Title

CO2 and acid-base sensing

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Chapter 2: CO_2 sensing

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Abstract

Carbon dioxide (CO_2) and its hydration products hydrogen (H^+), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions collectively contribute to the acid/base (A/B) status of aqueous solutions, and have major effects on the physiology of organisms. Correspondingly, organisms have developed the ability to sense specific A/B disturbances that routinely arise from metabolic and environmental sources, and to coordinate a variety of homeostatic responses. A common requirement for all homeostatic mechanisms is the ability to sense specific A/B disturbances and to coordinate appropriate responses. This chapter synthetizes our knowledge concerning the sensory pathways that allow fish to sense A/B disturbances of both metabolic and environmental origin and the ensuing downstream physiological responses that promote homeostasis in different organs. We focus largely on the peripheral, and to a lesser extent, the central

24 sites of CO_2/H^+ detection, emphasizing the cellular sites and molecular
25 mechanisms of A/B sensing.
26
27 **Keywords**: Carbon dioxide, soluble adenylyl cyclase, adcy10, chemosensor,
28 chemoreception, pH sensor, ocean acidification, neuroepithelial cell,
29 hypercapnia, acidosis, alkalosis.
30
31 **Abbreviations**
32 A/B: acid/base
33 BT: bicarbonate transporter
34 CA: carbonic anhydrase
35 Ca^{2+}: calcium
36 cAMP: 3' 5' cyclic adenosine monophosphate
37 CO_3^{2-}: carbonate
38 cGMP: 3' 5' cyclic guanosine monophosphate
39 CNAC: cyclic nucleotide-activated ion channel
40 CO_2: carbon dioxide
41 DAG: diacylglycerol
42 f_V: ventilation frequency
43 f_H: cardiac frequency
44 GPCR: G-protein coupled receptor
45 GPR4: G-protein coupled receptor 4
46 Guanylyl cyclase: GC

47 H^+: hydrogen ion

48 HCO_3^-: bicarbonate

49 HCN: hyperpolarization-activated cyclic nucleotide-modulated ion channels

50 IP_3: inositol 1,4,5-triphosphate

51 NBC: Na^+/HCO_3^- cotransporter

52 NEC: neuroepithelial cell

53 NKA: Na^+/K^+-ATPase

54 NKCC: $Na^+/K^+/2Cl^-$ cotransporter

55 OGR1: ovarian G-protein coupled receptor 1

56 O_2: Oxygen

57 PCO_2: CO_2 partial pressure

58 pHi: intracellular pH

59 pHe: extracellular pH

60 PLC: phospholipase C

61 PO_2: O_2 partial pressure

62 PKA: protein kinase A

63 PKC: protein kinase C

64 RTN: retrotrapezoid nucleus

65 sAC: soluble Adenylyl Cyclase

66 TASK: Twik-related acid-sensitive K^+ channel

67 tmAC: transmembrane Adenylyl Cyclase

68 VHA: V-type H^+-ATPase

69

Introduction

In aqueous solutions, carbon dioxide (CO_2) establishes a reversible equilibrium with hydrogen (H^+), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. Together with non-bicarbonate buffering, the levels of these molecules define the acid/base (A/B) status of the fluid. Maintaining A/B homeostasis in physiological fluids is essential for life because the concentration of [H^+] (~pH) greatly affects protein folding and function. Additionally, A/B conditions affect (and are affected by) various physiological processes such as metabolism, pH buffering, biomineralization, neurotransmission, oxygen (O_2) delivery, and feeding and digestion. Fish A/B status can also be affected by environmental factors, the most prominent being elevated CO_2 (hypercapnia) and the associated reduction in pH. Environmental hypercapnia can be found at night in densely populated environments such as kelp forest, coral reefs, mangroves, estuaries and tide pools (Duarte et al., 2013; Hofmann et al., 2011; Kline et al., 2012; Truchot and Duhamel-Jouve, 1980) due to organismal aerobic respiration that produces CO_2 as it depletes O_2. Thus, environmental hypercapnia often is associated with hypoxia. Indeed, fish might also experience environmental hypercapnia and hypoxia during upwelling events (Frieder et al., 2012). Chronic environmental hypercapnia may occur in recirculating aquaculture systems (Ellis et al., 2017), and at much lower levels, as ocean acidification develops (Duarte et al., 2013; Raven et al., 2005; Sabine et al., 2004) .

Need for acid/base sensing

Because virtually every physiological process is affected by A/B status, organisms have developed a variety of homeostatic responses to compensate for A/B disturbances that arise from metabolic and environmental sources. A common requirement for all homeostatic responses is the ability to sense A/B disturbances in the first place. In a broad sense, this means sensing acidosis and alkalosis from a specific set point. In addition, an A/B sensing mechanism must be able to differentiate between A/B disturbances of metabolic or respiratory origin, and if those are from environmental or internal sources. Furthermore, the A/B set point can differ between subcellular compartments, cell types and organs, as well as between fish living in different environments, or having different metabolic capacities and breathing modes. Another consideration is that A/B set points may change as a function of temperature according to the "alphastat hypothesis" (reviewed in Somero, 1986), and have the potential to dynamically adjust upon prolonged exposure to changed A/B conditions. Clearly, A/B sensing is a complex process involving multiple molecular sensors and feedback loops, much of which remains unexplored.

Physiologically relevant sites of A/B sensing

Peripheral CO_2 sensing

Cardiorespiratory reflexes

The most commonly reported cardiorespiratory response to elevated ambient CO_2 levels in adult fish is hyperventilation, an increase in the volume of water flowing over the gills (Dejours, 1973). Hypercapnic hyperventilation has

116 been reported in agnathans (Pacific hagfish *Eptatretus stoutii* , Perry et al.,
117 2009b), chondrichthyans (spotted dogfish *Scyliorhinus stellaris*, Randall et al.,
118 1976; Atlantic big skate *Raja ocellata*, Graham et al., 1990; spiny dogfish
119 *Squalus suckeleyi*, Perry and Gilmour, 1996) and a variety of actinopterygians
120 including holosteans (spotted gar *Lepisosteus oculatus*, Smatresk and Cameron,
121 1982), chondrosteans (white sturgeon *Acipenser transmontanus*, Crocker et al.,
122 2000) and teleosts (e.g. rainbow trout *Oncorhynchus mykiss*, Janssen and
123 Randall, 1975; Smith and Jones, 1982; Atlantic salmon *Salmo salar*, Perry and
124 McKendry, 2001; common carp *Cyprinus carpio*, Soncini and Glass, 2000) and
125 zebrafish *Danio rerio* (Vulesevic et al., 2006). Increases in ventilation are
126 mediated by adjustments to ventilation frequency (f_V) and/or amplitude (a
127 determinant of respiratory stoke volume, which is analogous to tidal volume in
128 air-breathers). Although relatively few species have been examined, hypercapnic
129 hyperventilation typically is associated with increases in breathing amplitude (see
130 Gilmour and Perry, 2007). However, the response patterns are highly variable
131 with some fish (e.g. zebrafish) responding to elevated CO_2 by increasing
132 ventilation amplitude exclusively (Vulesevic and Perry, 2006) with others (e.g.
133 tambaqui) solely adjusting f_V (Sundin et al., 2000). From an energetics
134 perspective, hyperventilatory responses mediated largely by increases in
135 ventilation amplitude are thought to be more efficient (Perry and Wood, 1989).
136 Minimizing the costs associated with hyperventilation is particularly important in
137 water breathers given the high metabolic costs associated with moving water
138 across the gills (Jones and Schwarzfeld, 1974). It was shown recently in air-

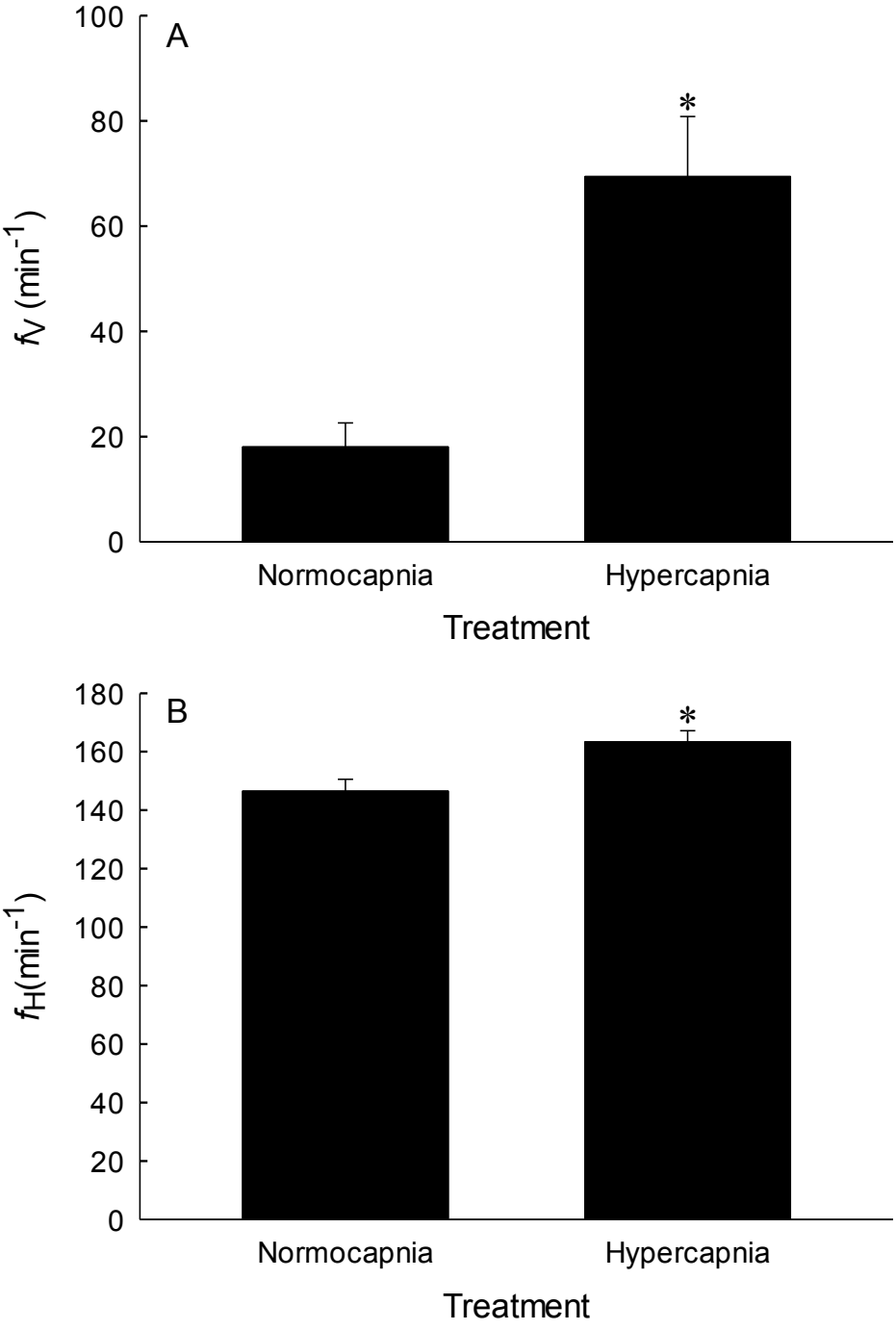
breathing *Pangasius hypophthalmus* that intra-arterial injections of lactate produce dose-dependent increases in gill ventilation amplitude and frequency, and at higher doses, stimulate air breathing (Thomsen et al., 2018). These responses, however, were independent of changes in pH.

