

RESEARCH PAPER

Soluble adenylyl cyclase is an acid-base sensor in rainbow trout red blood cells that regulates intracellular pH and haemoglobin–oxygen binding

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Abstract

Aim: To identify the physiological role of the acid-base sensing enzyme, soluble adenylyl cyclase (sAC), in red blood cells (RBC) of the model teleost fish, rainbow trout.

Methods: We used: (i) super-resolution microscopy to determine the subcellular location of sAC protein; (ii) live-cell imaging of RBC intracellular pH (pH_i) with specific sAC inhibition (KH7 or LRE1) to determine its role in cellular acid-base regulation; (iii) spectrophotometric measurements of haemoglobin–oxygen (Hb–O₂) binding in steady-state conditions; and (iv) during simulated arterial-venous transit, to determine the role of sAC in systemic O₂ transport.

Results: Distinct pools of sAC protein were detected in the RBC cytoplasm, at the plasma membrane and within the nucleus. Inhibition of sAC decreased the setpoint for RBC pH_i regulation by ~0.25 pH units compared to controls, and slowed the rates of RBC pH_i recovery after an acid-base disturbance. RBC pH_i recovery was entirely through the anion exchanger (AE) that was in part regulated by HCO₃[−]-dependent sAC signaling. Inhibition of sAC decreased Hb–O₂ affinity during a respiratory acidosis compared to controls and reduced the cooperativity of O₂ binding. During in vitro simulations of arterial-venous transit, sAC inhibition decreased the amount of O₂ that is unloaded by ~11%.

Conclusion: sAC represents a novel acid-base sensor in the RBCs of rainbow trout, where it participates in the modulation of RBC pH_i and blood O₂ transport through the regulation of AE activity. If substantiated in other species, these

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findings may have broad implications for our understanding of cardiovascular physiology in vertebrates.

KEYWORDS

ADCY10, band 3, bicarbonate, Bohr effect, cAMP, erythrocyte, fish, phosphodiesterase, POPDC, SLC4A1, teleost

1 | INTRODUCTION

Soluble adenylyl cyclase (sAC, ADCY10) is an enzymatic source of cyclic adenosine monophosphate (cAMP), a ubiquitous signaling molecule in all cells.¹ Unlike G-protein regulated transmembrane adenylyl cyclases (tmACs; ADCY 1-9), the enzymatic activity of sAC is directly stimulated by bicarbonate (HCO_3^-) ions,² making it a putative sensor of intracellular acid-base status. sAC is primarily found in the cytoplasm, but has also been described in mitochondria and the nucleus of various mammalian cell lines.³ Therefore, the microdomain model predicts that cAMP is produced in close proximity to its downstream targets, and is spatially contained by phosphodiesterase (PDE) activity.⁴ The downstream targets of sAC-produced cAMP are an area of active research and include: (i) protein kinase A (PKA) that modulates protein function by phosphorylation; (ii) exchange protein directly activated by cAMP (EPAC) that regulates protein function through the rat sarcoma-associated protein (RAP) pathway⁵; (iii) cyclic nucleotide gated channels that regulate cellular ion gradients and membrane potential⁶⁻⁸; and (iv) the novel Popeye domain containing (POPDC) family that may regulate membrane protein trafficking and tight junction formation.⁹

Since its discovery in mammals, sAC has been found in several other eukaryote taxa, including diatoms,¹⁰ ascidians,^{11,12} cnidarians,¹³ echinoderms,¹⁴ agnathans,^{15,16} elasmobranchs,¹⁷ and teleosts.¹⁸ Functional studies in fishes have described a role of sAC as an acid-base sensor in the gills of the spiny dogfish (*Squalus acanthias*), where it mediates the recovery from a metabolic alkalosis¹⁷; in the intestine of marine teleosts, where it regulates HCO_3^- secretion and water and ion absorption^{19,20}; in the hearts of the basal Pacific hagfish (*Eptatretus stoutii*) and lampreys (*Lampetra* spp.), where it controls hypoxic heart rates^{15,16}; and in the sperm of Atlantic salmon (*Salmo salar*), where it mediates capacitation.²¹ A recent molecular characterization of sAC in the model teleost rainbow trout (*Oncorhynchus mykiss*) revealed the expression of at least six (and potentially over 20) alternative mRNA splice variants with broad tissue and subcellular distributions that may have diverse, but yet uncharacterized functions.¹⁸ The red blood cells (RBCs) of rainbow trout showed robust

expression of a single sAC isoform of ~110 kDa, which is in line with a previous report of sAC in dogfish RBCs.⁴

Within RBCs, haemoglobin (Hb) is the principal oxygen (O_2) carrier in the blood of most vertebrates. In many teleost fishes, the binding of O_2 to Hb is highly pH-sensitive, where acidic conditions decrease Hb- O_2 affinity through the Bohr effect^{22,23} and Hb- O_2 carrying capacity through the Root effect.²⁴ Teleosts take advantage of this exceptional pH-sensitivity of Hb to enhance the unloading of O_2 at their eyes,^{25,26} swim bladders,²⁷ and swimming muscles.^{28,29} However, during systemic acidoses, adrenergically stimulated sodium-proton exchangers (β -NHE) on the RBC membrane must be activated to safeguard RBC pH_i and Hb- O_2 affinity. Other pathways involved in pH_i and volume regulation in fish RBCs are the anion exchanger (AE), sodium-potassium-chloride co-transporters (NKCC) and potassium-chloride co-transporters (KCC). In fishes, the regulation of RBC pH_i and ion homeostasis have largely been studied by bulk measurements across time scales that exceed the dynamic exchanges of gases and ions in the circulation (i.e., minutes for circulatory transit and seconds for capillary transit).³⁰⁻³² In human RBCs, live-cell imaging experiments with pH-sensitive fluorophores revealed that RBC intracellular pH (pH_i) regulation is rapid and depends entirely on the AE.³³ Full recovery of pH_i after ammonia pre-pulses (a technique to induce an intracellular acidosis) was achieved in <2 min,³³ which is more than double the rate observed in mammalian mesangial cells,³⁴ type-1 carotid body cells from rats,³⁵ or ventricular myocytes from guinea pigs.³⁶ However, live-cell measurements of pH_i regulation in the nucleated RBCs of fishes have not been attempted. Given the importance of RBC pH_i for systemic O_2 transport in teleosts, there is a critical need to characterize the cellular pathways of pH_i sensing and regulation over physiologically relevant time scales.

The aquatic environment is notoriously prone to hypoxia (low O_2) and hypercapnia (elevated PCO_2) that can lead to respiratory acid-base disturbances, whereas exhaustive exercise and feeding on large meals can produce a metabolic acidosis and alkalosis, respectively.^{37,38} In addition, water-breathers normally have low internal PCO_2 , resulting in a lower HCO_3^- buffer capacity. Therefore, fishes regularly experience large fluctuations

in blood pH and $[\text{HCO}_3^-]$ that far-exceed the range observed in air-breathers³⁹; for instance, rainbow trout can tolerate plasma $[\text{HCO}_3^-]$ ranging from <2 to ~50 mM.^{40–42} It has been suggested that sAC may act as a sensor of acid-base conditions inside RBCs that mediates pH-sensitive O_2 transport,¹⁸ but functional data are currently lacking. The HCO_3^- -sensitivity (EC_{50}) of sAC activity varies across vertebrate species, and was found to match typical blood $[\text{HCO}_3^-]$ of ~5 mM in dogfish,¹⁷ ~7 mM in rainbow trout¹⁸ and ~25 mM in mammalian sAC.² These enzymatic characteristics would allow sAC to modulate cellular cAMP concentrations in response to small changes in RBC $[\text{HCO}_3^-]$. In addition, the abundant presence of carbonic anhydrase (CA) within RBCs⁴³ rapidly establishes an equilibrium between the acid-base equivalents HCO_3^- , H^+ and CO_2 , potentially allowing intracellular sAC to function as a general sensor of metabolic and respiratory acid-base disturbances in the blood, which has been demonstrated in other cell types.⁴⁴

Based on these considerations, we hypothesized that sAC functions as an intracellular acid-base sensor in the RBCs of rainbow trout and plays a role in mediating pH-sensitive Hb- O_2 transport. To test this idea, we used two highly specific small-molecule sAC inhibitors, KH7 and LRE1⁴⁵ that do not affect tmAC activity^{46–48} and have

well-validated effects on rainbow trout sAC.¹⁸ To address the putative acid-base sensing functions of sAC, we (i) characterized the subcellular localization of sAC protein using immunocytochemistry and confocal super-resolution microscopy, (ii) studied the kinetics of RBC pH_i regulation by confocal live-cell imaging with the pH-sensitive fluorophore SNARF-1 and ammonia pre-pulses to induce acid-base disturbances, and (iii) tested the effects of sAC inhibition on the Hb- O_2 binding kinetics in live RBCs by spectrophotometry in steady state and cycling gas conditions that mimic circulatory transit.

2 | RESULTS

2.1 | Immunocytochemistry

Representative super-resolution immunofluorescence images depicting the subcellular distribution of sAC protein in the RBCs of rainbow trout ($N=6$) are presented in Figure 1A. Images with a larger subset of cells are shown in the supplement (Figure S1). Within RBCs, sAC protein was present in distinct locations that are highlighted in a signal-intensity profile throughout the cell (Figure 1B): (i) the cytoplasm, where the sAC signal had a patchy

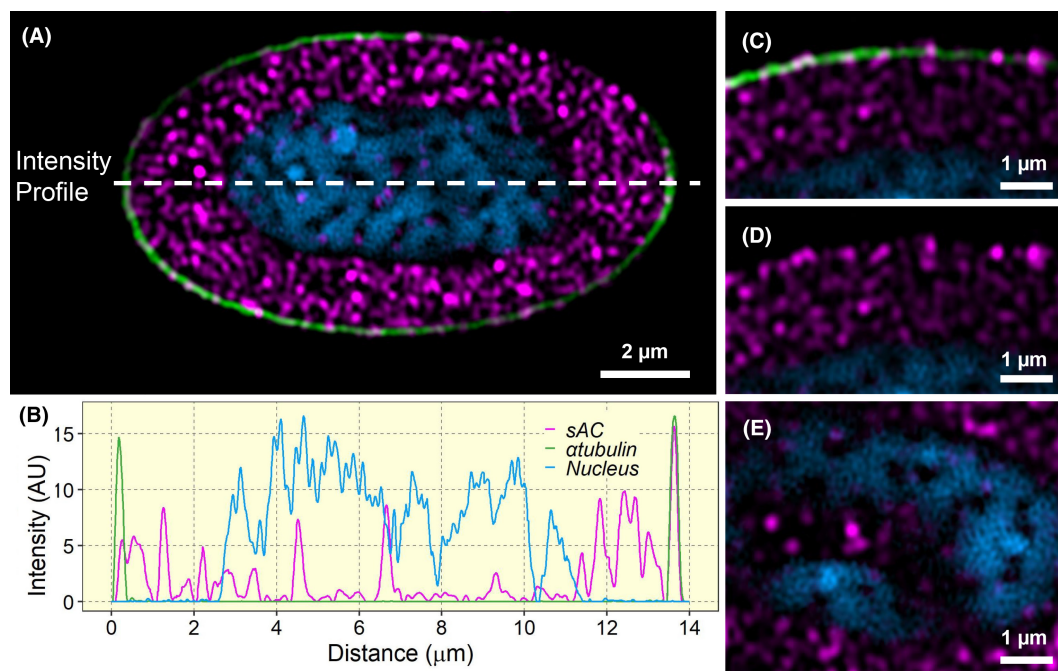


FIGURE 1 (A) Immunocytochemical localization of soluble adenylyl cyclase (sAC) in rainbow trout red blood cells (RBC). Blood was incubated in tonometers for 1 h and fixed cells were immuno-stained with an anti-sAC antibody (magenta) raised against the C1 subunit of the rainbow trout protein, with an anti- α -tubulin antibody to visualize the marginal band (green) and with DAPI to visualize the cell nucleus (blue). (B) Signal intensity profiles (arbitrary units; AU) for sAC, α -tubulin and DAPI, along a transect through the RBC (see panel A for orientation). Distinct pools of sAC protein were detected (C) in association with the plasma membrane; (D) in the RBC cytoplasm; and (E) within the nucleus. Results are representative of $N=6$ fish, and images showing a larger subset of cells and no-primary antibody controls are available in the supplement (Figure S1).

distribution; (ii) associated with the plasma membrane; and (iii) within the nucleus. However, we found no evidence of sAC protein in the mitochondria of rainbow trout RBCs (see [Figure S2](#)). These patterns in the sub-cellular distribution of RBC sAC are detailed further by 3D reconstructions of optical sections throughout single cells (see [Video S1](#)).

