site of calcification (ECM: extracellular calcifying medium). Together with Ca^{2+} transport into the ECM and vesicular transport of amorphous $CaCO_3$ (not shown), the alkaline pH in the SCM promotes coral skeleton formation. DIC: dissolved inorganic carbon ($CO_2 + HCO_3^- + CO_3^{2-}$). The pH of extracellular and intracellular compartments is noted to the left (sun and moon indicating

day- and nighttime pH for seawater respectively). Photosynthesis and calcification are linked by translocation of photosynthetic products to the site of calcification (i.e. oxygen and sugars) and calcification byproducts (H^+) to host cells. **(B)** Schematic of a coral host cell containing an algal symbiont to illustrate the CCM. The alga is not drawn to scale to allow for clarity but usually occupies >90% of a host cell's volume. BT: HCO_3^- transporter; CA: carbonic anhydrase; VHA: V-Type H^+-ATPase. **(C)** VHA immunostaining (red signal) of a symbiont-containing coral gastrodermal cell showing abundant VHA presence in the symbiosome membrane. The other proteins involved in transport of ions and other molecules are omitted for simplicity, and in many cases their identities are unknown.



Graphical abstract

A

$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+$

pKa_1 ~3.6 (CA)

pKa_2 ~6.3

pKa_3 ~10.3

B

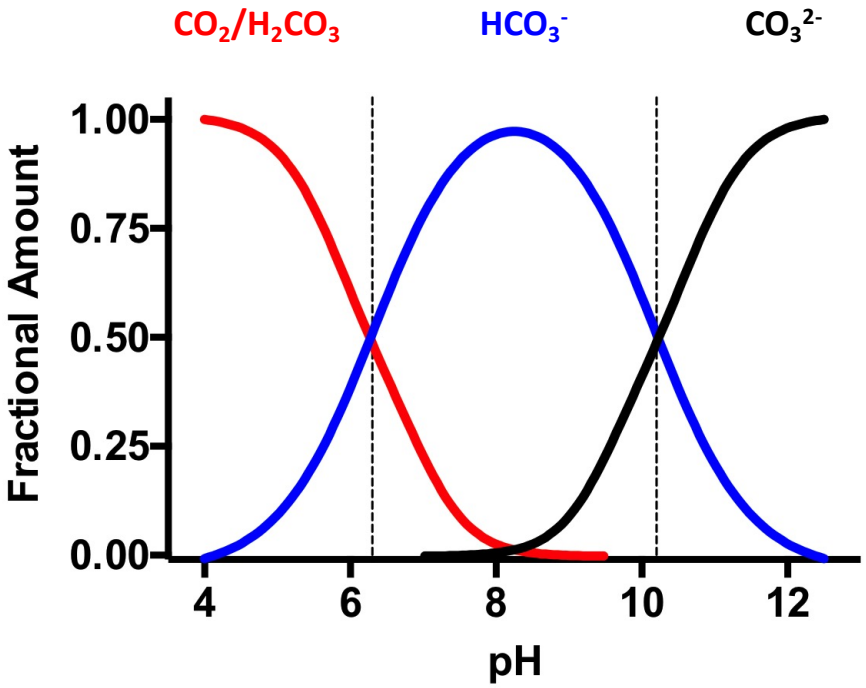


Figure 1

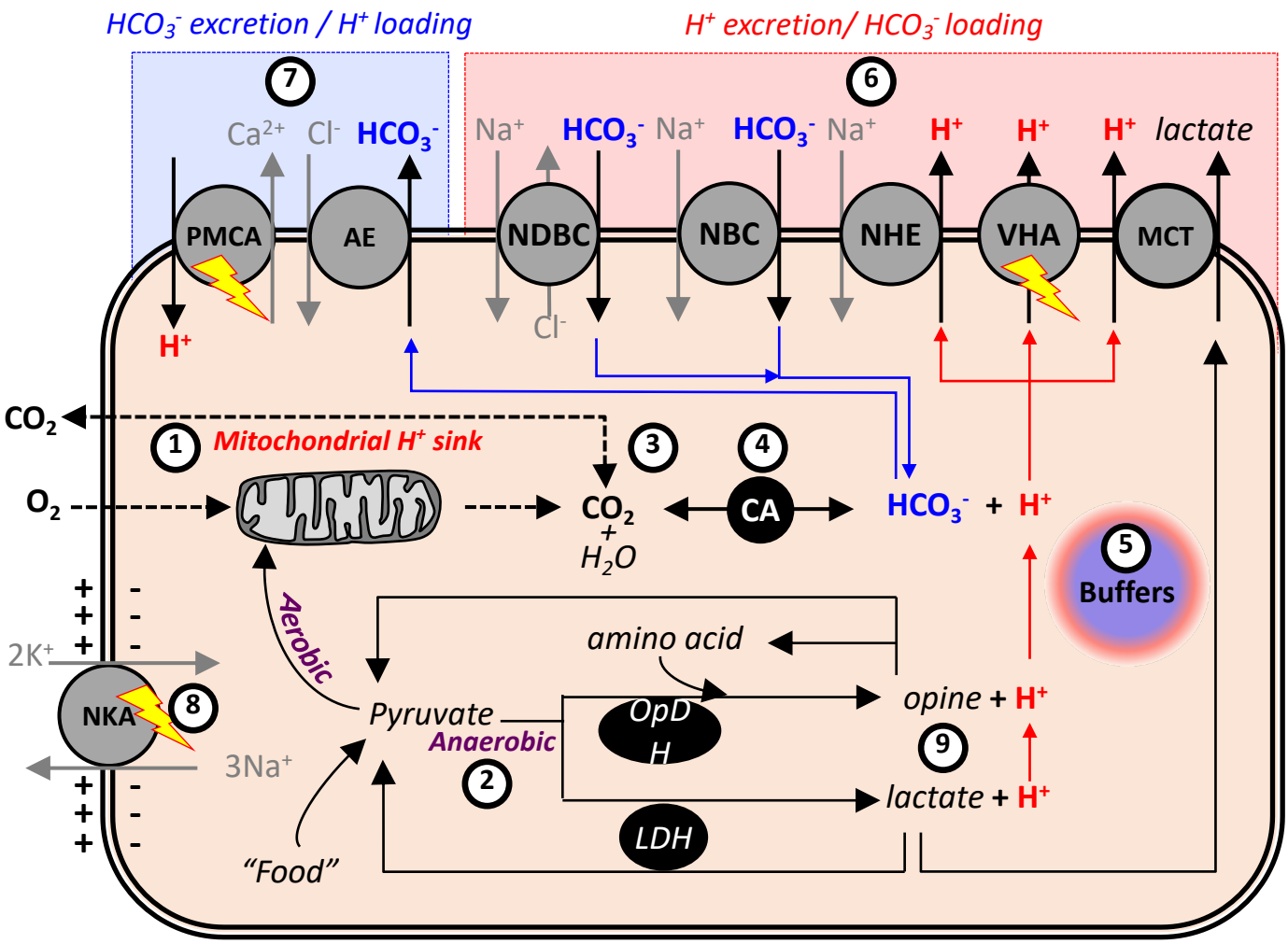

HCO3- excretion / H+ loading
H+ excretion/ HCO3- loading
7
6
Ca2+
Cl-
HCO3-
Na+
HCO3-
Na+
HCO3-
Na+
H+
H+
H+
lactate
PMCA
AE
NDBC
NBC
NHE
VHA
MCT
H+
Cl-
CO2
1
Mitochondrial H+ sink
3
4
O2
CO2
+
H2O
CA
HCO3- + H+
5
Buffers
2K+
Aerobic
amino acid
NKA
8
Pyruvate
Anaerobic
OpD
H
opine + H+
9
3Na+
2
lactate + H+
LDH
"Food"