Although less well-studied, hypercapnia may also initiate cardiovascular reflexes in adult fish including a reduction in heart rate (bradycardia) and an elevation of blood pressure (reviewed by Gilmour and Perry, 2007). In rainbow trout, the bradycardia is mediated by increased parasympathetic input to the heart while the elevated blood pressure is a consequence of increased systemic vascular resistance owing to neuronal mediated sympathetic peripheral vasoconstriction linked to α-adrenergic receptor stimulation (Perry et al., 1999). Despite bradycardia, cardiac output generally remains constant or may even increase slightly in the few species that have been examined (see Table 3.2 in Gilmour and Perry, 2007) owing to increasing stroke volume. The specific mechanisms underlying the rise in stroke volume during hypercapnia are unclear but may involve the stimulatory effects of circulating catecholamines (e.g. Perry et al., 1987), increased sympathetic nerve activity (Perry et al., 2009a), elevated central venous pressure (Perry et al., 2009a) and the increased filling time associated with the bradycardia (Starling's law of the heart).

Of the three principal cardiorespiratory reflexes evoked by hypercapnia (hyperventilation, bradycardia and increased systemic vascular resistance), only the hyperventilatory response appears to impart obvious physiological benefit to gas transfer across the gill. By analogy to branchial O_2 transfer (Perry et al.,

2009a), increasing water flow over the gill when metabolically produced CO_2 is elevated will increase the blood-to-water diffusion gradient and cause arterial blood PCO_2 to be lower than it otherwise would be without hyperventilation (Gilmour, 2001; Perry and Wood, 1989). The underlying mechanism facilitating the lowering of arterial PCO_2 during hyperventilation is a reduction in the residence time during which inspired water is in contact with the respiratory surfaces, thereby decreasing the accumulation of CO_2 into the inspired water during gill passage and lowering the average PCO_2 in the ventilatory flow. Clearly, respiratory acidosis is unavoidable during exposure to hypercapnic water but theory predicts that hyperventilation will lessen the extent of the acidosis by removing metabolically produced CO_2 and hence lowering arterial PCO_2. It has long been held that bradycardia, with or without an accompanying fall in cardiac output, increases branchial gas transfer efficiency (Randall and Daxboeck, 1984). With a single exception, however (Short et al., 1979), previous studies have failed to provide any empirical evidence in support of a beneficial role of bradycardia on gas transfer during hypoxia (Iversen et al., 2010; Mackenzie et al., 2009; Perry and Desforges, 2006; Taylor and Barrett, 1985) or hypercapnia (Perry and Desforges, 2006). In rainbow trout, the increase in blood pressure during hypercapnia has no obvious benefit on branchial CO_2 transfer (Perry and Desforges, 2006) despite theoretical arguments linking elevated blood pressure to increased diffusive conductance (Farrell et al., 1980). Thus, it remains to be determined whether fish derive any benefit from the cardiovascular responses associated with hypercapnia.

185 Unlike for hypoxia, little is known about the cardiorespiratory reflexes
186 associated with hypercapnia in developing (larval) fish. Indeed, to our knowledge,
187 the only species that has been studied during larval stages is zebrafish. Unlike
188 the majority of species that have been examined as adults, the larval zebrafish (5
189 - 7 days post fertilization) experiences tachycardia when exposed to hypercapnia
190 (Miller et al., 2014). The hypercapnic tachycardia appears to be mediated by
191 activation of cardiac β_1-adrenergic receptors by catecholamines originating from
192 sympathetic neurons (Miller et al., 2014). Although data are lacking, it is possible
193 that the cardiac response to hypercapnia in adult zebrafish is bradycardia, similar
194 to other species (see above). Unlike adults, which increase gill ventilation during
195 hypercapnia exclusively by adjusting ventilation amplitude (see above), larval
196 zebrafish hyperventilate via marked increases in breathing frequency when
197 exposed to elevated ambient CO_2 (Koudrina, 2017; Figure 1). Currently, it is not
198 possible to measure ventilation amplitude in zebrafish larvae.



199

200 **Figure 1.** The effect of acute (30 min) hypercapnia (1.5% CO_2 in air; PCO_2 =

201 11.25 mmHg) on A) ventilation frequency (f_V) and B) cardiac frequency (f_H) in

202 larval zebrafish (*Danio rerio*; N = 10) at 4 days post fertilization. Each larva was

exposed to normoxic, normocapnic conditions for a 20 min acclimation/control period. Baseline f_V and f_H measurements were collected for the last 2 min of this normocapnic period. To evaluate the cardiorespiratory responses to hypercapnia, f_V and f_H were assessed for each 2 min interval during the 30 min exposure period, and the peak (maximum) response was identified. Significant differences from the normocapnic period are indicated by asterisks ($P<0.05$; paired Student's t-test). Previously unpublished data from Koudrina (2017).

Sites of CO_2 chemoreception

It was originally held that the cardiorespiratory reflexes initiated by hypercapnia were mediated indirectly via hypoxemia (Randall, 1982; Smith and Jones, 1982) arising from the adverse effects of respiratory acidosis on lowering blood O_2 carrying capacity (the Root effect). However, it is now well established that increased ambient CO_2 can be sensed directly via peripheral chemoreceptors (Abdallah et al., 2012; Abdallah et al., 2015; Perry and Abdallah, 2012, Qin et al., 2010). Based largely on indirect *in vivo* evidence gathered from numerous adult species, these peripheral chemoreceptors appear to be activated by changes in water CO_2 tension rather than by accompanying changes in water pH (Gilmour et al., 2005; Miller et al., 2014; Perry and McKendry, 2001; Reid et al., 2000; Sundin et al., 2000). The CO_2 receptors are localized predominantly to the gills but occasionally may also reside on surfaces of the orobranchial cavity or other peripheral sites (Burleson and Smatresk, 2000; Florindo et al., 2004; McKendry et al., 2001; Milsom et al., 2002; Perry and Reid, 2002; Reid et al.,

226 2000; Sundin et al., 2000). In some species (e.g. rainbow trout), the
227 cardiorespiratory responses appear to be mediated largely by CO_2
228 chemoreceptors associated with the first gill arch (Perry and Reid, 2002) while in
229 others, the receptors contributing to the reflex responses are distributed across
230 all four gill arches (Reid et al., 2000; Sundin et al., 2000; reviewed by Milsom,
231 2012).

232 The bulk of available evidence suggests that the branchial CO_2
233 chemoreceptors respond preferentially to external changes in CO_2 tensions and
234 are largely insensitive to changes in internal (blood) CO_2 levels (Gilmour et al.,
235 2005; reviewed by Gilmour and Perry, 2007). Thus, it has been suggested that
236 the branchial CO_2 chemoreceptors are oriented in such a manner as to
237 exclusively detect changes in ambient CO_2 levels (Gilmour and Perry, 2007).
238 Despite the predominance of evidence implicating external sites of CO_2 sensing
239 in fish, there are data, albeit equivocal, to support the existence of CO_2 receptors
240 able to monitor internal CO_2 levels or acid-base status in teleosts (Wood and
241 Munger, 1994) and elasmobranchs (Graham et al., 1990; Wood et al., 1990).
242 The results of intra-arterial injections of acid, bicarbonate or CO_2-equilibrated
243 solutions have yielded conflicting results. Most frequently such injections have
244 been without effect (Burleson and Smatresk, 2000; Florindo et al., 2004; Gilmour
245 and Perry, 1996; Gilmour et al., 2007; Lopes et al., 2010; McKendry et al., 2001;
246 Reid et al., 2000; Sundin et al., 2000). There have been some studies, however,
247 where such injections in rainbow trout did stimulate ventilation (Aota et al., 1990;
248 Gilmour and Perry, 1996; Janssen and Randall, 1975; Mckenzie et al., 1991;

McKenzie et al., 1993; Reid *et al*., 2000). Although these data suggest the existence of internal receptors that monitor changes in arterial CO_2/H^+ levels, the sites remain unknown and may even be centrally located (i.e. within the brain) (see section on Central CO_2/H^+ Chemoreceptors: Cardiorespiratory reflexes and underlying cellular mechanisms). In rainbow trout following exhaustive exercise in normoxic water, ventilation remains elevated even though arterial PO_2 is normal. CO_2 that has accumulated during the exhaustive exercise takes longer to clear and hence arterial PCO_2 remains elevated, and pH reduced at this time (Perry et al., 1989; Tufts and Perry, 1998; Wood, 1991; Wood and Munger, 1994; Wood and Perry, 1985). Administering carbonic anhydrase (CA) to reduce the post-exercise acidosis and enhance CO_2 clearance reduced this hyperventilation (Wood and Munger, 1994). Similarly, when dogfish (*S. stellaris*) were exposed to hyperoxia the hypoventilatory response appeared to be fine-tuned by blood acid-base status (Heisler et al., 1988). Interestingly, in a study designed to examine the possible role of central acid-base stimuli in the increase in ventilation induced by hypercapnia in the skate, there was a better correlation between the changes in ventilation and changes in arterial pH rather than with cerebral-spinal fluid pH, the intracellular pH (pHi) of brain tissue, or arterial PCO_2 (Wood et al., 1990).