2.2 | Live-cell imaging

The loading of RBCs with the pH-sensitive fluorophore SNARF-1 yielded a robust signal that was stable well-beyond the 30 min experiments ([Figure 2A](#)). Using confocal microscopy, we were able to record RBC pH separately in the cytoplasm and the nucleus. Throughout this study, we report RBC pH_i as that measured in the cytoplasm of the cells, which is most relevant for the function of proteins involved in RBC acid-base regulation (e.g., membrane transporters), and O_2 and CO_2 transport (e.g., Hb and CA). However, the kinetics of nuclear pH regulation were also considered in our statistical analysis and are reported whenever significant differences were detected between the two cellular compartments. RBC pH_i was stable during the initial perfusion with control saline, but large changes were observed during ammonia pre-pulses ([Figure 2B](#)). As expected, perfusion with 30 mM NH_4Cl induced a sharp increase in RBC pH_i that remained elevated until switching back to the control saline (without NH_4Cl), when RBC pH_i decreased and undershot the baseline pH_i . A corresponding pattern was observed when the data were expressed as RBC $[\text{H}^+]_i$ ([Figure 2B](#), right y-axis). After this induced intracellular acidosis, control RBCs were able to recover pH_i and $[\text{H}^+]_i$ in less than 1 min; however, the kinetics of RBC pH_i regulation were clearly affected by the inhibition of sAC with KH7 and LRE1.

[Figure 3A](#) shows the baseline RBC pH_i averaged over the 2 min of perfusion with control saline before the ammonia pre-pulse. A significant main effect of treatment ($p < 0.001$) indicates that sAC inhibition affected baseline RBC pH_i , which on average was 7.87 ± 0.07 in control, 7.62 ± 0.03 in KH7- and 7.63 ± 0.06 in LRE1-treated RBCs; a similar pattern was observed when the data were expressed as RBC $[\text{H}^+]_i$ ([Figure S3](#)). This was not due to differences in the acid-base conditions of the perfusion salines, as no significant differences were observed between treatments and average values were: control saline pH 8.09 ± 0.00 ($p = 0.347$), total CO_2 (TCO_2) 5.19 ± 0.04 mM ($p = 0.702$); and high ammonia saline pH 8.10 ± 0.00 ($p = 0.710$), TCO_2 4.79 ± 0.07 mM ($p = 0.446$), and temperature for all salines $14.8 \pm 0.3^\circ\text{C}$

($p = 0.382$). On average pH in the nucleus was 7.68 ± 0.07 in control, 7.56 ± 0.03 in KH7- and 7.56 ± 0.05 in LRE1-treated RBCs and a significant compartment effect ($p = 0.003$) indicated these values were significantly different from those in the cytoplasm. When data from all treatments were pooled, baseline RBC pH_i was significantly higher ($p = 0.001$; [Figure 3B](#)) and $[\text{H}^+]_i$ was lower than in the nucleus ($p < 0.001$; [Figure S3B](#)). A non-significant treatment $\times$ compartment interaction ($p = 0.194$) indicated that sAC inhibition had similar effects on RBC pH_i and nuclear pH.

[Figure 4](#) shows the minimum RBC pH_i that was induced by the ammonia pre-pulses. A significant main effect of treatment indicates that drug additions to the saline affected the degree of intracellular acidification ($p < 0.001$). In RBCs treated with KH7 the minimum RBC pH_i was significantly lower compared to controls, whereas a trend ($p = 0.067$) indicated differences between DMSO- and LRE1-treated cells. The same pattern was observed when the data were expressed as the change in pH_i relative to baseline RBC pH_i (data not shown), or the maximal RBC $[\text{H}^+]_i$, where KH7-treated RBCs had significantly higher values compared to controls and a trend ($p = 0.088$) indicated the same for LRE1 ([Figure S4](#)).

[Figure 5A](#) shows the recovery of RBC pH_i after intracellular acidification due to the ammonia pre-pulses. A significant main effect of treatment indicates that drug additions affected the rate of RBC pH_i recovery ($p < 0.001$; [Figure 5B](#)). The initial slope of the RBC pH_i recovery was 1.26 ± 0.21 pH units min^{-1} in controls and decreased significantly in RBCs treated with KH7 (0.75 ± 0.06) or LRE1 (0.82 ± 0.07). In addition, a significant compartment effect ($p = 0.031$) indicated different rates of pH recovery between the cytoplasm and the nucleus. On average across treatments, pH_i in the cytoplasm recovered significantly faster ($p = 0.003$) than that in the nucleus ([Figure 5C](#)). A non-significant treatment $\times$ compartment interaction ($p = 0.915$) indicated that sAC inhibition had similar effects on RBC pH_i and nuclear pH. Despite the higher $[\text{H}^+]_i$ in KH7 and LRE1-treated cells, the rate of $[\text{H}^+]_i$ recovery after ammonia pre-pulses was not significantly different from controls ([Figure S5](#)). However, when the treatments are compared at similar RBC pH_i during the initial recovery phase (pH_i 7–7.5), control RBCs maintained significantly larger H^+ fluxes compared to KH7- or LRE1-treated cells, as indicated by non-overlapping 95% CIs ([Figure 5D](#)).

A separate series of experiments was performed to identify the membrane transporters involved in RBC pH_i recovery and the underlying cellular signaling pathways downstream of sAC. [Figure 6A](#) shows the kinetics of RBC pH_i recovery after an acid-base disturbance. Specific inhibitors of membrane transporters were only

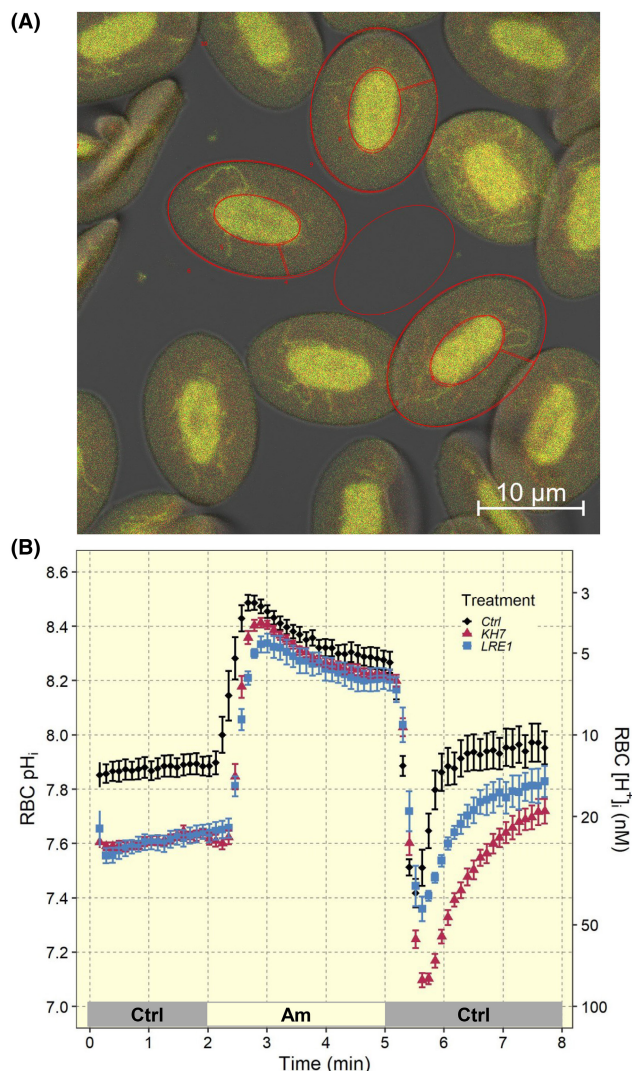


FIGURE 2 (A) Live-cell imaging of red blood cells (RBC) from rainbow trout loaded with the pH-sensitive fluorophore SNARF-1, showing the cell nucleus and mitochondria surrounding it (see Figure S2 for additional validation). For each run, fluorescence was averaged over three RBCs, recording separately from the cytoplasmic and nuclear compartments and subtracting the background signal (indicated as red areas of interest in panel A). (B) Measurements of RBC intracellular pH (pH_i ; in the cytoplasm) as a function of time (min). RBCs were perfused with a physiological saline (pH 8.00, $15^\circ C$, 21 kPa PO_2 , 0.3 kPa PCO_2) containing either: 0.25% DMSO as a carrier control, or the specific small-molecule sAC inhibitors KH7 ($25 \mu M$) or LRE1 ($50 \mu M$). An acid-base disturbance was induced by ammonia pre-pulses (replacing 30 mM NaCl with NH_4Cl) for 3 min. The right y-axis shows the corresponding changes in RBC intracellular proton concentration ($[H^+]_i$; nM; in the cytoplasm). All data represent means $\pm$ SEM for $N=6$ fish (and $N=5$ for LRE1).

added to the control saline during the recovery period to prevent confounding effects on acid loading during ammonium pre-pulses. The inhibition of the AE (with DIDS) significantly decreased the rate of RBC pH_i

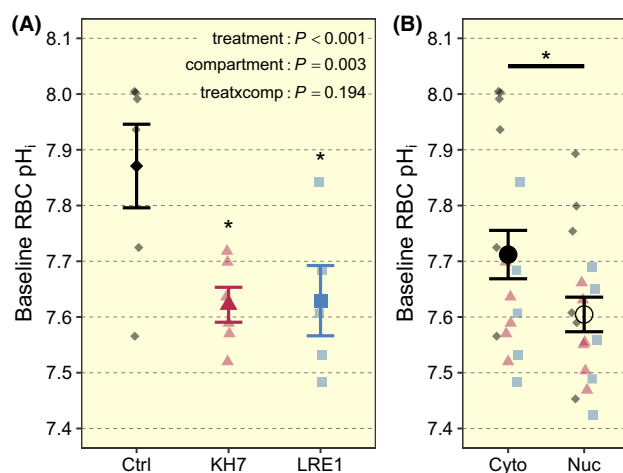


FIGURE 3 Baseline red blood cell (RBC) intracellular pH (pH_i ; in the cytoplasm) representing average values for the initial period (0–2 min) before the ammonia pre-pulses shown in Figure 2. (A) A significant main effect of treatment indicates that baseline RBC pH_i was affected by drug additions: (i) 0.25% DMSO as a carrier control, (ii) the specific small molecule sAC inhibitors KH7 ($25 \mu M$) and (iii) LRE1 ($50 \mu M$). Post-hoc analysis identified significant differences from the control that are indicated by * ($p < 0.05$). (B) On average baseline RBC pH_i was significantly (by ~ 0.1 pH units) higher in the cytoplasmic compartment compared to the nucleus of the cell ($p = 0.001$). All data are shown as means $\pm$ SEM with individual values for $N=6$ fish (and $N=5$ for LRE1).

recovery compared to controls (Figure 6B). In contrast, the inhibition of RBC NHE (with DMA), or KCC and NKCC (with bumetanide) had no significant effects on RBC pH_i recovery. The same pattern was observed when the data were expressed as the rate of RBC $[H^+]_i$ recovery, that was significantly slower in DIDS-treated cells (Figure S6) and when the recovery was expressed as H^+ fluxes (Figure 6C).