Figure 2

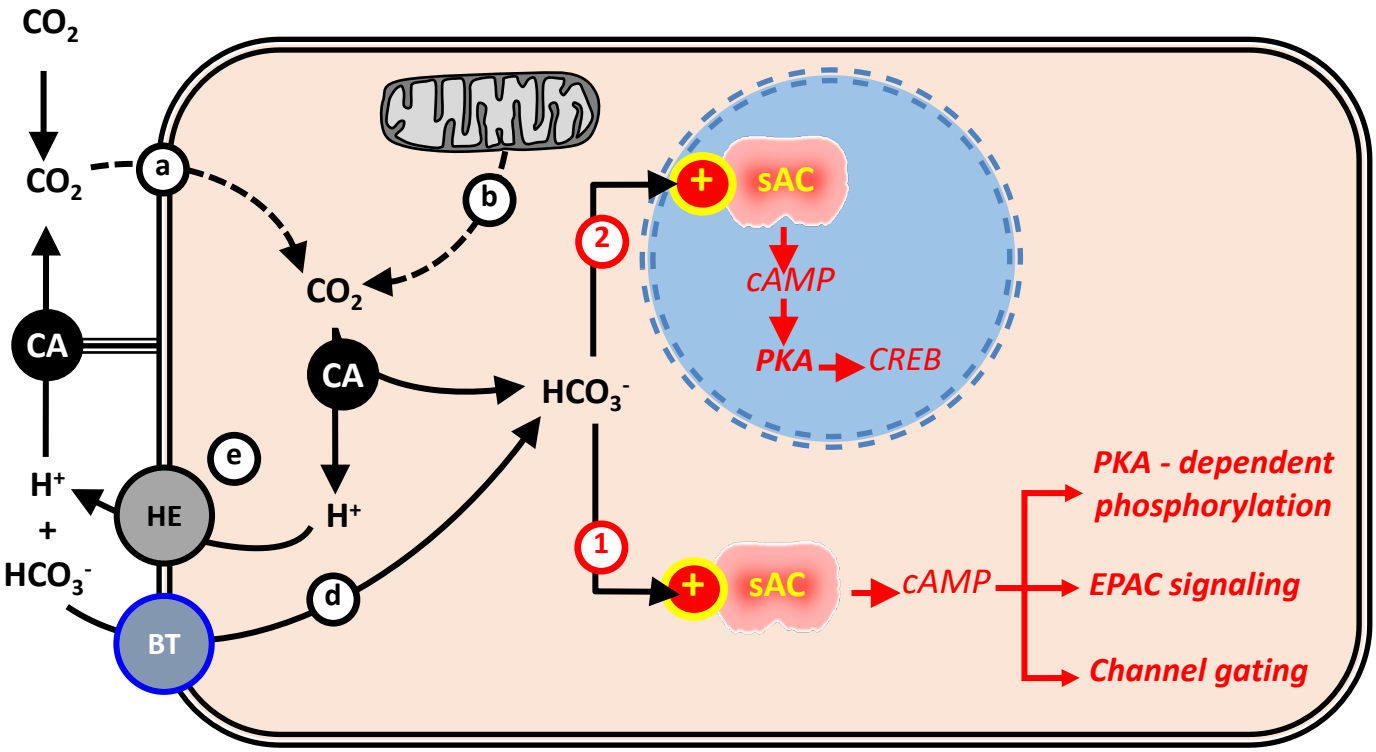

CO_2
CO_2
a
b
CO_2
CA
CA
HCO_3^-
e
H^+
+
HCO_3^-
HE
H^+
d
BT
2
sAC
cAMP
PKA
CREB
1
sAC
cAMP
PKA - dependent phosphorylation
EPAC signaling
Channel gating