It is important to note that although several studies reported cardiorespiratory changes (bradycardia, hypertension, and hyperventilation) during experimentally evoked internal acidosis (see above), blood O_2 status was not monitored. Therefore, activation of blood-oriented O_2-chemoreceptor reflexes owing to acid-induced hypoxemia (Root effect) cannot be ruled out.

As stated by Gilmour and Perry (2007), “the role of arterial acid–base status in regulating cardiorespiratory function remains uncertain as do the location and stimulus specificity of any chemoreceptors involved in mediating such responses.”

Neuroepithelial cells

Although direct data exist only for a limited number of species (zebrafish, rainbow trout, and channel catfish *Ictalurus punctatus*), it is widely accepted that a single cell type, the neuroepithelial cell (NEC) is the predominant peripheral chemoreceptor in fish (see reviews by Jonz, 2018; Jonz and Nurse, 2008;; Jonz et al., 2015; Jonz et al., 2016; Milsom, 2012; Perry and Tzaneva, 2016; Porteus et al., 2012; Zachar and Jones, 2012). Originally described in a variety of fish species and proposed as O_2 chemoreceptors in rainbow trout gill more than 35 years ago (Dunel-Erb et al., 1982), it was not until 2004 that the gill filament NECs of zebrafish were characterized definitively as O_2 sensors (Jonz et al., 2004). Shortly thereafter, an O_2 sensory function was attributed to the NEC’s of channel catfish gills (Burleson et al., 2006). In rainbow trout, the NEC’s detect elevated ammonia levels (Zhang et al., 2011) and their stimulation by elevation of internal ammonia is thought to contribute to hyperventilation, which aids in whole body ammonia clearance. It is likely that the ventilatory responses to lactate mentioned earlier in *Pangasius* also arise from a sub-population of NECs distributed throughout the gills. Several NEC types have been identified that respond to different stimuli or groups of stimuli, including O_2, CO_2, H^+, NH_4^+, CO, NO and H_2S (Perry and Tzaneva, 2016).

295 Thus in zebrafish, a subset of the gill filament NEC's are dual sensors of
296 O_2 and CO_2 (Abdallah et al., 2015; Qin et al., 2010) and appear to be functionally
297 analogous to the chemosensory cells of the mammalian carotid body, the glomus
298 (Type I) cells, which are also known to be bimodal sensors of O_2 and CO_2.
299 Although functionally analogous to the carotid body glomus cells, the zebrafish
300 NECs may not be homologous to the glomus cells of mammals (Hockman et al.,
301 2017). Instead, the NECs of zebrafish appear to arise from embryonic endoderm
302 whereas the glomus cells are derived from embryonic ectoderm cells of the
303 neural crest (Hockman et al., 2017). Therefore, contrary to our earlier
304 understanding of the evolution of vertebrate peripheral chemoreceptors (Milsom
305 and Burleson, 2007), it is possible that the fish gill NECs are not the evolutionary
306 precursors of the glomus cells of the mammalian carotid body as thought
307 previously. Rather, it is possible that the NEC's of fish are the evolutionary
308 precursors of the pulmonary neuroendocrine cells of the lung airways of higher
309 vertebrates (reviewed by Cutz and Jackson, 1999), which also are derived from
310 endoderm during development.
311 The number of adult species in which NECs have been identified in the gill
312 of water-breathers or the skin of amphibious fishes is increasing steadily
313 (Coolidge et al., 2008; Dunel-Erb et al., 1982; Porteus et al., 2012; Porteus et al.,
314 2014; Regan et al., 2011; Saltys et al., 2006; Zaccone et al., 2006; Zaccone et
315 al., 2017). Indeed, NEC's may be ubiquitous in fish although their location may
316 vary between the gill in water-breathers and the skin or other air-breathing
317 organs in amphibious or air-breathing species (Zaccone *et al*., 2006). Moreover,

318 the location of NECs may differ depending on species and developmental age.
319 For example, in zebrafish larvae, the NECs initially are situated on the skin (Jonz
320 and Nurse, 2005) but as the gills develop, the numbers of cutaneous NECs
321 decline while the numbers in the gills increase, eventually becoming the
322 predominant site of NEC localization at about two weeks post-fertilization
323 (Coccimiglio and Jonz, 2012; Jonz and Nurse, 20003). In adult fish, the branchial
324 NECs are found preferentially within the distal region of filaments where they lie
325 in close proximity to the efferent filament artery (the blood vessel that exits the
326 gill filament) and thus on the leading edge of the filament incident to the inspired
327 water current flowing over the gills (Hughes, 1966; Hughes and Morgan, 1973).
328 As such, the filament NECs are situated ideally to sense respiratory gases both
329 from the external environment (inspired water) and the blood leaving the gill
330 (Jonz and Nurse, 2003). In several species examined to date, the NECs also are
331 found scattered on the respiratory lamellae (Bailly et al., 1982; Coolidge et al.,
332 2008; Dunel-Erb et al., 1982; Jonz and Nurse, 2003; Tzaneva and Perry, 2010)
333 (Table 1). Lamellar NECs are smaller and appear more spherical than filament
334 NECs (Jonz and Nurse, 2003). On the lamellae, the NECs are usually exposed
335 to the external environment making them suitable for sensing external changes in
336 respiratory gases (Jonz and Nurse, 2003; Tzaneva and Perry, 2010).
337
338 **Table 1.** A summary of neuroepithelial cell distribution and their intracellular
339 neurochemicals in adult and larval fish.

Species and developmental	**NEC**	**Neuroche**	**References**

stage	location	micals	
Lesser spotted dogfish (*Scyliorhinus canicula*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
European eel (*Anguilla anguilla*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
European perch (*Perca fluviatilis*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
Smallmouth bass (*Micropterus dolmieu*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
Black bullhead catfish (*Ictalurus melas*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
Rainbow trout (*Oncorhynchus mykiss*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
Pikeperch (*Sander lucioperca*); adult	Gill filament	Serotonin	Dunel-Erb et al., 1982
Zebrafish (*Danio rerio*); adult	Gill filament, lamella	Serotonin	Jonz and Nurse, 2003
Zebrafish (*Danio rerio*); larva	Skin	Serotonin	Jonz and Nurse, 2006
Zebrafish (*Danio rerio*); larva	Skin	Hydrogen sulphide	Porteus et al., 2014
Zebrafish (*Danio rerio*); adult, larva	Skin, gill filament	Serotonin, nitric oxide	Porteus *et al*., 2015
Goldfish (*Carassius auratus*); adult	Gill filament, lamella	Serotonin	Saltys et al., 2006
Trairão (*Hoplias lacerdae*), adult	Gill filament, lamella	Serotonin	Coolidge et al., 2008
Traira (*Hoplias malabaricus*), adult	Gill filament, lamella	Serotonin	Coolidge et al., 2008
Mangrove rivulus (*Kryptolebias marmoratus*), adult	Gill filament, skin	Serotonin, acetylcholine?	Regan et al., 2011
Sockeye salmon (*Oncorhynchus nerka*), adult	Gill filament	Serotonin	Porteus et al., 2012
Medaka (*Oryzias latipes*), adult	Gill filament	Serotonin	Porteus et al., 2012
Mudskipper, (*Periophthalmodon schlosseri*), adult	Gill filament, lamella	Serotonin, acetylcholine?, catecholamines?	(Zaccone et al., 2017)
Brown trout (*Salmo trutta*)	Gill filament	Serotonin	(Zaccone et al., 1992)
Brown bullhead catfish (*Ictalurus nebulosus*), adult	Gill filament	Serotonin	Zaccone et al., 1992

African lungfish (*Protopterus annectens*), adult	Gill lamella	Serotonin	Zaccone et al., 1992
Bowfin (*Amia calva*), adult	Gill filament, lamella	Serotonin, acetylcholine?	Porteus et al., 2015

Cellular mechanisms of CO_2 sensing by NECs

In zebrafish NECs, the cellular responses to hypercapnia and hypoxia are similar, consisting of membrane depolarization (Qin et al., 2010) and an associated increase in the levels of intracellular free Ca^{2+} ($[Ca^{2+}]i$) (Abdallah et al., 2015). Unlike in mammalian glomus cells, however, the increase in $[Ca^{2+}]i$ in zebrafish NECs exposed to high CO_2, is derived exclusively from intracellular storage compartments with no contribution from extracellular stores (Abdallah et al., 2015). Similar to hypoxia, the membrane depolarization associated with hypercapnia is derived from an inhibition of K^+ flux through background K^+ channels (Qin et al., 2010) that are influenced by changes in pHi and possibly extracellular pH (pHe). Thus, Qin et al. (2010) demonstrated that membrane depolarization persisted during isohydric hypercapnia (i.e. increased PCO_2 at constant pH) and that its rate of development and final magnitude were decreased by CA inhibition using acetazolamide (Maren, 1967).

Interestingly, the sensing mechanisms underlying membrane depolarization (Qin et al., 2010) and the increase in $[Ca^{2+}]i$ (Abdallah et al., 2015) are dissimilar. For example, while the membrane depolarization appears to be linked to changes in pHi, the increase in $[Ca^{2+}]i$ is associated with changes in pHe. Abdallah et al. (2015) observed that isohydric hypercapnia or intracellular acidification (without a change in pHe) did not affect cytosolic Ca^{2+} levels.