Figure 7A shows the kinetics of RBC pH_i recovery in the presence of specific inhibitors of PDEs (IBMX), PKA (H89), and EPAC1 (CE3F4). IBMX treatment resulted in a significantly higher RBC pH_i after the ammonia pre-pulse, compared to control RBCs. In addition, IBMX caused a significant increase in the rate of RBC pH_i recovery compared to controls, whereas neither H89 nor CE3F4 affected the rate of RBC pH_i recovery under the tested conditions (Figure 7B). A significant compartment effect ($p = 0.023$) indicated that, on average across treatments, the rate of RBC pH_i recovery was significantly faster in the cytoplasm ($p = 0.009$) compared to the nucleus (Figure 7C). A non-significant treatment $\times$ compartment interaction ($p = 0.969$) indicated that the inhibition of PDEs with IBMX had similar effects on RBC pH_i and nuclear pH. When the data were expressed as the rate of RBC $[H^+]_i$ recovery, there was no treatment effect, (Figure S7), which is supported by the calculated H^+ fluxes during RBC pH_i

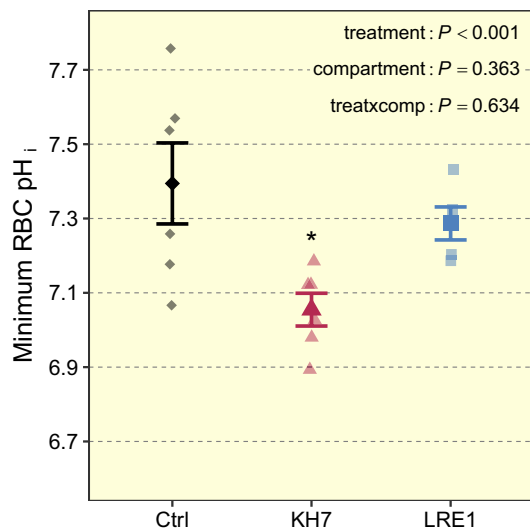


FIGURE 4 The minimum red blood cell (RBC) intracellular pH (pH_i ; in the cytoplasm) during the intracellular acidosis induced by the ammonia pre-pulses shown in Figure 2. A significant main effect of treatment indicates that the minimum RBC pH_i was affected by drug additions: (i) 0.25% DMSO as a carrier control, (ii) the specific small molecule sAC inhibitors KH7 (25 μ M) and (iii) LRE1 (50 μ M). Post-hoc analysis identified significant differences from the control that are indicated by * ($p < 0.05$). All data are shown as means $\pm$ SEM with individual values for $N = 6$ fish (and $N = 5$ for LRE1).

recovery, where 95% confidence intervals overlap in all treatments (Figure 7D).

2.3 | Haemoglobin–oxygen binding

The Hb- O_2 binding characteristics of rainbow trout whole blood are shown in Figure 8. The PO_2 at which Hb is 50% saturated (P_{50}) describes the affinity of Hb- O_2 binding (Figure 8A). Hb P_{50} was 4.0 kPa in control blood (0.3 kPa CO_2) treated with DMSO, and increased significantly ($p < 0.001$) to 7.2 kPa during a respiratory acidosis (1 kPa CO_2). KH7 had no effect on Hb P_{50} in normocapnia, but a significant drug $\times$ CO_2 interaction ($p = 0.009$) indicated that during a respiratory acidosis Hb P_{50} was higher in KH7 (9.1 kPa) compared to DMSO-treated RBCs. The Hill coefficient (n_H) describes the cooperativity of Hb- O_2 binding (Figure 8B) and was significantly lower in KH7 treated blood compared to controls ($p < 0.001$), and during a respiratory acidosis compared to normocapnia ($p < 0.001$). However, there was no significant drug $\times$ CO_2 interaction, indicating that KH7 had similar effects on n_H in both CO_2 treatments.

To address the physiological significance of RBC sAC under in vivo-like conditions, blood was exposed to cycling gas tensions (O_2 and CO_2) that mimic circulatory transit in

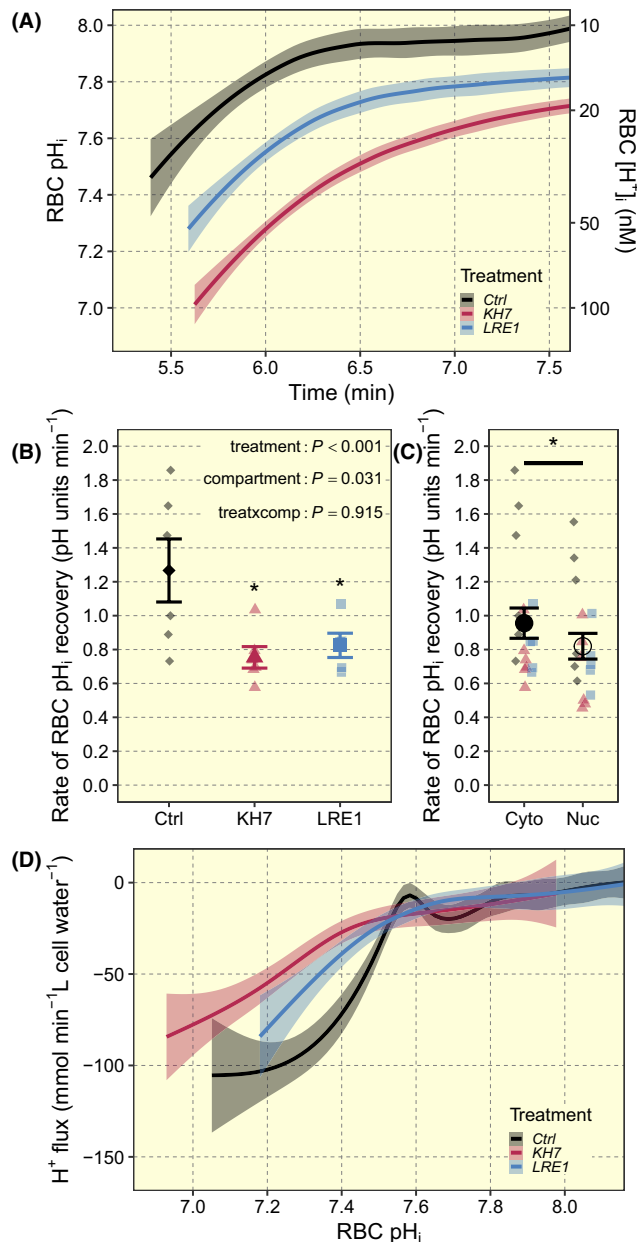


FIGURE 5 (A) Kinetics of red blood cell (RBC) intracellular pH (pH_i ; in the cytoplasm) recovery after acidification with an ammonia pre-pulse (means $\pm$ 95% CIs) in the presence of: (i) 0.25% DMSO as a carrier control, and (ii) the specific small molecule sAC inhibitors KH7 (25 μ M) and (iii) LRE1 (50 μ M). The right y-axis shows the corresponding changes in RBC intracellular proton concentration ($[H^+]$; nM; in the cytoplasm). (B) Average initial rates of RBC pH_i recovery were significantly affected by sAC inhibition. Post-hoc analysis identified significant differences from the Ctrl that are indicated by * ($p < 0.05$). (C) On average RBC pH_i recovery was significantly faster in the cytoplasm compared to the nucleus ($p < 0.004$). Data are means $\pm$ SEM with individual values for $N = 6$ fish (and $N = 5$ for LRE1). (D) H^+ fluxes during recovery as a function of RBC pH_i (means $\pm$ 95% CIs).

rainbow trout, and Hb- O_2 saturation was measured in real-time (Figure 9A). As expected, Hb- O_2 saturation decreased

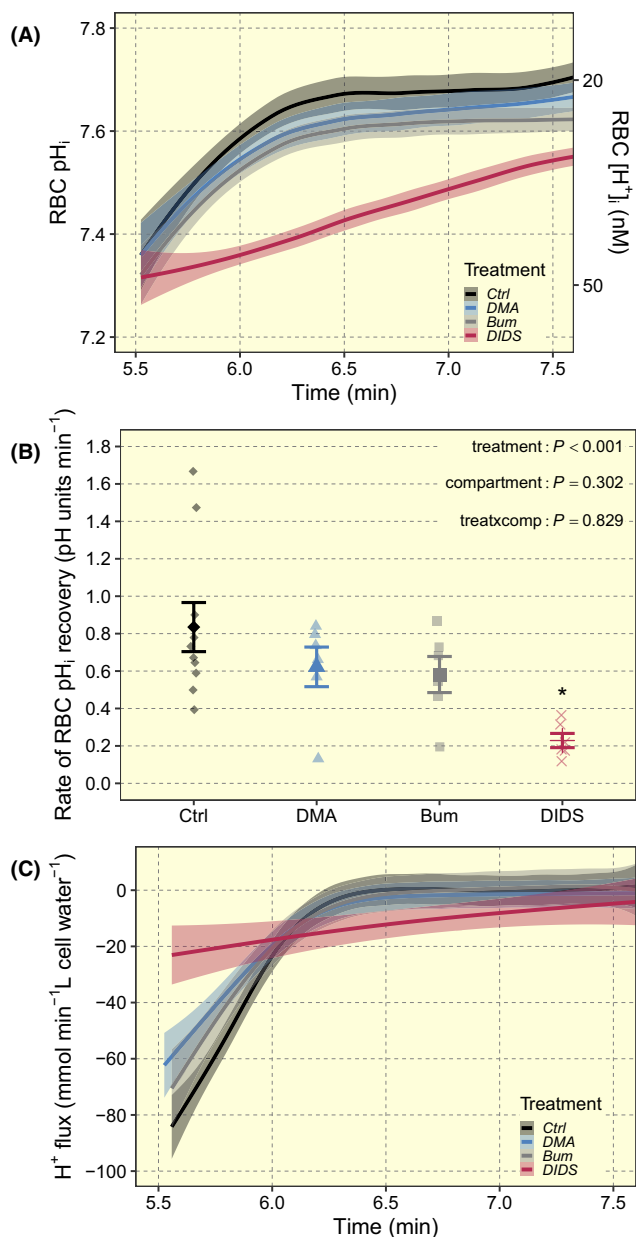


FIGURE 6 (A) Kinetics of red blood cell (RBC) intracellular pH (pH_i ; in the cytoplasm) recovery after acidification with an ammonia pre-pulse (means $\pm$ 95% CIs). The right y-axis shows the corresponding changes in RBC intracellular proton concentration ($[\text{H}^+]_i$; nM; in the cytoplasm). Inhibitors of membrane transporters were added after the ammonia pre-pulse: (i) 0.25% DMSO as a carrier control, (ii) dimethyl amiloride (DMA; 30 μM), an inhibitor of sodium-proton exchangers (NHE), or (iii) bumetanide (Bum; 10 μM) an inhibitor of potassium-chloride co-transporters (KCC) and sodium-potassium-chloride co-transporters (NKCC); and (iv) an anion exchanger inhibitor (DIDS; 10 μM). (B) The average initial rate of RBC pH_i recovery was affected by drug additions as indicated by a significant treatment effect. Post-hoc analysis identified significant differences from the Ctrl that are indicated by * ($p < 0.05$). Data are shown as means $\pm$ SEM with individual values for $N = 6$ fish (and $N = 10$ for Ctrl). (C) H^+ fluxes during RBC pH_i recovery as a function of time (means $\pm$ 95% CIs).