Figure 3

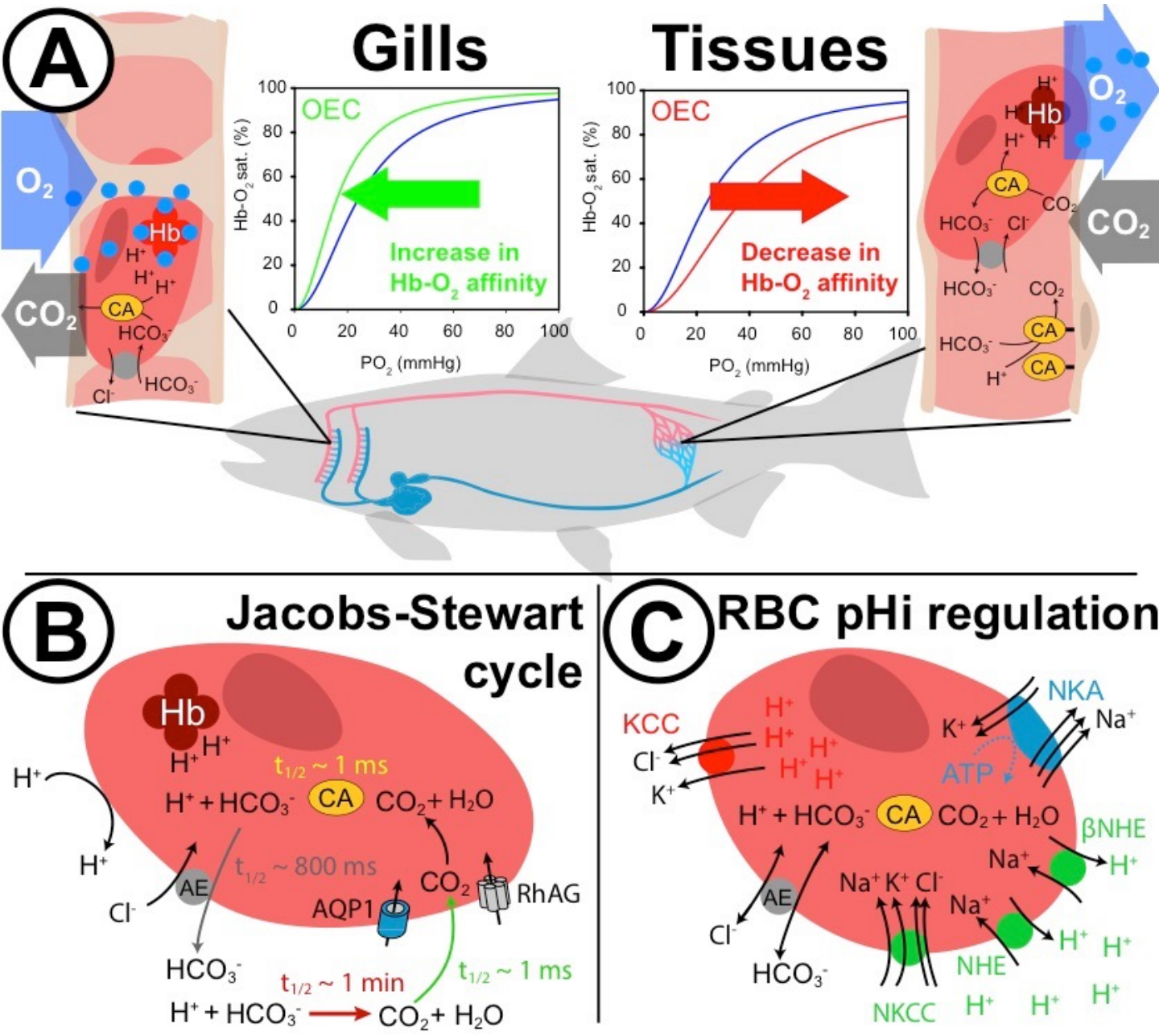
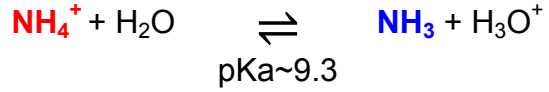