Moreover, the rise in $[Ca^{2+}]i$ was unaffected by CA inhibition. Therefore, in zebrafish NECs, there can be uncoupling between membrane depolarization and $[Ca^{2+}]i$ changes. The results also suggest that several categories of K^+ channels are present in NECs with some responding to changes in pHi and others responding to changes in pHe. The channels being controlled by pHe are likely members of the Twik-related acid-sensitive K^+ channel (TASK) family (Peña-Munzenmayer et al., 2014). Zebrafish have two specific TASK-2 channel paralogs encoded by kcnk5a (TASK-2) and kcnk5b (TASK2b). Using antisense gene silencing (morpholinos), it was demonstrated that the increases in cardiac and breathing frequencies associated with hypercapnia in zebrafish larvae were blunted in fish experiencing either TASK-2 and/or TASK-2b knockdown (Koudrina, 2017).

The NECs of adult zebrafish gill (Qin et al., 2010) and skin of 4 dpf larvae (Miller et al., 2014) exhibit CA immunoreactivity. The slowing of CO_2-mediated intracellular acidification and attenuation of the membrane depolarization in zebrafish NECs experiencing inhibition of CA activity is reflected by a decreased reflex cardiovascular response to hypercapnia in larvae *in vivo*. For example, Miller et al. (2014) demonstrated that inhibition of CA activity using acetazolamide or following gene knockdown of the cytosolic CA isoform (CAc; Esbaugh et al., 2005) led to a blunting of the tachycardia that is typically observed in zebrafish larvae exposed to hypercapnia. No studies, however, have yet addressed the role of CA in the ventilatory response of fish to hypercapnia.

Although direct data are lacking, it is believed that the increased levels of $[Ca^{2+}]i$ accompanying hypercapnic activation of NECs leads to the exocytosis of neurotransmitter(s) and activation of afferent neurons that ultimately promotes the ensuing downstream cardiorespiratory reflex responses. The specific neurotransmitter(s) secreted by NECs during hypercapnic stimulation is unknown but the most likely candidate is serotonin (5-HT) given that it is the principal neurochemical contained within serotonergic NECs of the gill and skin (reviewed by Porteus et al., 2012; see Table 1).

We must emphasize that although it is largely assumed based on *in vitro* studies and indirect correlative data from intact fish (Miller et al., 2014) that the NECs are functioning *in vivo* as CO_2 chemoreceptors at least in zebrafish, there is little, if any, direct evidence to support this view. The absence of direct *in vivo* data, in part, reflects the technical limitations associated with conducting electrophysiological recordings on NECs *in situ* and the absence of a viable loss-of-function model whereby cardiorespiratory responses to elevated CO_2 can be assessed in the absence of functional NECs.

Central CO_2/H^+ sensing

Cardiorespiratory reflexes

The evidence suggests that for most fish, CO_2-initiated cardiorespiratory reflexes arise exclusively from stimulation of branchial chemoreceptors sensitive primarily to changes in the CO_2 tension of water flowing over the gills, and not in blood perfusing the gills (see section on “Peripheral CO_2 sensing:

406 Cardiorespiratory reflexes, sites of chemoreception and underlying cellular
407 mechanisms"). There are data, however, that also suggest that changes in
408 arterial levels of CO_2/pH contribute to cardiorespiratory reflexes, particularly in
409 air-breathing fish. Much of the data is equivocal and remains open to
410 interpretation.

411 *Hagfish and lampreys*

412 Exposure to acute hypercapnia causes an increase in ventilation in
413 hagfish (Perry et al., 2009b). There are no studies, however, specifically
414 addressing whether the sites of respiratory chemoreception are peripheral or
415 central in this group. In studies using the isolated brainstem-spinal cord
416 preparation of lamprey (*Lampetra fluviatilis*), the periodic respiratory discharge
417 indicative of 'fictive' breathing (discharge in respiratory motorneurons) decreased
418 when the bicarbonate concentration of the bathing medium was increased in 4 of
419 7 preparations (concentrations not given). It also increased in 3 of 8 preparations
420 when half the bicarbonate was titrated with HCl (Rovainen, 1977). The data were
421 sufficiently equivocal that it was difficult to draw any firm conclusion. However,
422 the data do suggest that, while there were pH sensitive cells in the isolated
423 lamprey brain that could induce respiratory discharge, they most likely did not
424 account for the hypercapnic ventilatory responses seen in intact lamprey.

425 *Water breathing bony fishes*

426 Studies have been carried out in several species where the PCO_2/pH of
427 the extradural fluid surrounding the brain or within the fourth ventricle was
428 manipulated, yielding equivocal results. In studies on tench (*Tinca tinca*) the

injection of small volumes (0.2 – 1.0 μl) of pH 7.6 solution into areas of the posterior medulla from which respiratory related activity could be recorded, increased the amplitude of respiratory movements in some instances, and caused cessation of respiratory movements in others (Hughes and Shelton, 1962). In rainbow trout and tambaqui (*Colossoma macropomum*), such perfusions were completely without effect (Burleson et al., 1992; Reid et al., 2000).

The sum of these data suggests that central respiratory chemoreceptors sensitive to changes in CO_2/H^+ are unlikely in strictly water breathing fish.

Air breathing bony fishes

The evidence for the existence of central CO_2/pH chemoreceptors in air breathing teleost fish is also equivocal. In many of these species, gill ventilation initially is stimulated by low levels of environmental CO_2 but progressive hypercapnia leads to an inhibition of gill ventilation and a stimulation of air breathing (Johansen, 1970; Shelton et al., 1986; Smatresk, 1988). In other species, aquatic hypercapnia fails to produce any changes in gill ventilation (Johansen, 1966; McMahon and Burggren, 1987; Thomsen et al., 2017) or in air-breathing frequency (Lomholt and Johansen, 1974). In clown knifefish (*Chitala ornata*), aquatic hypercapnia induced a significant increase in air-breathing frequency without having any effect on gill ventilation (Perry et al., 2008), as did injection of CO_2-enriched gas into the air-breathing organ (Tuong et al, 2018; Tuong et al., 2019).

Similar to the rainbow trout described above (Peripheral Chemoreseption - Sites of Chemoreception), during recovery from the acidosis incurred as a result of exhaustive exercise in spotted gar, both branchial ventilation and air-breathing remained elevated and the prolonged recovery (4–8 h) was tightly correlated to removal of the post-exercise acidosis (Burleson et al., 1998).

In the jeju (*Hoplerythrinus unitaeniatus*), complete branchial denervation eliminated all ventilatory responses (gill and air breathing) to hypercapnia (Bojink et al., 2010) indicating that CO_2/H^+ sensitive receptors reside exclusively in the gills. In the clown knifefish, however, exposure to both hypercapnia and acetazolamide (to increase CO_2 retention and elevate arterial PCO_2) post-denervation of the gills still produced significant air-breathing responses (Tuong et al., 2019).

The existence of central CO_2/H^+ chemoreceptors in bowfin (*Amia calva*) has also been investigated obtained by perfusing mock extradural fluid containing elevated levels of CO_2 or $[H^+]$ through the cranial space in the medullary region of conscious animals. However, these perfusions were without effect on air breathing or gill ventilation (Hedrick et al., 1991). It has also been shown that superfusion of the isolated brainstem-spinal cord preparation from the Alaska blackfish (*Dallia pectoralis*) with artificial cerebrospinal fluid with elevated levels of $CO_2/[H^+]$ had no effect on fictive air-breathing (Hoffman et al., 2009).

Interestingly, two other studies using similar isolated brainstem-spinal cord preparations in longnose gar (*Lepisosteus osseus*) (Wilson et al., 2000) and Siamese fighting fish (*Betta splendens*) (Corcoran et al., 2007) did report

474 increases in fictive air breathing (but not fictive gill breathing) in response to
475 increases in superfusate $CO_2/[H^+]$.
476 *Air Breathing lobe-finned fishes*
477 This group includes all of the extant lungfishes (*Protopterus*, *Lepidosiren*
478 and *Neoceratodus* species). Some species use air breathing to supplement O_2
479 uptake while the gills (and/or skin) remain the primary site for CO_2 excretion,
480 other species rely completely on air breathing for gas exchange (Graham, 1997).
481 As the reliance on air-breathing increases, the functional surface area of the gills
482 is reduced with a consequent increase in arterial PCO_2 (Perry et al., 2009a).
483 While it has been shown that the African lungfish *Protopterus annectens*
484 responds to aerial hypercapnia with pronounced pulmonary hyperventilation
485 (Babiker, 1979), similar treatment had no effect on ventilation in other species
486 (the slender lungfish *P. dolloi*, and the marbled lungfish *P. aethiopicus* as well as
487 the South American lungfish, *Lepidosiren paradoxa*) (Burggren, 1979; Jesse et
488 al., 1967; Johansen et al., 1967, Johansen et al., 1968; Lomholt and Johansen,
489 1974; Perry et al., 2008; Sanchez and Glass, 2001) even though it produced a
490 respiratory acidosis (Perry et al., 2005). While these results suggest that this
491 group of fishes also lacks any internal CO_2/H^+ sensitive respiratory
492 chemoreceptors, the story may not be this straightforward. It has also been
493 shown that *L. paradoxa* possesses CO_2 sensitive airway receptors (DeLaney et
494 al., 1974). These receptors when stimulated by elevated CO_2 inhibit ventilation.
495 Thus, giving lungfish CO_2 to breathe, to simulate elevated levels of arterial CO_2
496 arising either from uptake from hypercapnic water or from metabolically produced