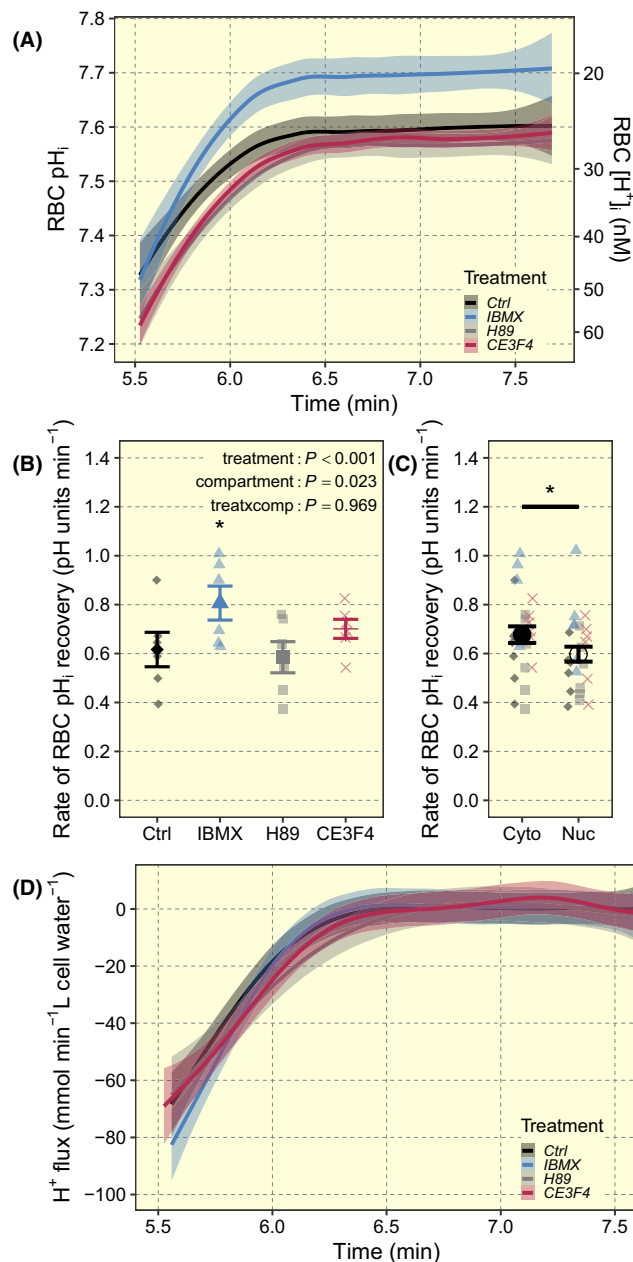


FIGURE 7 (A) Kinetics of red blood cell (RBC) intracellular pH (pH_i ; in the cytoplasm) recovery after acidification with an ammonia pre-pulse (means $\pm$ 95% CIs) in the presence of: (i) 0.25% DMSO as a carrier control, (ii) 3-Isobutyl-1-methylxanthine (IBMX; 50 μM), an inhibitor of phosphodiesterases (PDE), (iii) H89 (1 μM) an inhibitor of protein kinase A (PKA), and (iv) CE3F4 (25 μM) an inhibitor of exchange protein directly activated by cAMP 1 (EPAC1). The right y-axis shows the corresponding changes in RBC intracellular proton concentration ($[\text{H}^+]_i$; nM; in the cytoplasm). (B) Average initial rates of RBC pH_i recovery were significantly affected by drug additions. Post-hoc analysis identified significant differences from the Ctrl that are indicated by * ($p < 0.05$). (C) On average the rate of RBC pH_i recovery was significantly faster in the cytoplasm compared to the nucleus ($p = 0.009$). Data are shown as means $\pm$ SEM with individual values for $N = 6$ fish (and $N = 10$ for Ctrl). (D) H^+ fluxes during RBC pH_i recovery (means $\pm$ 95% CIs).

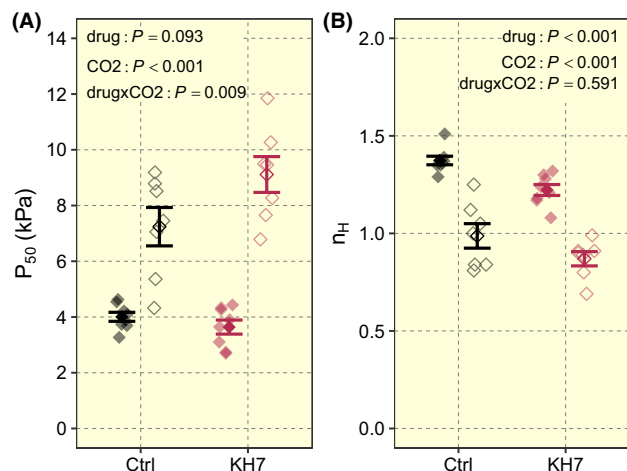


FIGURE 8 Haemoglobin (Hb)–oxygen (O₂) binding characteristics of rainbow trout whole blood. (A) Hb–O₂ binding affinity, measured as the partial pressure of O₂ at which Hb is 50% saturated (P_{50}). (B) The cooperativity of Hb–O₂ binding is described by the Hill coefficient (n_H). Blood was equilibrated in tonometers for 1 h with either: (i) 0.25% DMSO as a carrier control; or (ii) the specific small molecule sAC inhibitor, KH7 (25 μ M). Measurements were performed at either 0.3 kPa mimicking resting arterial levels (solid symbols), or 1 kPa PCO₂ to simulate a respiratory acidosis (open symbols). All data are means $\pm$ SEM with individual values for $N = 7$ –8 fish.

significantly ($p < 0.001$; Figure 9B) from $93.1 \pm 0.8\%$ and $91.5 \pm 1.7\%$ for controls and KH7 in arterial conditions, to $34.3 \pm 2.0\%$ and $39.3 \pm 1.8\%$ in venous conditions, and then increased to $90.8 \pm 0.7\%$ and $89.2 \pm 1.9\%$ upon return to arterial conditions. Importantly, sAC inhibition with KH7 affected Hb–O₂ saturation and the effect was dependent on the gas conditions, as indicated by a drugxcondition interaction ($p = 0.006$). The difference between arterial and venous Hb–O₂ saturation (Δ Hb–O₂ sat.) represents the amount of O₂ that could be unloaded to a tissue (Figure 9C). When RBC sAC was inhibited with KH7, Δ Hb–O₂ sat. was significantly lower ($-52.2 \pm 1.7\%$; $p = 0.002$) compared to control RBCs ($-58.8 \pm 1.8\%$), indicating that O₂ unloading from Hb could be impaired at the tissues.

3 | DISCUSSION

3.1 | Subcellular distribution of sAC

Super-resolution imaging of rainbow trout RBCs revealed sAC protein throughout the cytoplasm as well as in association with the plasma membrane and the nucleus (Figure 1A), where these different pools of sAC may fulfill dedicated physiological functions.^{7,49} The subcellular distribution of sAC can inform on its spatial association

with downstream targets and other proteins within RBCs, to infer potential signaling pathways. sAC in the RBC cytoplasm is likely closely associated with the abundant CA pool within RBCs, which establishes a rapid equilibrium between H⁺, HCO₃[−] and CO₂. This may enable cytosolic sAC to act as a general sensor of metabolic and respiratory acid-base disturbances in the blood. In addition, CA catalytic activity is actively modulated by cAMP-induced phosphorylation, such as shown in rainbow trout gill tissues.⁵⁰ A role of sAC in the HCO₃[−]-dependent phosphorylation of RBC CA remains to be tested, but could have important consequences for systemic gas transport. On the other hand, the pool of membrane sAC may be closely associated with membrane transporters, where it may work with tmACs to provide localized cAMP signals to activate membrane transport of ions and H⁺. The physiological functions of mitochondrial sAC in mammalian cells may include a regulation of the electron transport chain in response to endogenously produced CO₂.³ However, a potential absence of mitochondrial sAC in rainbow trout RBCs (Figure S2) raises new questions about the regulation of oxidative phosphorylation in RBCs and perhaps other cell types. Finally, nuclear sAC may be involved in cAMP-mediated gene expression and, as it may not be associated with CA, could function exclusively in sensing local [HCO₃[−]]. Our findings confirm the presence of localized pools of sAC throughout the RBCs of rainbow trout, and more research is needed to understand their individual physiological functions. Therefore, our subsequent experiments aimed at determining the role of membrane, cytosolic and nuclear sAC as putative acid-base sensors in RBCs.

3.2 | Sensory functions of sAC

Using live-cell imaging we were able to measure the RBC pH_i of single nucleated RBCs in real time (Figure 2A). At 15°C and a saline pH of 8.09, average RBC pH_i was 7.87 ± 0.07 measured in the cytoplasm (excluding the nucleus, which had a lower pH). These RBC pH_i values, are relatively high compared to some previous studies (e.g., pH_i 7.55–7.64 using the freeze-thaw technique),⁵¹ but are still within the range reported for rainbow trout (e.g., pH_i 7.86 at saline pH 8.19, using the DMO and Cl[−] distribution methods).⁵² Work in human RBCs using SNARF showed that higher pH_i values may also result after calibrations with the nigericin method,³³ but the suitability of other methods (e.g., null point calibration) for pH_i measurements in the separate compartments of nucleated RBCs remains to be tested. When RBC sAC was inhibited with either one of two specific sAC inhibitors (KH7 or LRE1), steady-state baseline pH_i was ~ 0.25 pH units lower

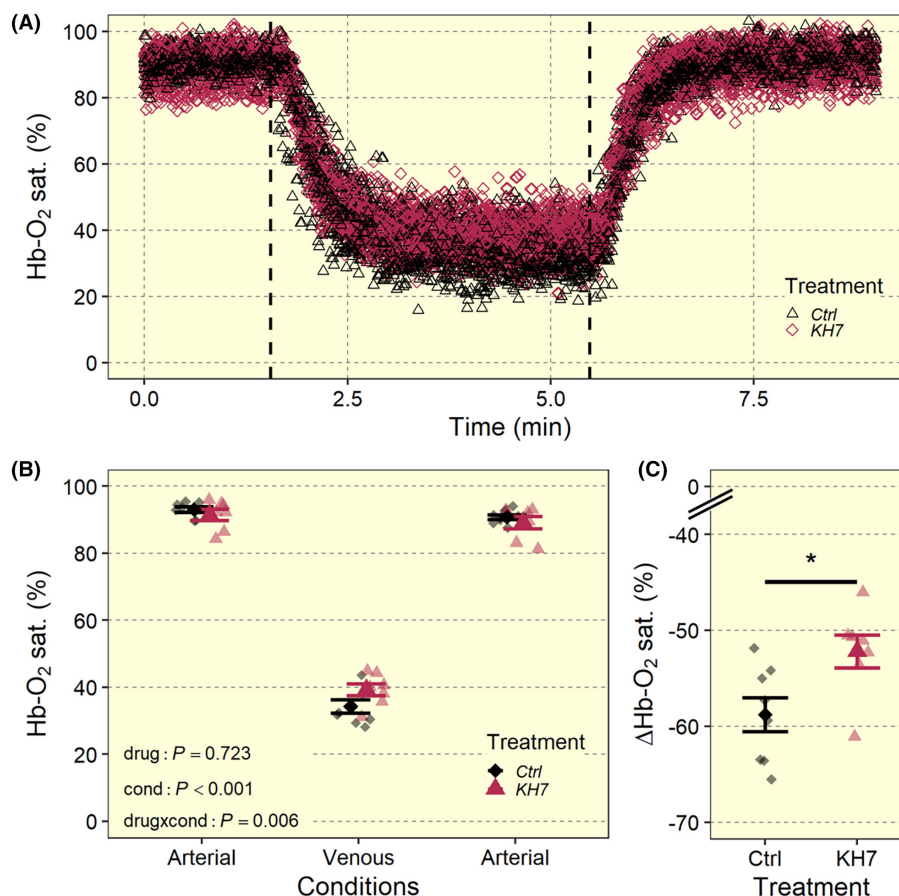


FIGURE 9 (A) Haemoglobin–oxygen saturation (Hb-O₂ sat.) measured spectrophotometrically in rainbow trout blood in vitro. Samples were exposed to cycling arterial (16 kPa PO₂, 0.3 kPa PCO₂ at 15°C) and venous conditions (5.3 kPa PO₂ and 1 kPa PCO₂) that mimic in vivo values (dashed lines indicate changes in gas tensions). Before these measurements, blood was equilibrated in tonometers for 1 h with either: (i) 0.25% DMSO as a carrier control; or (ii) the specific small molecule sAC inhibitor, KH7 (25 μM). (B) Average Hb-O₂ sat. during arterial and venous conditions were analyzed with a linear-mixed model that detected significant effects of drug treatments, conditions and their interaction (drug × cond). (C) The change in Hb-O₂ sat. (ΔHb-O₂ sat.) during transition from arterial to venous conditions, represents the amount of O₂ that can be unloaded to the tissues, and was significantly lower in RBCs treated with KH7 compared to Ctrl (p = 0.002). Raw data in (A) and means ± SEM with individual values for N = 7–8 fish in (B and C).

compared to that of DMSO-treated controls (Figures 2B and 3A). Conceptually, the stimulation of sAC activity by HCO₃[−] would lead to a localized production of cAMP, which is confined by the activity of PDEs that degrade the signaling molecule as it diffuses away from its origin.^{8,53} Indeed, the inhibition of PDEs (with IBMX) significantly increased RBC pH_i compared to controls (see Figure 7A), likely by magnifying the effects of sAC-produced cAMP on RBC pH_i. This framework may also explain the previous findings of a lower pH_i when human RBCs were suspended in a HCO₃[−]-free saline,³³ where putative sAC activity would likely be decreased. Combined, these data provide the first functional evidence for a pivotal role of sAC in sensing and mediating pH_i setpoints in vertebrate RBCs.