A
Gills
Tissues
O2
CO2
Hb
H+
CA
HCO3-
Cl-
OEC
Hb-O2 sat. (%)
PO2 (mmHg)
Increase in Hb-O2 affinity
Decrease in Hb-O2 affinity
B
Jacobs-Stewart cycle
t1/2 ~ 1 ms
t1/2 ~ 800 ms
t1/2 ~ 1 min
AE
AQP1
RhAG
C
RBC pHi regulation
KCC
NKA
ATP
βNHE
NHE
NKCC
Na+
K+

Figure 4

A


NH4+ + H2O
NH3 + H3O+
pKa~9.3

B

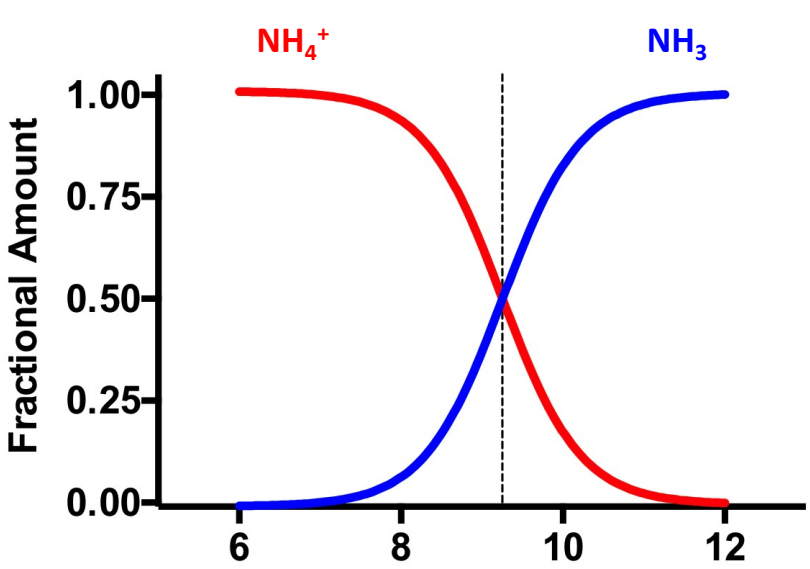

NH4+
NH3