497 CO_2, would mask the effects of any central CO_2/H^+ stimulation. In support of this,
498 it has been shown that these fish exhibit a “post-hypercapnic hyperpnea”
499 (Sanchez and Glass, 2001). That is, when animals subsequently return to
500 breathing normocapnic air, inspired CO_2 levels fall immediately while arterial
501 levels of CO_2 fall slowly as whole body CO_2 stores are eliminated. Thus, the
502 inhibitory effect of elevated airway CO_2 is removed, while systemic CO_2 levels
503 remain elevated, as does air-breathing frequency (see Milsom et al., 2004 for a
504 review of this phenomenon) suggesting that this species, at least, does possess
505 internal CO_2/H^+ receptors. Consistent with these data, it has been shown that
506 pulmonary ventilation increases in *L. paradoxa* in response to independent
507 changes in both CO_2 and pH of cerebrospinal fluid indicating that the internal
508 receptors reside in the central nervous system (Amin-Naves et al., 2007a; Amin-
509 Naves et al., 2007b; Sanchez et al., 2001). These are the only unequivocal data
510 in support of the presence of central CO_2/pH chemoreceptors. While similar
511 studies have yet to be performed on other lungfish species, the African lungfish
512 *P. annectens* also can adjust branchial and/or pulmonary ventilation
513 appropriately to correct blood acid–base disturbances arising from arterial
514 infusions of $NaHCO_3$ or NH_4Cl (reviewed in Perry and Gilmour, 2006). These
515 findings support the suggestion that central CO_2/H^+ sensitive central
516 chemoreceptors are common to all lungfish species.
517 *Cellular mechanisms of central CO_2 sensing*
518 Given the equivocal nature of the evidence in support of central CO_2/H^+
519 chemoreceptors in fish, it should not be surprising that there has been no work

done to date analyzing the possible mechanisms of central CO_2 sensing. The only group in which this has been studied in any detail is the mammals. In mammals, chemosensitive neurons are spread among numerous brain stem regions, and neurons from different regions have different levels of chemosensitivity. Recent evidence indicates that the retrotrapezoid nucleus (RTN) may be of particular importance. Two non-mutually exclusive mechanisms have been suggested to explain the sensitive response of RTN neurons to CO_2. Changes in pHi could excite RTN neurons directly mediated by the intrinsic acid sensitivity of subsets of potassium channels, or indirectly via specialized proton receptors and intracellular messengers. Alternately, CO_2 may activate RTN neurons by causing the surrounding glia to release ATP (reviewed in Guyenet 2012). The signaling mechanisms for chemosensitivity at other sites may also involve changes of pHe, $[Ca^{2+}]i$, gap junctions, oxidative stress, $[HCO_3^-]$, or PCO_2. The normal target for these signals is generally believed to be a variety of K^+ channels as well as Ca^{2+} channels (reviewed in Putnam et al., 2004).

Cellular Acid/Base sensing

The peripheral and central CO_2/H^+ chemosensing mechanisms described in the previous sections mediate cardiorespiratory processes through neural pathways. This section describes A/B sensing mechanisms that modulate cellular physiology in response to local fluctuations in CO_2, pH and $[HCO_3^-]$, without the need for neuronal or hormonal inputs. Some of these A/B sensing mechanisms can contribute to general cellular homeostasis, for example, by sensing

543 metabolic CO_2 and H^+ production and regulating pHi or gene expression. In
544 addition, A/B sensing mechanisms in specialized cells can modulate organ and
545 whole animal physiology. In fact, peripheral and central chemosensing must
546 ultimately rely on similar cellular A/B sensing mechanisms to trigger action
547 potentials in response to CO_2/H^+.

548 In fish, specialized ion-transporting cells (ionocytes) located in the gill are
549 responsible for regulating blood plasma A/B status. Broadly speaking, gill
550 ionocytes excrete excess H^+ in exchange for environmental Na^+ to compensate
551 systemic acidosis, and excrete excess HCO_3^- in exchange for Cl^- to compensate
552 systemic alkalosis. These processes also involve accumulation of HCO_3^- and H^+,
553 respectively. As explained in detail below, A/B sensing of blood plasma of
554 elasmobranch fishes is mediated by the HCO_3^--sensing enzyme soluble adenylyl
555 cyclase (sAC; adcy10) inside gill ionocytes (Roa and Treguerres 2016;
556 Tresguerres et al 2010). sAC is also present in gill ionocytes of bony fish where it
557 likely contributes to blood A/B and ionic regulation (Salmerón et al, unpublished);
558 however, experimental confirmation still awaits.

559 The end result of branchial H^+ and HCO_3^- excretion and absorption is the
560 maintenance of a relatively stable A/B status in blood plasma that lessens the
561 amount of energy necessary for regulating pH_i in the rest of the cells. However,
562 those cells must still regulate (and therefore, be able to sense), the A/B status in
563 the cytosol. These mechanisms have not been characterized in fish, but they are
564 most likely similar to those described in coral (Barott et al., 2017) and mammals
565 (Tresguerres et al., 2010a).

566 Additional sites that require A/B sensing in fish include extracellular
567 compartments such as the cerebrospinal fluid, otolith endolymph, and intestinal
568 fluid. With the exception of the latter where sAC has again been implicated in
569 sensing elevated HCO_3^- (Tresguerres et al 2010a), the A/B sensing mechanisms
570 remain unknown. Importantly, the A/B conditions in those external, extracellular,
571 and intracellular sites can vary widely, implying the presence of A/B sensing
572 mechanisms specifically tuned for each site.
573

574 ## Molecular A/B sensors

575 While the structure of all proteins is affected by pH to a certain extent, a
576 molecular A/B sensor must also be able to regulate and coordinate the activity of
577 downstream effector proteins in a manner conducive to a homeostatic response
578 (Figure 2).
579

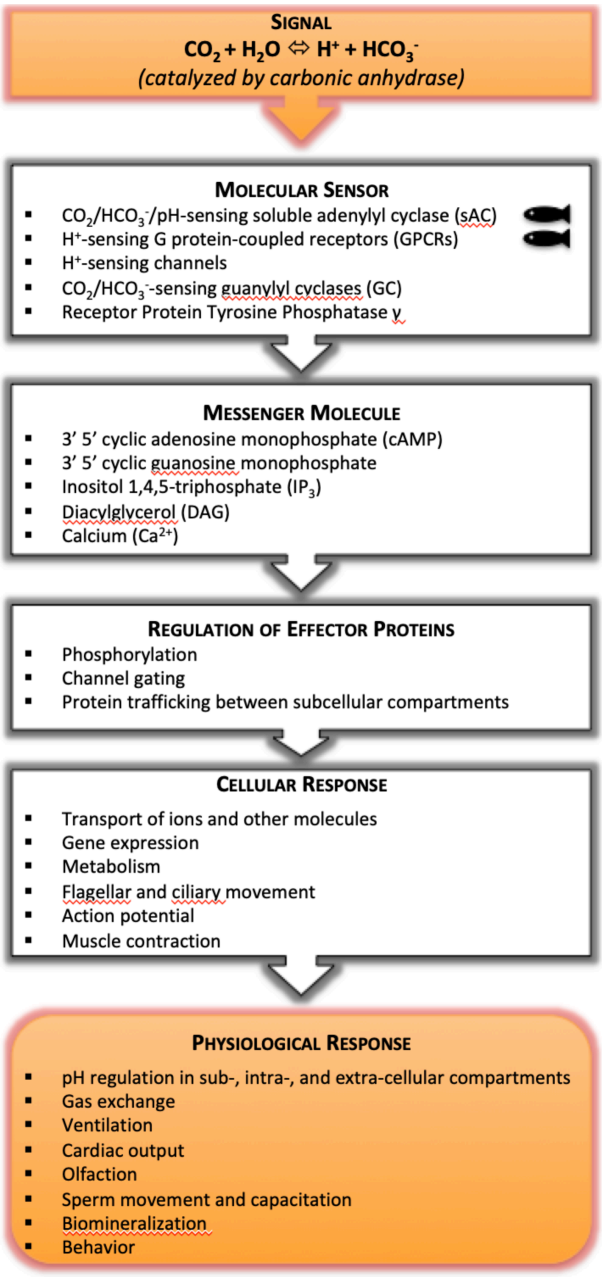


Figure 2. Generalized Acid/Base sensing mechanisms. The fish icons indicate Molecular Sensors that have been characterized in fish. The other elements in this figure (Messenger Molecules, Regulation of Effector Proteins, Cellular and Physiological Responses) are conserved throughout the Animal kingdom.

587 In animals, molecular A/B sensors fall into two broad categories: (1)
588 enzymes that induce the production of messenger molecules [e.g. cyclic
589 adenosine monophosphate (cAMP), cyclic guanylyl monophosphate (cGMP),
590 inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), Ca^{2+}] and whose activity is
591 modulated by one or more A/B parameter, and (2) pH-sensitive ion channels that
592 modulate the membrane potential of chemosensing neurons, and thus action
593 potentials and nerve impulse conduction. Multiple types of A/B molecular sensors
594 might cooperate together as part of a cellular A/B sensing mechanism to allow
595 the discrimination between the different types of A/B stress, and regulate A/B
596 status around specific set points in different organs, cells, and subcellular
597 compartments.

598 At 25°C in pure water, the reversible equilibrium between H_2O, CO_2, H^+,
599 HCO_3^- and CO_3^{2-} is pH-dependent and follows the equation:

$$CO_2 + H_2O \underset{\mathbf{CA}}{\overset{pK_1 = 3.6}{\Leftrightarrow}} H_2CO_3 \overset{pK_2 = 6.35}{\Leftrightarrow} HCO_3^- + H^+ \overset{pK_3 = 10.33}{\Leftrightarrow} CO_3^{2-} + 2H^+$$

600

601 As a result, at the physiological pH of most internal fluids the dominant
602 carbon species is by far HCO_3^-. Furthermore, although the interconversion
603 between CO_2 and H_2O and H_2CO_3 is relatively slow, catalysis by CA ensures the
604 almost instantaneous equilibration of CO_2 with HCO_3^- and H^+. An important
605 implication for the purposes of A/B sensing is that HCO_3^- and H^+ can be used as
606 proxies for CO_2 levels. Indeed, the vast majority of A/B molecular sensors
607 identified to date sense HCO_3^- or H^+. A variety of molecular A/B sensing

mechanisms have been identified in mammals, insects, plants, yeast and bacteria (Linder and Schultz, 2003; Steegborn, 2014; Tresguerres et al., 2010a; Tresguerres et al., 2011). However in fish, the only molecular A/B sensors identified to date are the CO_2/pH/HCO_3^- sensor sAC (Figure 3A) and the H^+-sensing G-protein coupled receptors (GPCRs) OGR1, GPR4, and G2A (Ichijo et al., 2016; Mochimaru et al., 2015) (Figure 3B).