We then used ammonia pre-pulses to induce a transient intracellular acidification that was reflected in corresponding changes in RBC pH_i and [H⁺]_i. Ammonia

pre-pulses have been shown to induce acid-base disturbances that activate sAC, such as in coral sperm, where cellular cAMP levels increased 10-fold.⁵⁴ When RBC sAC was inhibited with KH7, the cells became significantly more acidified than controls but also than LRE1-treated cells (Figure 4 and Figure S4). A detailed study of the recombinant sAC protein in rainbow trout found that 25 μM KH7 inhibits both the baseline and HCO₃[−]-stimulated activities of sAC, whereas 50 μM LRE1 has no effect on the baseline activity and only inhibits the HCO₃[−]-stimulated component.¹⁸ These differences in the mode of action of the two inhibitors can likely account for the greater effects of KH7 on RBC minimum pH_i compared to LRE1. After ammonia pre-pulses, control RBCs recovered pH_i by membrane transport of acid-base equivalents over the course of ~1 min (Figure 2B), but KH7 or LRE1-treated RBCs had significantly slower rates of RBC pH_i recovery (Figure 5). Surprisingly, the rate of [H⁺]_i recovery was not

significantly different between treatments (Figure S5), despite the larger trans-membrane H^+ gradients in the KH7 and LRE1 treated RBCs, where baseline pH_i was lower. However, when the H^+ fluxes during recovery were compared over similar ranges of RBC pH_i , it became clear that sAC inhibition led to significantly lower H^+ fluxes compared to control RBCs (Figure 5D), indicating an impairment of pH_i regulation. Combined, our data show that two sAC inhibitors with different modes of action significantly affected the setpoints for pH_i regulation and the kinetics of pH_i recovery in rainbow trout RBCs and thus support the hypothesis that sAC functions as an intracellular acid-base sensor in the regulation of cellular homeostasis over physiologically relevant time scales.

3.3 | Role of sAC in Hb-O₂ binding

We further addressed the physiological significance of sAC signaling by measuring Hb-O₂ binding characteristics under steady-state conditions. As expected, Hb P_{50} increased during a respiratory acidosis due to the Bohr effect, however, the change was greater in RBCs treated with KH7 compared to DMSO controls (Figure 8A). An effect of KH7 on Hb P_{50} was not observed in normocapnia, indicating that the mechanism is likely through changes in RBC pH_i . KH7 also decreased the cooperativity of Hb-O₂ binding (n_H) in both normocapnia and during a respiratory acidosis (Figure 8B). To determine the *in vivo* relevance of these findings, we exposed RBCs to cycling gas tensions that mimic those during circulatory transit in rainbow trout. In arterial conditions, Hb-O₂ saturation was >90% in all treatments and decreased significantly during the transition to venous conditions (Figure 9A). The fact that arterial Hb-O₂ saturation was apparently not affected by the effects of KH7 on baseline RBC pH_i , can likely be explained by the sigmoidal shape of the oxygen equilibrium curve, resulting in small effects of pH_i on Hb-O₂ saturation at high PO_2 ,⁵⁵ and the absence of a significant Root effect in rainbow trout over the tested range of RBC pH_i >7.2⁵⁶ (but also consider).⁵⁷ A significant interaction effect between drug treatments and the arterial and venous conditions (drug×condition) indicates that the inhibition of RBC sAC with KH7 affected the kinetics of O₂ unloading (Figure 9B). RBCs treated with DMSO unloaded $58.8 \pm 0.02\%$ of their O₂ during transition from arterial to venous conditions, which is in line with *in vivo* measurements in rainbow trout during strenuous exercise in a swim tunnel.⁵⁸ However, when RBC sAC was inhibited with KH7, O₂ unloading decreased significantly by ~11% (Figure 9C). This change in Hb-O₂ unloading could have significant systemic consequences if it occurred with every pass of the RBCs through the circulation and could be even greater considering the short times available for gas exchange at

the capillaries *in vivo*. Combined, these data support our hypothesis that sAC is also involved in mediating the unloading of O₂ from pH-sensitive Hb, in the highly dynamic acid-base environment of the microcirculation.

The finding that sAC inhibition compromised Hb-O₂ unloading in venous conditions may seem at odds with the increased Hb P_{50} during a respiratory acidosis. However, these measurements were performed at very different time scales that result in fundamentally different acid-base kinetics. The inhibition of sAC under steady-state conditions compromises the RBC's ability to maintain its pH_i setpoints, which is consistent with the reduction in baseline RBC pH_i observed during live-cell imaging (Figure 3) and an increase in Hb P_{50} during a respiratory acidosis (Figure 8A). However, *in vivo* acid-base conditions will never be in a steady state. At the tissue capillaries, metabolically produced CO₂ diffuses into the RBCs and is rapidly converted to H^+ and HCO_3^- by the abundant CA pool within the cells.⁴³ Based on *in vivo* measurements of arterial and venous RBC pH_i and PCO_2 ⁵¹ in rainbow trout and the pK_a at 15°C⁵⁹, the intracellular HCO_3^- concentration in RBCs increases from 7 to 12 mM during capillary transit, causing a significant stimulation of sAC activity ($EC_{50} \sim 7$ mM).¹⁸ A continuous hydration of CO₂ during capillary transit of the RBCs is only possible if the two products of the hydration reaction are rapidly removed from the cytoplasm. The H^+ will largely bind to oxygenation-dependent buffer groups on Hb, provided by the large Haldane effect of teleosts,⁶⁰ while HCO_3^- is exported into the plasma in exchange for Cl^- by the AE, a membrane transporter that is driven by the concentration gradients for the two ions.⁶¹ Conceptually, a delay in the activation of the AE or a decrease in its transport capacity would slow the CO₂ hydration within the RBCs due to the intracellular accumulation of HCO_3^- . This would reduce the intracellular availability of H^+ , which is a critical driver of O₂ unloading through the large Bohr effect in teleosts.⁶² Therefore, our findings in the dynamic acid-base conditions during simulated arterial-venous transit, link cAMP signaling by sAC with the activity of the AE pathway.

In fact, our transporter inhibition experiments indicate that the AE was the primary transporter involved in the recovery of RBC pH_i after ammonia pre-pulses (Figure 6), which is consistent with findings in human RBCs.³³ The incomplete inhibition of AE may be due to the lower DIDS concentration (10 μ M) compared to that used in previous studies (300 μ M),³³ which was necessary to exclude potential interactions with RBC sAC (see methods section). Importantly however, there were no detectable contributions of NHE, KCC or NKCC to RBC pH_i recovery, which indicates that the effects of sAC inhibition on RBC acid-base regulation are through a functional link with the AE. Specifically, we propose that: (i) high PCO_2 during capillary transit increases RBC HCO_3^- concentration and activates

sAC on the plasma membrane; (ii) localized cAMP signaling may regulate the rate of AE through post-translational modifications of the transporter; and (iii) the inhibition of this pathway with KH7 delays HCO_3^- export, the formation of H^+ and therefore, impairs pH-sensitive O_2 unloading from Hb (Figure 10A,B). If substantiated in vivo, a role of RBC sAC in mediating acid-base dependent Hb- O_2 binding, could fundamentally change our understanding of how vertebrates regulate O_2 delivery in the microcirculation.

3.4 | Downstream targets of sAC

Additional experiments were performed to identify the cellular pathways involved in cAMP signaling through RBC sAC (Figure 7). The mammalian AE protein has several tyrosine phosphorylation sites that modulate transporter activity,⁶³⁻⁶⁵ and also the binding of glycolytic enzymes, deoxy-haemoglobin⁶⁶ and CA^{67,68} to the cytoplasmic domain of the exchanger. The physiological role of these post-translational modifications and protein-protein interactions are still poorly understood,⁶⁹ but represent potential pathways by which sAC-produced cAMP could regulate RBC AE function. For instance, the sAC-mediated capacitation of human sperm depends on the activity of AE that is regulated by PKA-dependent

tyrosine phosphorylation.⁷⁰ In our experiments, we tested whether PKA and EPAC1 pathways played a role in regulating RBC pH_i recovery but found no evidence for their involvement under the tested conditions. Future studies using a wider range of inhibitors, concentrations and experimental conditions may help shed light on this issue, but alternative cAMP targets should also be considered. An involvement of EPAC2 and especially POPDC proteins is possible and would represent a novel avenue in the regulation of AE activity and RBC acid-base homeostasis.⁹ In fact, POPDC2 mRNA is present in published rainbow trout RBC transcriptomes,⁷¹ but its physiological function is still unknown. A lack of specific pharmacological POPDC inhibitors is currently hindering experimental work in this area, but a membrane-permeable disruptor peptide that was recently described⁷² has promise for future studies. Interestingly, the latter study also described interactions between POPDC proteins and PDE4 in mouse cardiomyocytes. In rainbow trout RBCs, the inhibition of PDEs (IBMX) significantly increased the rate of RBC pH_i recovery (Figure 7B), however, did not show an effect on $[\text{H}^+]_i$ recovery (Figure S7A) or H^+ fluxes (Figure 7D). Therefore, it seems that the effects of IBMX on pH_i recovery are not through changes in membrane transport, but rather due to the effects on pH_i setpoints. Based on our combined data, there is promise in exploring functional

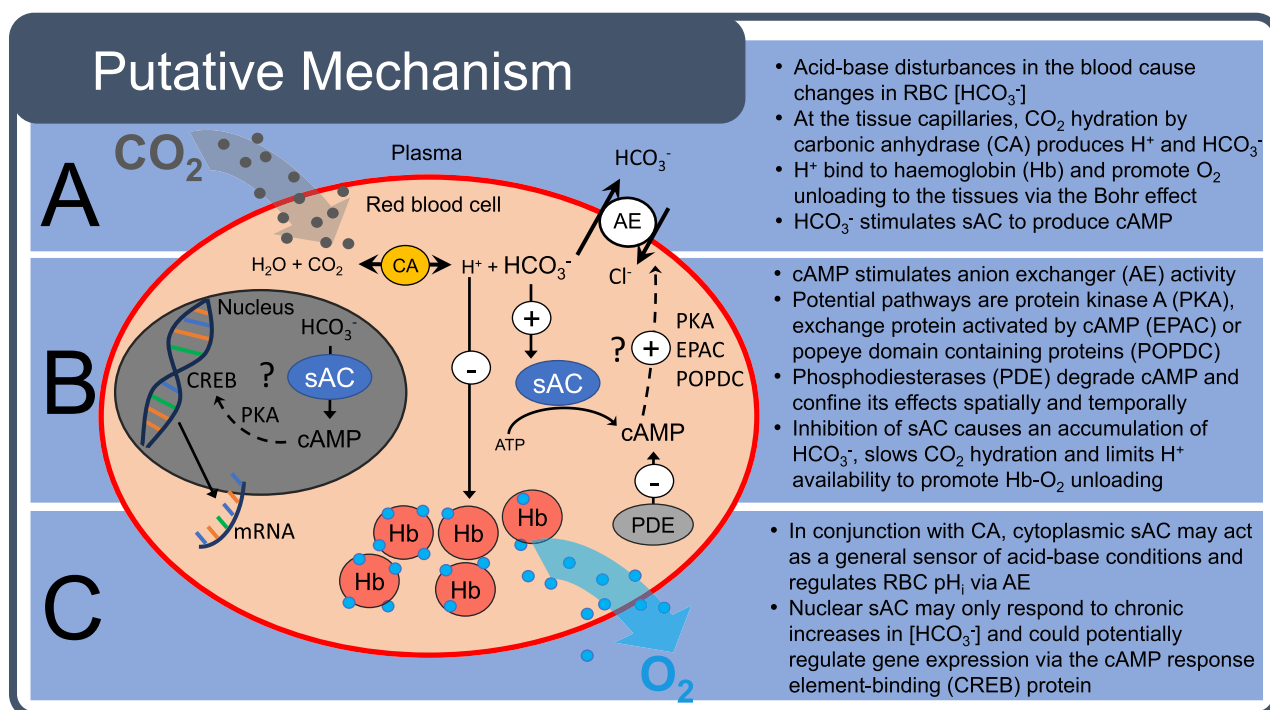


FIGURE 10 Putative mechanism of soluble adenylyl cyclase (sAC) signaling in rainbow trout red blood cells (RBC). (A) sAC is an intracellular acid-base sensor in RBCs that produces cAMP. (B) cAMP activates membrane transporters to regulate cellular pH_i and pH -sensitive Hb- O_2 transport. (C) If nuclear sAC regulates gene expression in response to chronic acid-base disturbances in fish, this may be an important mechanism of phenotypic plasticity after feeding, exercise, or elevated CO_2 .⁴

links between POPDC proteins and the regulation of the AE, RBC acid-base homeostasis and Hb-O₂ binding.