Fractional Amount
1.00
0.75
0.50
0.25
0.00
6
8
10
12

Figure 5

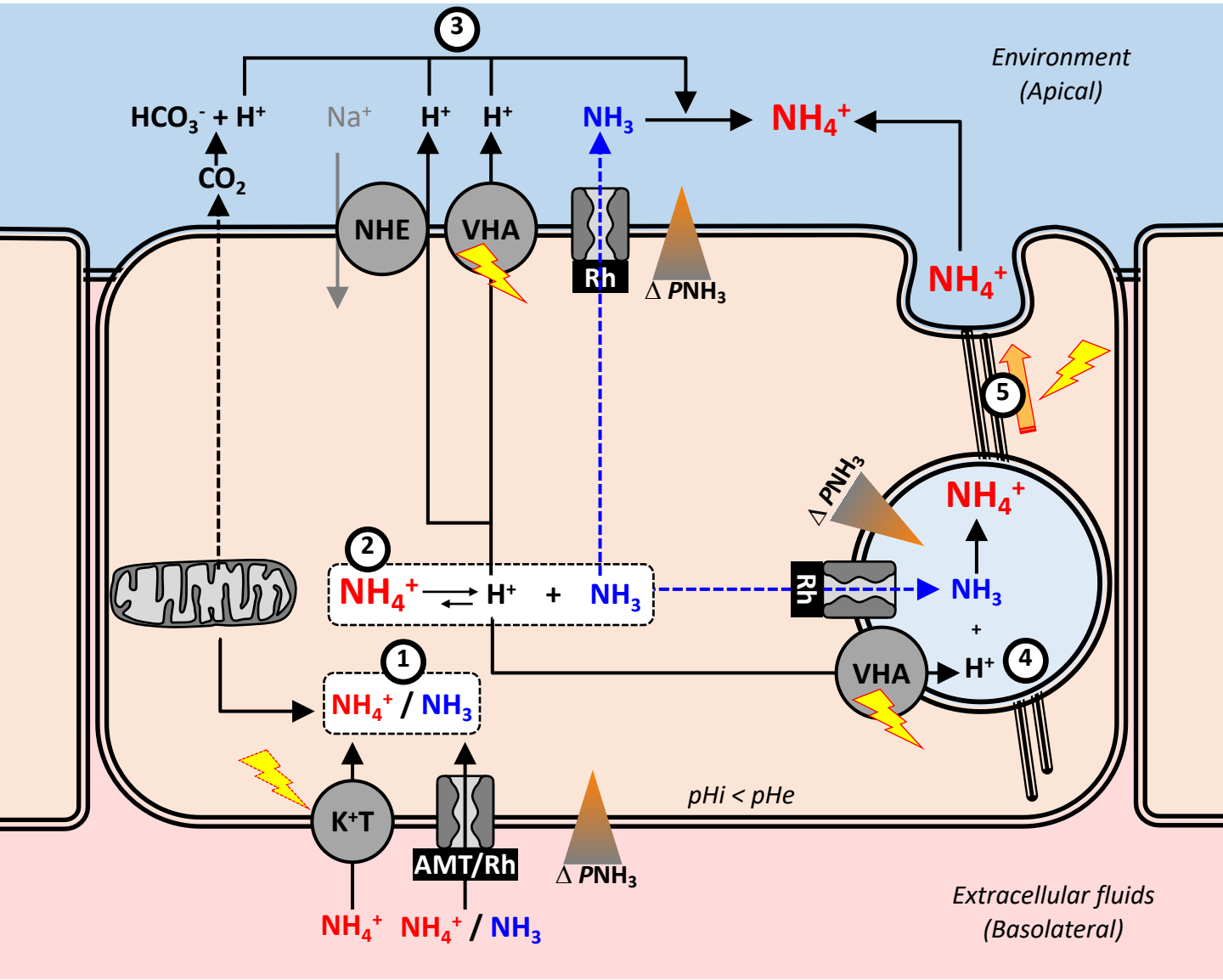

3
Environment
(Apical)
$HCO_3^- + H^+$
CO_2
Na^+
H^+
H^+
NH_3
NH_4^+
NHE
VHA
Rh
Δ PNH_3
NH_4^+
5
Δ PNH_3
NH_4^+
2
NH_4^+ ⇌ H^+ + NH_3
Rh
NH_3
+
VHA
H^+
4
1
NH_4^+ / NH_3
pHi < pHe
K^+T
AMT/Rh
Δ PNH_3
NH_4^+
NH_4^+ / NH_3
Extracellular fluids
(Basolateral)