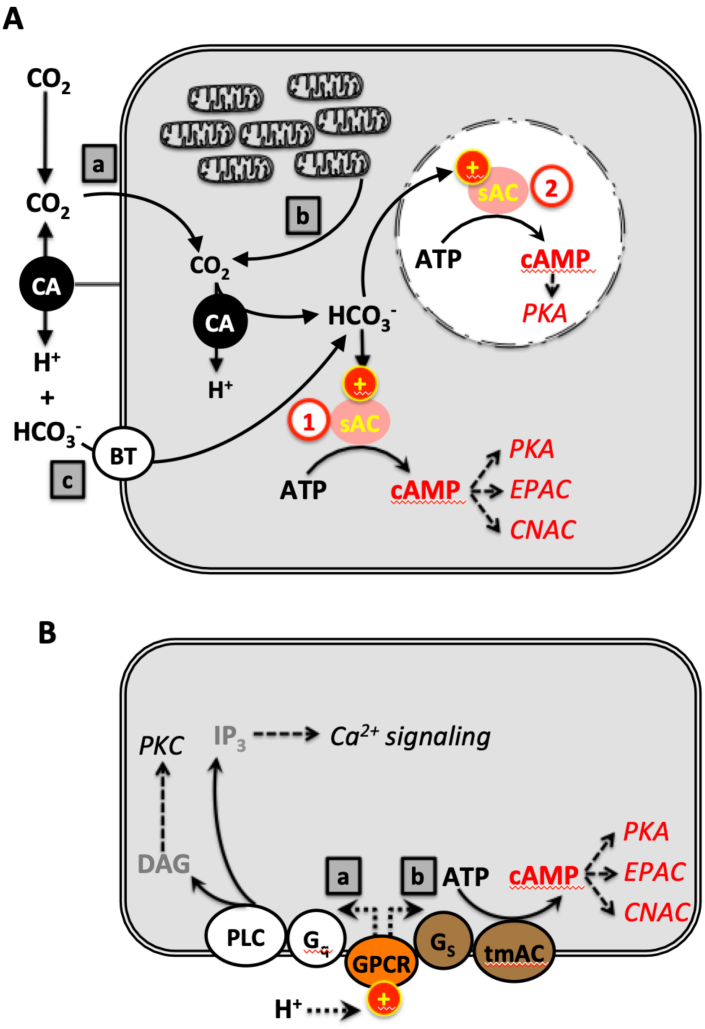


Figure 3. Cellular and molecular mechanisms characterized in fish. **A)** Soluble adenylyl cyclase (sAC) in the cytoplasm (1) and nucleus (2). sAC may be

618 stimulated by intracellular HCO_3^- derived from CO_2 from [a] external and [b]
619 metabolic origin after being hydrated in a reaction catalyzed by carbonic
620 anhydrase (CA). [c] sAC may also be stimulated by HCO_3^- that enters the cell
621 through HCO_3^- transporting proteins (BT). sAC-produced cAMP mediates
622 physiological functions in the nucleus and cytosol (and possibly other subcellular
623 compartments) through protein kinase A (PKA), exchange protein activated by
624 cAMP (EPAC), and cyclic nucleotide activated channels (CNAC). **(B)** H^+-sensing
625 G protein-coupled receptors (GPCRs). Extracellular H^+ (blood plasma, interstitial
626 fluids) activates H^+-sensing GPCRs. [a] If linked to protein Gq, it leads to
627 phospholipase C (PLC) activation, production of diacylglycerol (DAG) and inositol
628 triphosphate (IP_3), and modulation of downstream proteins by protein kinase C
629 (PKC) and Ca^{2+} release from intracellular stores, respectively. [b] If linked to
630 protein Gs, it stimulates transmembrane adenylyl cyclase (tmAC) to produce
631 cAMP leading to subsequent PKA, EPAC, and/or CNAC modulation.
632
633 **CO_2/pH/HCO_3^- sensing sAC**
634 sAC is a cAMP-producing enzyme that is directly activated by HCO_3^-
635 (Buck et al., 1999; Chen et al., 2000). However, due to the interrelationship with
636 CO_2 and H^+ explained above, sAC can also act as a sensor for both internal and
637 external CO_2 and pH (reviewed in Tresguerres et al, 2011) (Figure 3A). Originally
638 identified and characterized from rat testis, sAC is related to HCO_3^--sensing
639 adenylyl cyclases from cyano- (Buck et al., 1999; Chen et al., 2014; Steegborn,
640 2014) and chloroflexi bacteria (Kobayashi et al., 2004), and was later molecularly

641 and biochemically characterized in phylogenetic diverse organisms including
642 coral (Barott et al., 2017), sea urchin (Nomura et al., 2005), shark (Tresguerres
643 et al., 2010c), and bony fish (Salmerón et al. unpublished). Accordingly, sAC is
644 now accepted as an evolutionarily conserved pH/CO_2/HCO_3^- sensor.
645 *sAC in elasmobranch gills senses blood A/B*
646 In elasmobranch fishes, sAC is abundantly expressed in the cytoplasm of
647 acid- and base-secreting cells where it acts as a sensor of blood A/B status (Roa
648 and Tresguerres, 2016; Roa and Tresguerres, 2017, Tresguerres et al, 2010c).
649 The mechanism is shown in figure 4, and is as follows: during a post-feeding
650 blood alkalosis, plasma HCO_3^- is dehydrated into CO_2 by CAs in red blood cells
651 and at the basolateral membrane of gill pillar cells. CO_2 then diffuses into the
652 base-secreting cells, where it is rehydrated into HCO_3^- and H^+ by cytosolic CAs.
653 The elevated HCO_3^- is sensed by sAC, resulting in increased cAMP production
654 that triggers the translocation of vesicles containing V-type H^+-ATPase (VHA)
655 from the cell's cytoplasm to the basolateral membrane, and of vesicles containing
656 the anion exchanger pendrin to the apical membrane [although the involvement
657 of sAC in latter has not been directly established (Roa et al 2014)]. Cells with
658 apical pendrin and basolateral VHA are thus activated to secrete HCO_3^- and
659 absorb H^+ and Cl^-, which effectively counteracts the blood alkalosis. This A/B
660 sensing mechanism also takes place in isolated gill fragments (Tresguerres et
661 al., 2010c) and isolated gill cells (Roa and Tresguerres, 2016), indicating A/B
662 sensing takes place locally in each gill base-secreting cell and is therefore

independent of the peripheral and central chemosensors described in previous sections.

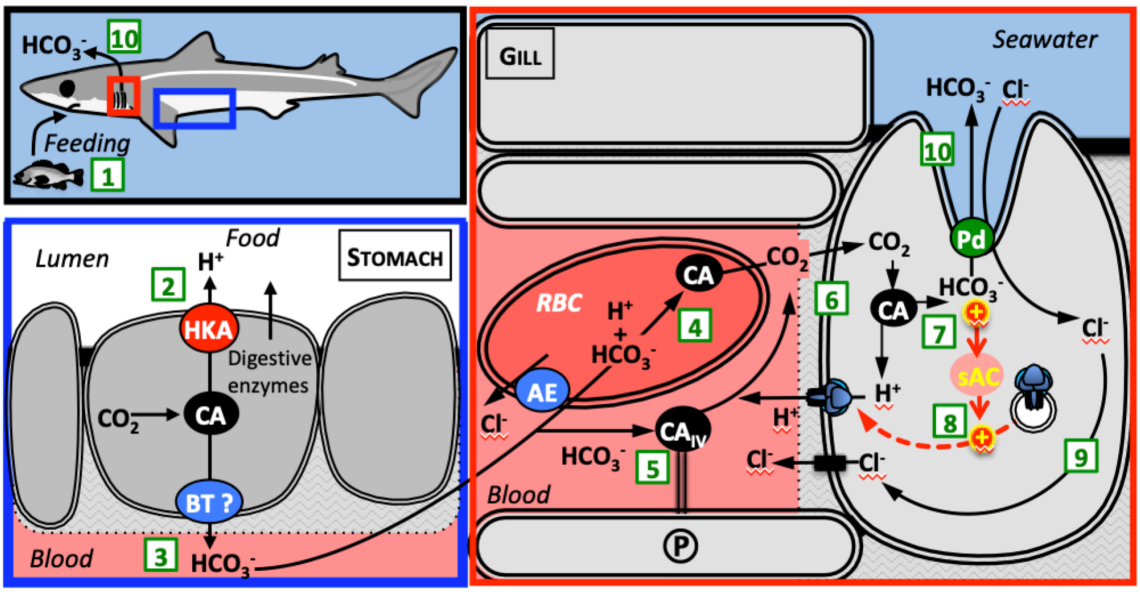


Figure 4. Sensing of blood alkalosis by soluble adenylyl cyclase in elasmobranch gill cells. **Black Box**: (1) Sharks feed opportunistically on a variety of fish and invertebrate prey. **Blue Box:** (2) Gastric H^+/K^+-ATPase (HKA), not to be confused with VHA, helps secrete HCl into the stomach lumen and, together with digestive enzymes, digest the food. (3) At the same time, HCO_3^- is absorbed into the blood through unidentified HCO_3^- transporters (BT), which induces a blood alkalosis. **Red Box:** HCO_3^- travels in blood plasma and also enters red blood cells (RBC) *via anion* exchangers (AE). (4) Inside RBCs, intracellular carbonic anhydrase (CA) hydrates HCO_3^- into CO_2. (5) In addition, extracellular CA IV 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^-