3.5 | Putative roles of nuclear sAC

The use of confocal microscopy during live-cell imaging enabled separate measurements of RBC p*H*_i in the cytoplasm and the nucleus. Across all experiments, nuclear pH was consistently ~0.1 pH units lower than pH in the cytoplasm (Figure 3B). A lower nuclear pH may contribute to the observed discrepancy between cytoplasmic p*H*_i measured by live-cell imaging and the freeze-thaw methods on cell homogenates. Furthermore, this finding is in line with a previous study that reported H⁺ gradients between the cytoplasm and the nucleus of two mammalian cancer cell lines.⁷³ Other studies on worm,⁷⁴ jellyfish⁷⁵ and plant models⁷⁶ did not detect differences between cytoplasmic and nuclear pH, and concerns have been raised about interference of densely packed DNA with SNARF measurements in the nucleus.⁷⁷ However, in our experiments we performed separate calibration curves for the nuclear and cytoplasmic compartments, which should account for any extrinsic influences on the fluorescent signal. Interestingly, we detected no compartment specific effects of sAC inhibition in any of the measurements describing RBC p*H*_i kinetics (treatment × compartment *p* > 0.05; Figures 3–5), thus indicating that sAC plays a similar role in regulating cytoplasmic and nuclear pH. The pH recovery was slower in the nucleus compared to the cytoplasm (Figures 5C and 7C and Figure S7B), which may suggest that nuclear pH simply follows whole cell p*H*_i regulation and perhaps that the nucleus has a higher buffer capacity compared to the cytoplasm. The lower pH in the nucleus will typically correspond to a lower [HCO₃[−]] (assuming that PCO₂ is equilibrated across the nuclear membranes) and therefore, more HCO₃[−] will be necessary to stimulate sAC. Therefore, nuclear sAC may respond to chronic acid-base disturbances rather than the recurring changes in HCO₃[−] concentrations that occur during circulatory transit.

In mammalian systems, nuclear sAC signaling has been shown to mediate the cAMP response element binding (CREB) pathway to regulate acid-base-dependent gene expression.^{78,79} When rainbow trout and other fishes are exposed to chronic hypercapnia, they compensate blood pH in the course of 48 h, by accumulating plasma HCO₃[−] to concentrations that can exceed normal levels by 10-fold.^{40,41} Also postprandial metabolic alkaloses can greatly increase plasma HCO₃[−] after feeding in rainbow trout^{80,81} and sharks, where sAC in gill cells has been shown to mediate the compensation for the acid-base disturbance.¹⁷ We propose that RBC nuclear sAC may sense chronic

increases in HCO₃[−] concentration and regulates gene expression through the cAMP-PKA-CREB pathway as part of an integrative physiological response to acid-base disturbances in fishes (Figure 10C). Transcriptional investigations of nucleated RBCs have shown important responses to environmental stressors⁸² and to RBC aging.⁸³ Therefore, studying the transcriptional response of nucleated vertebrate RBCs to acid-base disturbances, in combination with specific inhibitors of sAC and its downstream targets, may hold the key to detect putative mechanisms of acid-base dependent gene expression. Potentially, this approach may reveal novel cellular pathways of phenotypic plasticity in fishes, which has great relevance for understanding the effects of anthropogenic environmental change on aquatic ecosystems.

4 | CONCLUSION AND PERSPECTIVES

One of the fundamental functions of the vertebrate cardiovascular system is to match the supply of O₂ to the demand of the metabolizing tissues, but the cellular mechanisms that mediate this process are still poorly understood. Vertebrate RBCs experience highly variable gas tensions and acid-base conditions with every cycle through the circulation and are in a favorable position to detect localized imbalances before they have systemic consequences.^{84,85} The exchange of O₂ and CO₂ by RBCs in the microcirculation are dynamic processes that occur within a few seconds^{31,86} and may require a rapid control of cellular acid-base fluxes. The novel findings from our work provide the first functional evidence that sAC is an acid-base sensor in the RBCs of rainbow trout, is involved in the regulation of p*H*_i setpoints, the rate of acid-base fluxes through the AE, and may play a role in regulating gene expression in nucleated RBCs. In addition, proteomic evidence also supports the presence of a full-length 187 kDa sAC protein in both the cytosolic and membrane fractions of human RBCs.^{87,88} Therefore, an extension of our findings to other species may have broader implications for our fundamental understanding of cardiovascular physiology in vertebrates.

5 | MATERIALS AND METHODS

5.1 | Animals and husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1972) of both sexes were obtained from Thomas Fish Company (Anderson, CA, USA) and raised in a recirculating system with 10 L tank size. Artificial freshwater

(in μM : KCl 4, MgSO_4 6, NaHCO_3 64, CaCl_2 30, NaCl 16, pH: 7.00–7.20) was made according to previous studies on acid-base physiology in rainbow trout,⁸⁹ was kept at 15°C with dissolved oxygen levels above 90% and water quality was monitored daily. Fish were fed every day (Skretting, Classic Fry, Tooele, UT, USA), but feeding was withheld for approximately 24 h before sampling. At the time of experiments, the average body mass of the fish was 14.63 ± 2.19 g (mean $\pm$ SEM). Animal husbandry and the following experimental procedures were performed in compliance with all Institutional Animal Care and Use Committee (IACUC) guidelines and were approved by the Animal Care program at the University of California San Diego (Protocol No. S10320).

5.2 | Blood sampling

Rainbow trout were euthanized in 70 mg/L benzocaine and a terminal blood sample of $\sim 100 \mu\text{L}$ was taken by caudal puncture into a heparinized syringe. Blood was spun down in a table-top centrifuge (500g; 3 min) and the plasma was removed and stored at 4°C. The RBC pellet was rinsed three times with filter sterilized Cortland's saline to remove platelets and white blood cells (in mM: 131 NaCl, 5.1 KCl, 1.6 CaCl_2 , 0.9 MgSO_4 , 6 NaHCO_3 , 3 NaH_2PO_4 , 5.6 glucose; 300 mOsm, pH adjusted to 7.8 with NaOH).⁹⁰ The washed RBCs were resuspended in 20 mL of saline (haematocrit <1%) and left to equilibrate overnight on a tilt-shaker at 17°C to reverse the adrenergic effects of sampling stress, as described previously.⁹¹

5.3 | Live-cell imaging

Perfusate saline solutions were prepared fresh each day (in mM: 132.3 NaCl, 3 KCl, 1.5 CaCl_2 , 1.5 MgCl_2 , 10 HEPES, 2.8 glucose; 300 mOsm). To mimic arterial blood conditions perfusates were equilibrated to 0.3 kPa, 21 kPa PO_2 in N_2 ,⁵⁸ from a pre-mixed gas cylinder (Praxair, Danbury, CT, USA). During equilibration with the gas at 15°C, solutions were adjusted to pH 8.00 with 0.1 M NaOH until stable. A subsample of 50 μL RBC suspension was pipetted onto a 0.1% poly-L lysine coated glass coverslip and left to settle for 10 min in a humidified chamber. Thereafter, the cover slip was rinsed three times with saline to remove cells that did not settle. The pH-sensitive fluorophore SNARF-1-AM (Invitrogen S22801, Waltham, MA, USA) was made up in DMSO and aliquots were diluted with fresh saline for a final concentration of 20 μM . This SNARF solution was pipetted (50 μL) onto the cover slip with settled RBCs and incubated in a humidified chamber for 1 h. Thereafter, the cover slip was rinsed three

times with saline and quickly mounted into a closed imaging chamber (Warner Instruments RC43C, Hamden CT, USA). The chamber was perfused with saline at 2 mL min^{-1} at 15°C from header tanks that were continuously bubbled with the equilibration gas. Immediately before each run, the drugs were added to the perfusates and pH was measured at 15°C (Fisher Accumet 13-620-850 electrode, Hampton, NY, USA; and Denver Instruments UB-10 meter, Bohemia, NY, USA). TCO_2 was measured in triplicate using a Corning 965 (Corning Instruments, Corning, NY, USA) and HCO_3^- was calculated using the Henderson-Hasselbalch equation and published pK_a and solubility values.⁵⁹

At the beginning of each trial, RBCs were perfused for 12 min with control saline (with 0.03% DMSO). Image acquisition was started after 10 min of perfusion. To induce an acid-base disturbance RBCs were perfused with a high-ammonia saline for 3 min (Am; replacing 30 mM NaCl with NH_4Cl), and then perfusion was switched back to the control saline for 3 min before the end of the trial. This ammonia pre-pulse technique⁹² loads the cells with NH_3 and NH_4^+ , where the diffusible NH_3 gas enters more rapidly, reacts with intracellular H^+ to form NH_4^+ , and causes an initial increase in RBC pH_i . Upon return to the control solution NH_3 diffuses out of the cells leaving behind H^+ and some NH_4^+ . The result is an intracellular acidification that must be corrected by the cells through membrane transport of acid-base equivalents and provides a mechanistic tool to study the cellular mechanisms involved in acid-base homeostasis.

To study the role of sAC in sensing intracellular acid-base disturbances, the above experiments were entirely performed in the presence of 25 μM KH7 (Tocris Bioscience, Minneapolis, MN, USA) or 50 μM LRE1 (Sigma-Aldrich, St. Louis, MO, USA), two specific, small-molecule inhibitors of sAC.^{46,47} To test which membrane transporters are involved in the recovery of RBC pH_i , additional experiments were performed with dimethyl amiloride (DMA; 30 μM ; Sigma) an inhibitor of NHE, bumetanide (Bum; 10 μM ; Sigma) an inhibitor of NKCC and KCC, and with 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS; Thermo Fisher Scientific, Waltham, MA, USA) an inhibitor of the AE. At high concentrations (exceeding 50 μM), DIDS has been shown to inhibit the enzymatic activity of recombinant sAC from rainbow trout¹⁸ and therefore, the specific inhibition of AE in intact RBCs was targeted with a low concentration of 10 μM DIDS. RBCs were only perfused with these membrane transporter inhibitors after the ammonia pre-pulse, to avoid confounding effects on acid loading. Finally, an additional series of experiments was run to investigate the intracellular cAMP signaling pathways downstream of sAC. Therefore, RBCs were perfused with 3-Isobutyl-1-methylxanthine (IBMX;

50 μM ; Sigma), an inhibitor of PDEs, H89 (1 μM ; Cayman Chemical, Ann Arbor, MI, USA) an inhibitor of PKA, or CE3F4 (25 μM ; Sigma) an inhibitor of EPAC1. All inhibitor stocks were made up in DMSO and stored at -80°C , but DIDS was made fresh each morning and kept on ice until use, because preliminary trials indicated a rapid loss of potency of DIDS stock solutions.