Figure 6

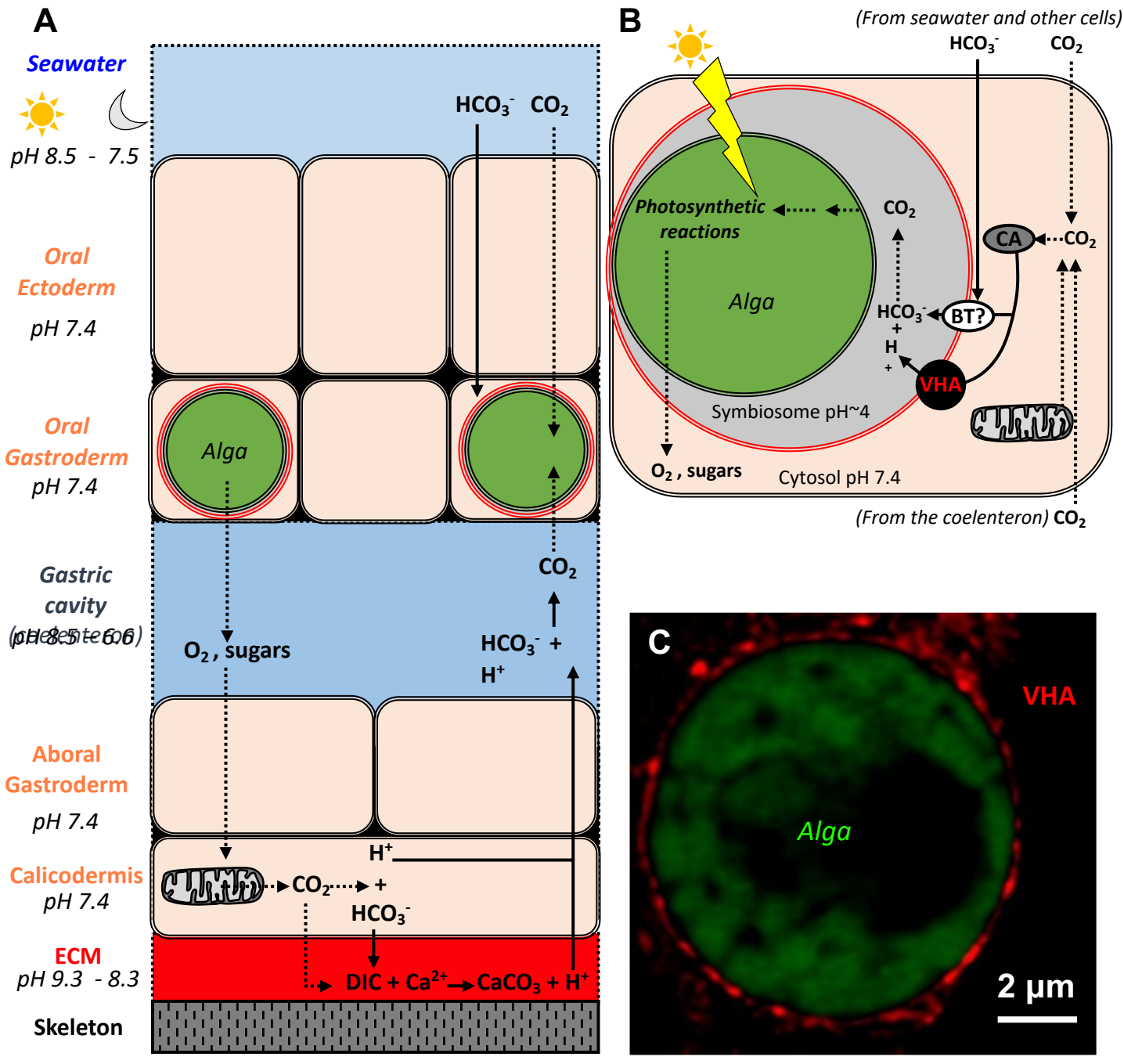

A
Seawater
pH 8.5 - 7.5
HCO3- CO2
Oral Ectoderm
pH 7.4
Oral Gastroderm
pH 7.4
Alga
Gastric cavity
pH 8.5 - 6.6
CO2
O2 , sugars
HCO3- + H+
Aboral Gastroderm
pH 7.4
Calicodermis
pH 7.4
H+
CO2 + HCO3-
ECM
pH 9.3 - 8.3
DIC + Ca2+ CaCO3 + H+
Skeleton
B
(From seawater and other cells)
HCO3- CO2
Photosynthetic reactions
CO2
CA
CO2
Alga
HCO3- + H+
BT?
VHA
Symbiosome pH~4
O2 , sugars
Cytosol pH 7.4
(From the coelenteron) CO2
C
VHA
Alga
2 µm

Figure 7