679 stimulates soluble adenylyl cyclase (sAC), which (8) triggers the translocation of
680 cytoplasmic vesicles containing VHA (blue icon) to the cell basolateral
681 membrane. VHA then secretes H^+ into the blood. (9) A putative basolateral
682 channel brings Cl^- from VHA-rich cells into the blood. (10) Intracellular HCO_3^- is
683 secreted to seawater in exchange for Cl^- via apical pendrin (Pd)-like anion
684 exchangers. The combined action of H^+ reabsorption by VHA and HCO_3^-
685 secretion by pendrin corrects blood alkalosis. Modified from Tresguerres (2016).
686 Based on Gilmour et al. (2007), Roa et al. (2014), Roa and Tresguerres (2016),
687 Tresguerres et al. (2005, 2006c, 2007b, 2010), Wood et al. (2005, 2009). Water
688 molecules have been omitted for simplicity. The shaded areas surrounding
689 epithelial gastric and branchial cells signify connective tissue and other cell types
690 that might separate them from the blood space.
691
692 Some unknown aspects of this A/B sensing mechanism include the
693 processes that connect sAC-produced cAMP to VHA translocation (i.e. PKA or
694 EPAC, regulation of vesicle movement along microtubules), and how gill acid-
695 and base-secreting cells discriminate between the different types of A/B stress.
696 In this regard, it has been hypothesized that the coordinated action of sAC and
697 H^+ sensing GPCRs (described in the next section) may stimulate base-secreting
698 cells during metabolic alkalosis and inhibit them during acidosis, while
699 simultaneously having the opposite modulatory effect on acid-secreting cells
700 (Roa and Tresguerres, 2016). However, this model requires experimental
701 confirmation.

sAC in other elasmobranch tissues

In elasmobranchs, sAC has also been reported in rectal gland, cornea, intestine, skeletal and cardiac muscle (Roa and Tresguerres, 2017), and red blood cells (Tresguerres et al., 2014). However, the physiological roles of A/B sensing by sAC in those organs are still unknown. Immunohistochemical analysis has found sAC can be present in the nucleus of cells from diverse organs. Furthermore, cell nuclei isolated from gill and rectal gland demonstrated HCO_3^--stimulated cAMP production that is inhibited by pharmacological sAC inhibition (Roa and Tresguerres, 2017), suggesting that sAC regulates gene expression in response to A/B stress by phosphorylation of gene transcription factors as reported for mammals (Zippin et al., 2004).

sAC in bony fish

Genes coding for sAC are also present in bony fish (Tresguerres, 2014; Tresguerres et al., 2014). Recent research has found that rainbow trout possesses multiple sAC splice variants and protein isoforms (Salmerón et al., unpublished). Interestingly, some of the sAC isoforms are preferentially located in the cytoplasm while others are found in the nucleus or associated with the Golgi apparatus. This suggests different sAC isoforms sense A/B and regulate specific physiological functions in each subcellular compartment.

sAC has also been detected in the head, midpiece and flagella of Atlantic salmon sperm (Schalburg et al., 2018), and most likely regulates sperm flagellar movement and capacitation as described in mammals (Hess et al., 2005) and sea urchin (Beltrán et al., 2007; Nomura et al., 2005).

sAC in fish intestine modulates NaCl-driven water absorption

In marine bony fishes, sAC is able to sense CO_2/ HCO_3^- levels inside intestinal ionocytes and modulate transepithelial NaCl-driven water absorption (Figure 5). Intestinal water absorption is essential for osmoregulation in marine fishes, and depends on massive HCO_3^- secretion into the intestinal lumen where it can reach concentrations in excess of 100 mM (Wilson et al., 2002). This unique A/B physiology prompted studies about the potential regulatory roles of HCO_3^- and sAC on intestinal NaCl and water transport. The evidence supporting a role for sAC includes immunohistochemical detection using heterologous antibodies against shark and rat sAC (Carvalho et al., 2012; Tresguerres et al., 2010b), and a reduction in transepithelial NaCl and water absorption upon sAC inhibition (Carvalho et al., 2012; Tresguerres et al., 2010b). sAC in intestinal ionocytes is stimulated by HCO_3^- that enters from blood plasma *via* Na^+/HCO_3^- cotransporters (NBCs) and by HCO_3^- derived from CA-catalyzed hydration of metabolic CO_2. Additionally, the HCO_3^- that stimulates sAC might be derived from CO_2 buildup in the intestinal lumen. Thus, sAC in intestinal ionocytes might integrate sensory inputs for CO_2/HCO_3^- from three compartments (plasma, cells and lumen). The downstream pathway is not completely understood, but is has been proposed that sAC-produced cAMP activates PKA to modulate the activities of apical $Na^+/K^+/2Cl^-$ cotransporters (NKCCs) and basolateral Na^+/K^+-ATPases by phosphorylation (Carvalho et al., 2012; Tresguerres et al., 2010b) (Figure 5).

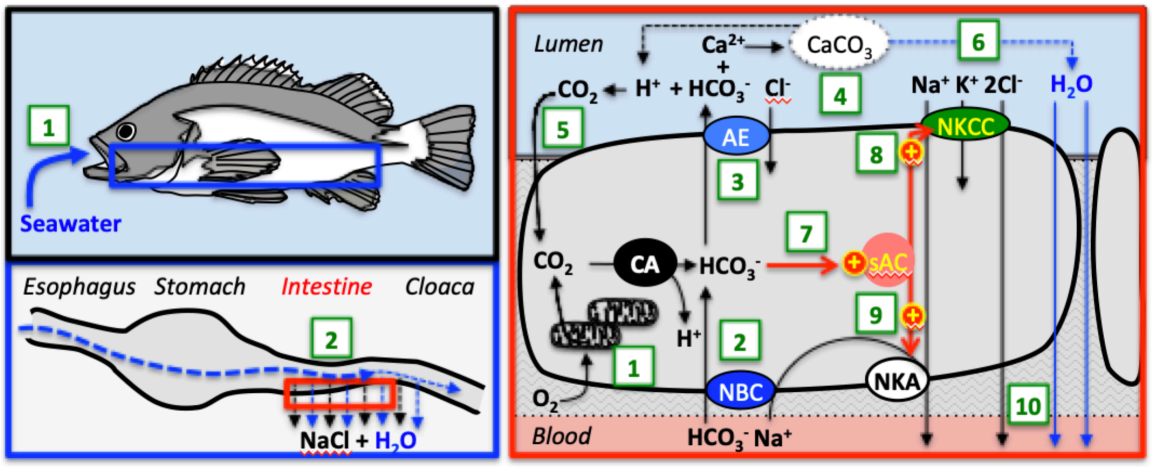


Figure 5. Acid/Base sensing by soluble adenylyl cyclase in the intestine of marine teleost fish. (1) Marine teleosts drink large amounts of seawater to counteract dehydration. (2) NaCl-mediated water absorption takes place in the intestine. In intestinal cells, the mechanism is as follows: (1) HCO_3^- is generated from carbonic anhydrase (CA)-catalyzed hydration of metabolic CO_2, and (2) imported into the cell via Na^+/HCO_3^- exchangers (NBC). (3) An apical Anion Exchanger (AE) excretes HCO_3^- into the intestinal lumen. (4) The high luminal [HCO_3^-] precipitates with Ca^{2+} (and Mg^{2+}) from the ingested seawater. (5) Precipitation of carbonates generates H^+, resulting in formation of CO_2 that diffuses into the intestinal cells and is hydrated to HCO_3^- and (6) reduces the osmolality of the fluid within the intestinal lumen. Intracellular HCO_3^- derived from those three sources stimulates soluble adenylyl cyclase (sAC). The exact downstream mechanisms are unknown, but it has been hypothesized that cAMP produced by sAC stimulates protein kinase A, which in turns activates apical $Na^+/K^+/2Cl^-$ cotransporters (NKCC) and /or Na^+/K^+-ATPase (NKA). Overall, this results in transepithelial NaCl and H_2O absorption. Modified from Tresguerres et al. (2010b). Based on Carvalho et al. (2012), Taylor et al. (2010), Tresguerres et

766 al. (2010b), Wilson et al. (2002).

767

768 *sAC in hagfish heart modulates cardiac frequency*

769 Immunohistochemical detection and pharmacological experiments have
770 also established a role for sAC in regulating Pacific hagfish cardiac frequency in
771 response to HCO_3^- fluctuations (Wilson et al., 2016). In isolated hagfish systemic
772 hearts, this mechanism contributes to bradycardia during anoxia and to
773 tachycardia during the early phase of normoxia following anoxia. *In vivo*, this
774 mechanism may induce similar responses when hagfish are feeding in anoxic
775 environments.

776 sAC was immunohistochemically detected throughout the hagfish
777 systemic heart, although not in all cells (Wilson et al., 2016). In cardiomyocytes,
778 sAC signal was more intense in defined regions (presumably sacomeric bands).
779 Based on the effect of pharmacological sAC inhibition on heart beat rate, sAC is
780 likely to be present in pacemaker cells (although these have not been identified
781 in hagfish yet). The mechanisms downstream of sAC are also unknown, but may
782 include hyperpolarization-activated cyclic nucleotide-modulated ion channels
783 (HCNs) in addition to PKA phosphorylation of multiple targets Figure 6). Similar
784 to bony fish intestine, sAC in hagfish heart likely senses HCO_3^- derived from
785 plasma that enters cardiac cells through NBCs, and from metabolic CO_2
786 production. The levels of both HCO_3^- sources decrease as a result of anaerobic
787 metabolism during anoxia, and experience a sharp peak at the onset of aerobic
788 metabolism when normoxic conditions return (Cox et al., 2011). Because the

hagfish heart lacks innervation (Greene, 1902), sAC-mediated control of cardiac rate might reflect an ancestral characteristic potentially present in basal animals and vertebrate larval stages.