Imaging was with a confocal laser-scanning microscope (Zeiss Observer Z1 with LSM 800) with a 63x Plan-Apochromat objective and ZEN blue edition software v.2.6 (Carl Zeiss AG, Oberkochen, Germany). SNARF was excited with an argon laser at 488 nm and emitted fluorescence was recorded simultaneously at 586 and 639 nm every 1.3 s at 1024×1024 pixels and a 2 Airy unit pin-hole. The accumulation of SNARF in intracellular compartments allowed differentiating the nucleus (validated against 4',6-diamidino-2-phenylindole stained RBCs; DAPI Thermo Fisher; Waltham, MA, USA) and the mitochondria (see Figure S2 for validation). RBC pH_i was measured in three cells per run and separately for the nucleus and the cytoplasm by drawing regions of interest around these compartments (measurements in mitochondria were not possible due to the rapid movement in live RBCs; see Video S2). The ratio of the fluorescence signal at both wavelengths was used to generate the raw experimental data and the background signal was subtracted. At the end of each run, calibration curves were generated by perfusing the cells with high-potassium salines (in mM: 140 KCl, 1.5 CaCl₂, 1.5 MgCl₂, 10 HEPES, 2 NaCl; 300 mOsm) of known pH (measured at 15°C as described above), containing 10 μM nigericin (Thermo Fisher) to permeabilize the cells.³³ This calibration method was chosen (over a null-point calibration) in an attempt to improve the calibration results for the nuclear compartment by permeabilizing both plasma and nuclear membranes. Six calibration points were generated for each run, to determine the linear relationship between pH and the absorbance ratio of each region of interest. These individual calibration curves were then used to calculate RBC pH_i separately for each cell and compartment and the data of the three cells was averaged for each run. We validated our measured RBC pH_i values against the expected pH_i versus pH_e relationship ($\text{pH}_i = 0.858 \times \text{pH}_e + 0.899$) that we determined experimentally using the freeze-thaw method⁹³ on a separate group of freshwater rainbow trout, and that matches previously reported data using the DMO ($\text{pH}_i = 0.725 \times \text{pH}_e + 1.80$) or Cl^- distribution ($\text{pH}_i = 0.821 \times \text{pH}_e + 1.05$) methods.⁵²

5.4 | Haemoglobin–oxygen binding

RBCs were sampled and stored overnight as described above. The cells were rinsed three times and re-suspended

in Cortland's saline at 5% haematocrit. Aliquots of 300 μL were loaded into individual tonometers and equilibrated at 15°C to 21 kPa PO_2 and either 0.3 or 1% PCO_2 to induce a respiratory acidosis and treated either with 0.03% DMSO as a carrier control or 25 μM KH7 to inhibit sAC. After 1 h of equilibration, 2 μL of blood were loaded into a Blood Oxygen Binding System (BOBS Loligo, Viborg, Denmark) for the spectrophotometric measurement of Hb- O_2 binding characteristics. Oxygen equilibrium curves (OEC) were generated by stepwise increases in PO_2 (0.5, 1, 2, 4, 8, 16, 21 kPa) at 15°C . The absorbance of the blood was recorded once per second at 430 and 390 nm, which represent the O_2 dependent and O_2 independent (isosbestic) wavelengths in the absorption spectrum of Hb. The ratio between the recordings at these two wavelengths was used to generate the raw data. To correct for linear drift, two calibration steps were performed at the beginning and end of the run, each equilibrating blood to gas tensions that yielded full Hb oxygenation (99.7 kPa PO_2 , 0.3 kPa PCO_2) and deoxygenation (0 kPa PO_2 , 0.3 kPa PCO_2). These calibrations allowed the calculation of Hb- O_2 saturation from the ratio-metric raw data, as described previously.⁹¹

A separate trial was run to assess the effects of sAC inhibition on Hb- O_2 binding under dynamic in vivo-like conditions. RBCs were prepared as described above, equilibrated in tonometers (0.3 kPa PCO_2 and 21 kPa PO_2) and treated either with 0.25% DMSO as a carrier control or 25 μM KH7 to inhibit sAC. Samples of 2 μL were loaded into the BOBS and gas tensions were cycled every 4 min between arterial (0.3 kPa PCO_2 and 16 kPa PO_2) and venous conditions (1 kPa PCO_2 and 5.3 kPa PO_2) that mimic maximal exercise in rainbow trout.^{51,94}

5.5 | Immunocytochemistry

For RBC fixations, the above tonometry trials were repeated with cells resuspended in saline, as plasma proteins can interfere with the quality of antibody staining. RBCs were fixed and immunostained as previously described,⁹¹ with custom-made purified rabbit polyclonal antibodies raised against the C1 subunit of the rainbow trout sAC protein¹⁸ (Genscript Biotech Corporation, Piscataway, NJ, USA), monoclonal mouse antibodies against tetrahymena α -tubulin (DSHB12G10, Developmental Studies Hybridoma Bank, Iowa City, USA), cytochrome c oxidase IV (COX IV; mAbcam33985; Abcam, Boston, MA, USA) and the α -F1 subunit of ATP synthase (ATP5A, 15H4C4; Abcam), and DAPI (Thermo Fisher). Secondary antibodies were goat-anti-rabbit Alexa Fluor 647 or Alexa Fluor 568 for sAC, and goat-anti-mouse Alexa Fluor 488 for α -tubulin, ATP5A and COX IV (Invitrogen). Super-resolution images were acquired with the Zeiss AiryScan

detector system, on cells resuspended in PBS with a mounting medium (Invitrogen ProLong P36980) or embedded in 1% low-melt ultrapure agarose (Invitrogen) to prevent movement.⁹⁵ Three-dimensional (3-D) processing of optical sections and movie rendering was with the Imaris software v.9.0. (Oxford Instruments, Abingdon, UK).

5.6 | Data analysis and statistics

Data were analyzed in RStudio 2023.03.0 using Rv.4.0.4⁹⁶ and the ggplot2 package to generate all figures.⁹⁷ RBC baseline pH_i was calculated from the first 2 min of each recording. RBC minimum pH_i represents the lowest value recorded after the ammonia pre-pulse. RBC pH_i recovery rates were calculated by fitting a linear regression to the first 10 data points after the minimum pH_i and calculating the slopes for each run. H^+ fluxes were calculated by multiplying the pH_i recovery slope with the RBC buffer capacity as previously determined for human RBCs using the SNARF method.³³ For the Hb- O_2 binding experiments, the last ten Hb- O_2 saturation readings from each gas equilibration step were averaged and used for statistical analysis. The Hb- O_2 binding affinity (P_{50}) and cooperativity (n_H) were calculated from the parameters of a nonlinear Hill function that was fit to each run. Parametric assumptions were tested with the Shapiro–Wilkinson's and Levene's test for normality and homogeneity of variance, respectively. The main effects of drug additions (control, KH7, LRE1, and DIDS; or control, IBMX, H89, and CE3F4; or control, DMA, and Bum) were tested with a linear mixed model with fish as a random effect (repeated measures design; lme function in R). If significant main effects were detected, post-hoc analysis was performed using pairwise *t*-tests with a Benjamini–Hochberg correction of the significance level. The same analyses were applied to the measurements of Hb- O_2 binding, for the main effects of drug (control vs. KH7), conditions (arterial vs. venous) and their interaction. All data are reported as means $\pm$ SEM, for $N=6$ fish (unless stated otherwise) and conform to good publishing practice in physiology according to Acta Physiologica guidelines [Acta Physiol (Oxf). 2022;234(1):e13741. 10.1111/apha.13741.].

AUTHOR CONTRIBUTIONS

Till S. Harter: Conceptualization; investigation; validation; visualization; writing – original draft; formal analysis; data curation; supervision; methodology; writing – review and editing. **Emma A. Smith:** Investigation; formal analysis; writing – review and editing. **Cristina Salmerón:** Writing – review and editing; conceptualization. **Angus B.**

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All raw data and analysis scripts (R) are publicly available through figshare (10.6084/m9.figshare.26298889).

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REFERENCES

1. Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. *Proc Natl Acad Sci USA*. 1999;96(1):79–84.
2. Chen Y, Cann MJ, Litvin TN, et al. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science*. 2000;289(5479):625–628.

3. Zippin JH, Chen Y, Nahirney P, et al. Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. *FASEB J*. 2003;17(1):82-84.
4. Tresguerres M, Barott KL, Barron ME, Roa JN. Established and potential physiological roles of bicarbonate-sensing soluble adenylyl cyclase (sAC) in aquatic animals. *J Exp Biol*. 2014;217(5):663-672.
5. Cheng X, Zhenyu J, Tsalkova T, Mei F. Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Biochim Biophys Sin Shanghai*. 2008;40:651-662.
6. Cooper DMF. Regulation and organization of adenylyl cyclases and cAMP. *Biochem J*. 2003;375(3):517-529.
7. Zaccolo M. cAMP signal transduction in the heart: understanding spatial control for the development of novel therapeutic strategies. *Br J Pharmacol*. 2009;158(1):50-60.
8. Zaccolo M, Di Benedetto G, Lissandron V, Mancuso L, Terrin A, Zamparo I. Restricted diffusion of a freely diffusible second messenger: mechanisms underlying compartmentalized cAMP signalling. *Biochem Soc Trans*. 2006;34(4):495-497.
9. Brand T, Schindler R. New kids on the block: the Popeye domain containing (POPDC) protein family acting as a novel class of cAMP effector proteins in striated muscle. *Cell Signal*. 2017;40:156-165.
10. Matsuda Y, Nakajima K, Tachibana M. Recent progresses on the genetic basis of the regulation of CO₂ acquisition systems in response to CO₂ concentration. *Photosynth Res*. 2011;109(1):191-203.
11. Shiba K, Inaba K. Distinct roles of soluble and transmembrane adenylyl cyclases in the regulation of flagellar motility in ciona sperm. *Int J Mol Sci*. 2014;15(8):13192-13208.
12. Shiba K, Inaba K. The role of soluble adenylyl cyclase in the regulation of flagellar motility in ascidian sperm. *Biomol Ther*. 2023;13(11):1594.
13. Barott KL, Barron ME, Tresguerres M. Identification of a molecular pH sensor in coral. *Proc Biol Sci*. 2017;284(1866):20171769.
14. Nomura M, Beltrán C, Darszon A, Vacquier VD. A soluble adenylyl cyclase from sea urchin spermatozoa. *Gene*. 2005;353(2):231-238.
15. Lo M, Shahriari A, Roa JN, Tresguerres M, Farrell AP. Differential effects of bicarbonate on severe hypoxia- and hypercapnia-induced cardiac malfunctions in diverse fish species. *J Comp Physiol B*. 2021;191(1):113-125.
16. Wilson CM, Roa JN, Cox GK, Tresguerres M, Farrell AP. Introducing a novel mechanism to control heart rate in the ancestral Pacific hagfish. *J Exp Biol*. 2016;219(20):3227-3236.
17. Tresguerres M, Parks SK, Salazar E, Levin LR, Goss GG, Buck J. Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis. *Proc Natl Acad Sci USA*. 2010;107(1):442-447.
18. Salmerón C, Harter TS, Kwan GT, et al. Molecular and biochemical characterization of the bicarbonate-sensing soluble adenylyl cyclase from a bony fish, the rainbow trout *Oncorhynchus mykiss*. *Interface Focus*. 2021;11(2):20200026.
19. Carvalho ES, Gregório SF, Power DM, Canário AV, Fuentes J. Water absorption and bicarbonate secretion in the intestine of the sea bream are regulated by transmembrane and soluble adenylyl cyclase stimulation. *J Comp Physiol B*. 2012;182(8):1069-1080.
20. Tresguerres M, Levin LR, Buck J, Grosell M. Modulation of NaCl absorption by [HCO₃⁻] in the marine teleost intestine is mediated by soluble adenylyl cyclase. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(1):R62-R71.
21. von Schalburg KR, Gowen BE, Leong JS, Rondeau EB, Davidson WS, Koop BF. Subcellular localization and characterization of estrogenic pathway regulators and mediators in Atlantic salmon spermatozoal cells. *Histochem Cell Biol*. 2018;149(1):75-96.
22. Bohr C, Hasselbalch K, Krogh A. Über einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensäurespannung des blutes auf dessen Sauerstoffbindung übt. *Skand Arch Physiol*. 1904;16:402-412.
23. Krogh A, Leitch I. The respiratory function of the blood in fishes. *J Physiol*. 1919;52(5):288-300.
24. Root RW. The respiratory function of the blood of marine fishes. *Biol Bull*. 1931;61(3):427-456.
25. Damsgaard C, Lauridsen H, Harter TS, et al. A novel acidification mechanism for greatly enhanced oxygen supply to the fish retina. *eLife*. 2020;9:e58995.
26. Wittenberg JB, Wittenberg BA. Active secretion of oxygen into the eye of fish. *Nature*. 1962;194(4823):106-107.
27. Pelster B. Buoyancy at depth. In: Randall D, Farrell A, eds. *Fish Physiology*. Academic Press; 1997:195-238.
28. Harter TS, Zanuzzo FS, Supuran CT, Gamperl AK, Brauner CJ. Functional support for a novel mechanism that enhances tissue oxygen extraction in a teleost fish. *Proc Biol Sci*. 2019;286(1903):20190339.
29. Rummer JL, McKenzie DJ, Innocenti A, Supuran CT, Brauner CJ. Root effect hemoglobin may have evolved to enhance general tissue oxygen delivery. *Science*. 2013;340(6138):1327-1329.
30. Harter TS, May AG, Federspiel WJ, Supuran CT, Brauner CJ. The time-course of red blood cell intracellular pH recovery following short-circuiting in relation to venous transit times in rainbow trout, *Oncorhynchus mykiss*. *Am J Physiol Regul Integr Comp Physiol*. 2018;315(2):R397-R407.
31. Honig CR, Feldstein ML, Frierson JL. Capillary lengths, anastomoses, and estimated capillary transit times in skeletal muscle. *Am J Phys*. 1977;233(1):H122-H129.
32. Hughes GM, Horimoto M, Kikuchi Y, Kakiuchi Y, Koyama T. Blood flow velocity in microvessels of the gill filaments of the goldfish (*Carassius auratus* L.). *J Exp Biol*. 1981;90:327-331.
33. Swietach P, Tiffert T, Mauritz JMA, et al. Hydrogen ion dynamics in human red blood cells. *J Physiol*. 2010;588(24):4995-5014.
34. Boyarsky G, Ganz MB, Cragoe EJ, Boron WF. Intracellular-pH dependence of Na-H exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells. *Proc Natl Acad Sci USA*. 1990;87(15):5921-5924.
35. Buckler KJ, Vaughan-Jones RD. Application of a new pH-sensitive fluoroprobe (carboxy-SNARF-1) for intracellular pH measurement in small, isolated cells. *Pflugers Arch*. 1990;417(2):234-239.
36. Lagadic-Gossmann D, Buckler KJ, Vaughan-Jones RD. Role of bicarbonate in pH recovery from intracellular acidosis in the Guinea-pig ventricular myocyte. *J Physiol*. 1992;458(1):361-384.
37. Milligan CL, Wood CM. Intracellular and extracellular acid-base status and H⁺ exchange with the environment after exhaustive exercise in the rainbow trout. *J Exp Biol*. 1986;123:93-121.
38. Wood CM, Kajimura M, Mommsen TP, Walsh PJ. Alkaline tide and nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*). *J Exp Biol*. 2005;208(14):2693-2705.
39. Heisler N. Acid-base regulation in fish. In: Hoar WS, Randall DJ, eds. *Fish Physiology*. Academic Press; 1984:315-401.