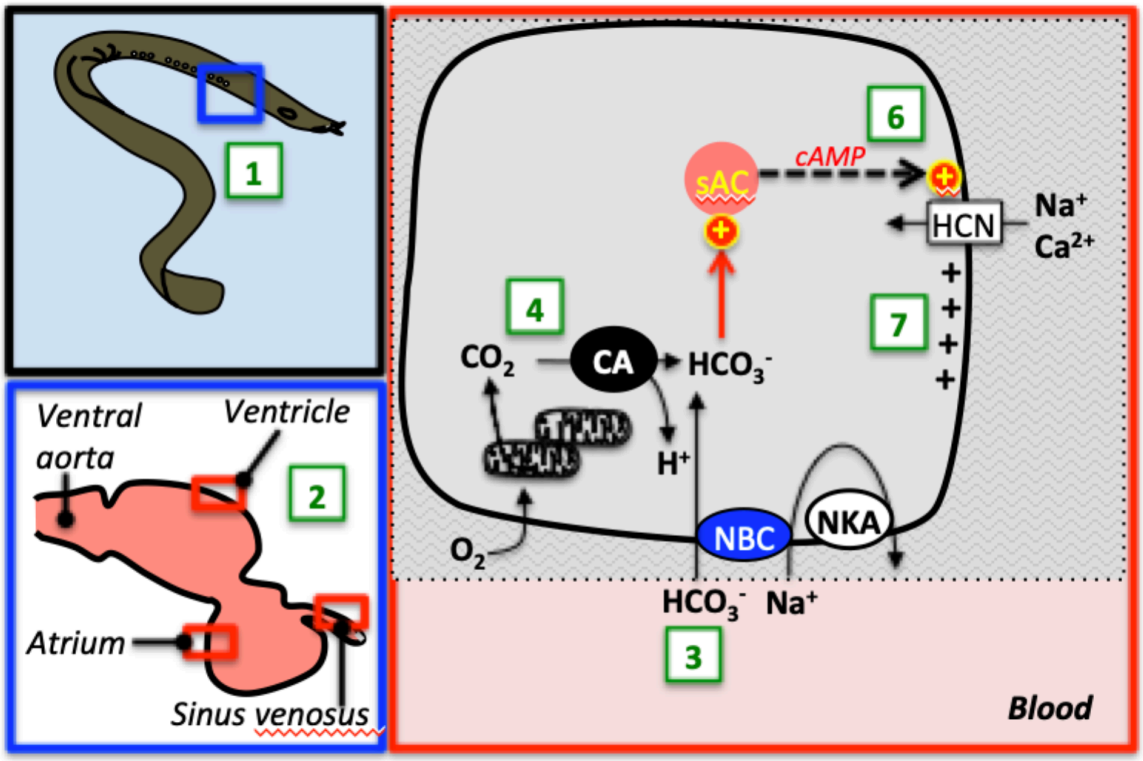


Figure 6. Acid/Base sensing by soluble adenylyl cyclase in hagfish heart. (1) The hagfish systemic heart lacks innervation so it relies on local mechanisms to regulate heart beat frequency. (2) Soluble adenylyl cyclase is present in many cells (but not all) in the atrium, ventricle, and sinus venosus. The proposed mechanisms of sAC activation in atrial and ventricular cardiomyocytes involves (3) HCO_3^- entry from blood plasma via Na^+/HCO_3^- exchangers (NBC) and (4) HCO_3^- that is generated from carbonic anhydrase (CA)-catalyzed hydration of metabolic CO_2. (6) sAC-produced cAMP is proposed to activate hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels (a type of

803 cyclic nucleotide activated channel). (7) This leads to cell membrane
804 depolarization and Ca^{2+} influx which control pacemaker activity. Based on Green
805 (1903), Wilson et al. (2013, 2016).
806
807 *Emerging patterns of A/B sensing by sAC*
808 The few studies described above have revealed some interesting
809 characteristics about A/B sensing by sAC in fish and animals in general, as well
810 as raising novel questions. One of them is that sAC proteins are tuned to the
811 typical physiological HCO_3^- levels present in each species. This is evident in their
812 $EC_{50}{}^{HCO}{}_3^-$ (the concentration of HCO_3^- that results in half-maximal cAMP
813 production), which is ~5 mM in shark, ~10 mM in coral and trout, and ~20 mM in
814 hagfish and mammals (reviewed in Tresguerres et al., 2014). Because the EC_{50}
815 is at the midpoint of the steepest part of the HCO_3^-/cAMP dose response curve,
816 this implies that minor HCO_3^- fluctuations around a set point will result in
817 relatively large changes in cAMP production, which is exactly what one would
818 predict (and desire) for a physiological A/B sensor.
819 Another emerging pattern is that sAC can act as a sensor of metabolic
820 CO_2 production as reported in fish intestine and hagfish heart, but also in coral
821 cells (Barott et al., 2017). Intriguingly, sAC is also essential for maintaining pHi
822 homeostasis in coral. Considering that corals belong to the ancestral phylum
823 Cnidaria, a universal role of sAC in this fundamental physiological function
824 certainly is a possibility.

825 A third consideration concerns the interaction between sAC and the other
826 sources of cAMP, the traditional, hormone-activated transmembrane adenylyl
827 cyclases (tmACs). This relates to the model of intracellular cAMP-signaling
828 microdomains whereby cAMP from different pools of sACs and tmACs are
829 present in discrete subcellular regions and specifically regulate effector proteins
830 within each domain (reviewed in Tresguerres and Salmeron, 2018). Fish provide
831 several examples in support of the cAMP signaling microdomain model. Indeed,
832 sAC and tmACs have opposite effects on the VHA translocation in elasmobranch
833 gill cells and on NaCl and H_2O absorption and secretion across the intestine of
834 marine bony fish, and induce different responses on hagfish heart rate despite
835 both producing the same messenger molecule, cAMP. The cAMP signaling
836 microdomain model is also relevant for A/B sensing because some of the H^+-
837 sensing GPCRs described in the next section also signal through cAMP.
838 Finally, the mechanism of A/B sensing by sAC and its downstream
839 responses in fish gill, intestine and heart provide hints about how A/B sensing
840 might take place in other cell types, physiological functions, and A/B
841 disturbances, including those associated with ocean acidification.
842
843 **H^+ sensing GPCRs**
844 A subset of G-Protein Coupled Receptors (GPCR) act as H^+ sensors in
845 mammals (reviewed in Tresguerres et al., 2010a). These GPCRs are stimulated
846 by a drop in pHe (Ludwig et al., 2003); i.e. pH in blood plasma and interstitial

847 fluids. Some of those H^+-sensing GPCRs have been described in zebrafish,
848 specifically OGR1, GPR4 (Mochimaru et al., 2015), and G2A (Ichijo et al., 2016).
849 The molecular mechanism that confers mammalian and zebrafish GPCRs
850 their H^+-sensing properties relies on conserved histidine residues that, upon
851 protonation in the physiological pH range, induce a conformational change that
852 initiates a signaling cascade (Liu et al., 2010; Ludwig et al., 2003). If linked to Gs
853 protein, this leads to tmACs stimulation, cAMP production, and modulation of
854 target effector proteins by PKA phosphorylation, EPAC, and CNACs. However if
855 linked to Gq protein, H^+-sensing GPCRs activate phospholipase C leading to
856 diacylglycerol-PKC phosphorylation and to IP_3-mediated Ca^{2+} release from
857 intracellular stores and subsequent modulation of effector proteins (Figure 2B).
858 Based on heterologous expression in mammalian cell lines, zebrafish H^+-sensing
859 GPCRs can act through all of those pathways and differentially regulate gene
860 transcription (Ichijo et al., 2016; Mochimaru et al., 2015). Those *in vitro* studies
861 suggest zebrafish OGR1, GPR4, and G2A can regulate the expression of
862 different genes; however, these functions have not been studied in native
863 zebrafish cells.
864 Zebrafish have two G2A homologs (G2A-a and G2A-b). Their mRNAs are
865 widely present throughout tissues including brain, pituitary, eye, gill, heart, gas
866 bladder, gut, gallbladder, spleen, kidney, testis, ovary, muscle and scales (Ichijo
867 et al., 2016). GPR4 and OGR1 mRNAs are present in zebrafish embryos
868 (Mochimaru et al., 2015). However, the expression of OGR1 and GPR4 in adult
869 zebrafish tissues has not been reported.

In mammals, H^+-sensing GPCRs regulate multiple physiological process in response to systemic acidosis including (but not limited to) renal H^+ secretion, angiogenesis, and tumor growth (Codina et al., 2011; Sun et al., 2010; Wyder et al., 2011; Yang et al., 2007). In zebrafish, H^+-sensing GPCRs have been studied from a biomedical perspective. However, H^+-sensing GPCRs orthologs are present in genomic and transcriptomic databases of multiple fish species, and their H^+ sensing functions likely play important physiological roles during A/B disturbances.

Conclusions and Future Directions

Decades of research have identified peripheral and to a lesser extent, central sites of CO_2 sensing in diverse fish, and established evolutionary patterns and downstream whole animal cardiorespiratory responses. More recent research has identified molecular and cellular aspects of CO_2 sensing in peripheral NECs, as well as two molecular chemosensing enzymes that can regulate the activity of effector proteins *via* posttranslational modifications: CO_2/pH/HCO_3^--sensing sAC, and H^+-sensing GPCRs. Because these molecular sensors are responsive to physiologically relevant CO_2, HCO_3^- and pH levels and are widely expressed throughout fish tissues, they are poised to sense metabolic and environmental A/B disturbances and modulate multiple homeostatic responses. However, there is still much to learn including other organs and processes under regulatory control by those sensors, as well as additional A/B sensors. In this regard, promising candidates include the pHi sensor Pyk2,

893 extracellular CO_2 sensors that rely on CA in combination with HCO_3^--sensing
894 guanylyl cyclases or H^+ gated channels (reviewed in Tresguerres et al 2010a),
895 and the recently identified extracellular CO_2/HCO_3^- sensor Protein Tyrosine
896 Phosphatase γ (Zhou et al., 2016). However, these candidate CO_2-sensing
897 mechanisms are based on mammalian systems, so their potential fish
898 counterparts must be tuned to the different A/B physiology of fishes.
899 Another interesting area of future investigation is the potential interaction
900 between molecular chemosensors. As described throughout this chapter, some
901 of them can use the same signaling pathways and therefore precise regulatory
902 mechanisms must exist to ensure adaptive signal specificity. Moreover, the ability
903 to differentiate between different types of A/B stress and conditions (i.e.
904 metabolic and respiratory acidosis and alkalosis, compensated respiratory
905 acidosis, etc.) likely requires at least two different chemosensors (see Roa and
906 Tresguerres, 2016).
907 A final intriguing question is the potential for A/B sensing mechanisms to
908 adjust their set points or mediate homeostatic responses to chronic changes in
909 environmental CO_2 levels such as those experienced in fish aquaculture facilities
910 and expected ocean acidification. The answer to this question could determine
911 fish universal or species-specific vulnerability or resilience.
912
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