40. Larsen BK, Jensen FB. Influence of ionic composition on acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. *Fish Physiol Biochem.* 1997;16(2):157-170.
41. Shartau RB, Damsgaard C, Brauner CJ. Limits and patterns of acid-base regulation during elevated environmental CO₂ in fish. *Comp Biochem Physiol A Mol Integr Physiol.* 2019;236:110524.
42. Wood CM, Milligan CL, Walsh PJ. Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. *Am J Physiol Regul Integr Comp Physiol.* 1999;277(2):R482-R492.
43. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol Rev.* 1967;47(4):595-781.
44. Rossetti T, Jackvony S, Buck J, Levin LR. Bicarbonate, carbon dioxide and pH sensing via mammalian bicarbonate-regulated soluble adenylyl cyclase. *Interface Focus.* 2021;11(2):20200034.
45. Balbach M, Rossetti T, Ferreira J, et al. On-demand male contraception via acute inhibition of soluble adenylyl cyclase. *Nat Commun.* 2023;14(1):637.
46. Bitterman JL, Ramos-Espiritu L, Diaz A, Levin LR, Buck J. Pharmacological distinction between soluble and transmembrane adenylyl cyclases. *J Pharmacol Exp Ther.* 2013;347(3):589-598.
47. Ramos-Espiritu L, Kleinboelting S, Navarrete FA, et al. Discovery of LRE1 as a specific and allosteric inhibitor of soluble adenylyl cyclase. *Nat Chem Biol.* 2016;12(10):838-844.
48. Roa JN, Tresguerres M. Soluble adenylyl cyclase is an acid-base sensor in epithelial base-secreting cells. *Am J Physiol Cell Physiol.* 2016;311(2):C340-C349.
49. Steinberg SF, Brunton LL. Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu Rev Pharmacol Toxicol.* 2001;41(1):751-773.
50. Carrie D, Gilmour KM. Phosphorylation increases the catalytic activity of rainbow trout gill cytosolic carbonic anhydrase. *J Comp Physiol B.* 2016;186(1):111-122.
51. Brauner CJ, Thorarensen H, Gallagher P, Farrell AP, Randall DJ. CO₂ transport and excretion in rainbow trout (*Oncorhynchus mykiss*) during graded sustained exercise. *Respir Physiol.* 2000;119(1):69-82.
52. Nikinmaa M. Control of red cell pH in teleost fishes. *Ann Zool Fenn.* 1986;23:223-235.
53. Houslay MD, Milligan G. Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem Sci.* 1997;22(6):217-224.
54. Speer KF, Allen-Waller L, Novikov DR, Barott KL. Molecular mechanisms of sperm motility are conserved in an early-branching metazoan. *Proc Natl Acad Sci USA.* 2021;118(48):e2109993118.
55. Kobayashi M, Ishigaki K, Kobayashi M, Imai K. Shape of the haemoglobin-oxygen equilibrium curve and oxygen transport efficiency. *Respir Physiol.* 1994;95(3):321-328.
56. Berenbrink M, Koldkjær P, Hannah Wright E, Kepp O, José da Silva A. Magnitude of the Root effect in red blood cells and haemoglobin solutions of fishes: a tribute to August Krogh. *Acta Physiologica.* 2011;202(3):583-592. <https://doi.org/10.1111/j.1748-1716.2010.02243.x>
57. Vermette MG, Perry SF. Adrenergic involvement in blood oxygen transport and acid-base balance during hypercapnic acidosis in the Rainbow Trout, *Salmo gairdneri*. *Journal of Comparative Physiology B.* 1988;158(1):107-115. <https://doi.org/10.1007/bf00692734>
58. Brauner CJ, Thorarensen H, Gallagher P, Farrell AP, Randall DJ. The interaction between O₂ and CO₂ exchange in rainbow trout during graded sustained exercise. *Respir Physiol.* 2000;119(1):83-96.
59. Boutilier RG, Heming TA, Iwama GK. Physicochemical parameters for use in fish respiratory physiology. In: Hoar WS, Randall DJ, eds. *Fish Physiology*. Academic Press; 1984:403-426.
60. Brauner CJ, Randall D. The linkage between oxygen and carbon dioxide transport. In: Perry SF II, Tufts BL, eds. *Fish Physiology*. Academic Press; 1998:283-320.
61. Romano L, Passow H. Characterization of anion transport system in trout red blood cell. *Am J Physiol Cell Physiol.* 1984;246(3):C330-C338.
62. Lapennas GN. The magnitude of the Bohr coefficient: optimal for oxygen delivery. *Respir Physiol.* 1983;54(2):161-172.
63. Brunati AM, Bordin L, Clari G, Moret V. The Lyn-catalyzed Tyr phosphorylation of the transmembrane Band-3 protein of human erythrocytes. *Eur J Biochem.* 1996;240(2):394-399.
64. Brunati AM, Bordin L, Clari G, et al. Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites. *Blood.* 2000;96(4):1550-1557.
65. Puchulu-Campanella E, Turrini FM, Li Y-H, Low PS. Global transformation of erythrocyte properties via engagement of an SH2-like sequence in band 3. *Proc Natl Acad Sci USA.* 2016;113(48):13732-13737.
66. Low PS, Allen DP, Zioncheck TF, et al. Tyrosine phosphorylation of band 3 inhibits peripheral protein binding. *J Biol Chem.* 1987;262(10):4592-4596.
67. Vince JW, Reithmeier RAF. Carbonic anhydrase II binds to the carboxyl terminus of human band 3, the erythrocyte Cl⁻/HCO₃⁻-exchanger*. *J Biol Chem.* 1998;273(43):28430-28437.
68. Vince JW, Reithmeier RAF. Identification of the carbonic anhydrase II binding site in the Cl⁻/HCO₃⁻ anion exchanger AE1. *Biochemistry.* 2000;39(18):5527-5533.
69. Jennings ML. Cell physiology and molecular mechanism of anion transport by erythrocyte band 3/AE1. *Am J Physiol Cell Physiol.* 2021;321:C1028-C1059. doi:10.1152/ajpcell.00275.2021
70. Donà G, Tibaldi E, Andrisani A, et al. Human sperm capacitation involves the regulation of the Tyr-phosphorylation level of the anion exchanger 1 (AE1). *Int J Mol Sci.* 2020;21(11):4063.
71. Nombela I, Lopez-Lorigados M, Salvador-Mira ME, et al. Integrated transcriptomic and proteomic analysis of red blood cells from rainbow trout challenged with VHSV point towards novel immunomodulant targets. *Vaccine.* 2019;7(3):63.
72. Tibbo AJ, Mika D, Dobi S, et al. Phosphodiesterase type 4 anchoring regulates cAMP signaling to Popeye domain-containing proteins. *J Mol Cell Cardiol.* 2022;165:86-102.
73. Santos JM, Martínez-Zaguilán R, Facanha AR, Hussain F, Sennoune SR. Vacuolar H⁺-ATPase in the nuclear membranes regulates nucleo-cytosolic proton gradients. *Am J Physiol Cell Physiol.* 2016;311(4):C547-C558.
74. Gould MC, Stephano JL. Nuclear and cytoplasmic pH increase at fertilization in *Urechis caupo*. *Dev Biol.* 1993;159(2):608-617.
75. Robey RB, Ruiz O, Santos AVP, et al. pH-dependent fluorescence of a Heterologously expressed Aequorea green fluorescent protein mutant: in situ spectral characteristics and applicability to intracellular pH estimation. *Biochemistry.* 1998;37(28):9894-9901.

76. Shen J, Zeng Y, Zhuang X, et al. Organelle pH in the Arabidopsis endomembrane system. *Mol Plant*. 2013;6(5):1419-1437.
77. Freeman SA, Grinstein S, Orlowski J. Determinants, maintenance, and function of organellar pH. *Physiol Rev*. 2023;103(1):515-606.
78. Mardones P, Medina JF, Elferink RPJO. Activation of cyclic AMP signaling in Ae2-deficient mouse fibroblasts*. *J Biol Chem*. 2008;283(18):12146-12153.
79. Zippin JH, Farrell J, Huron D, et al. Bicarbonate-responsive "soluble" adenylyl cyclase defines a nuclear cAMP microdomain. *J Cell Biol*. 2004;164(4):527-534.
80. Bucking C, Wood CM. The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J Exp Biol*. 2008;211(15):2533-2541.
81. Cooper CA, Wilson RW. Post-prandial alkaline tide in freshwater rainbow trout: effects of meal anticipation on recovery from acid-base and ion regulatory disturbances. *J Exp Biol*. 2008;211(15):2542-2550.
82. Jeffries KM, Teffer A, Michaleski S, Bernier NJ, Heath DD, Miller KM. The use of non-lethal sampling for transcriptomics to assess the physiological status of wild fishes. *Comp Biochem Physiol B Biochem Mol Biol*. 2021;256:110629.
83. Götting M, Nikinmaa MJ. Transcriptomic analysis of young and old erythrocytes of fish. *Front Physiol*. 2017;8:1046. doi:[10.3389/fphys.2017.01046](https://doi.org/10.3389/fphys.2017.01046)
84. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, Sprague RS. Erythrocytes: oxygen sensors and modulators of vascular tone. *Phys Ther*. 2009;24(2):107-116.
85. Shiva S, Castro L, Brookes PS. Mitochondria and nitric oxide. *Nitric Oxide*. 3rd ed. Academic Press; 2017:137-156.
86. Swenson ER. Kinetics of oxygen and carbon dioxide exchange. In: Boutilier RG, ed. *Advances in Comparative and Environmental Physiology*. Springer-Verlag; 1990:163-209.
87. D'Alessandro A, Dzieciatkowska M, Nemkov T, Hansen KC. Red blood cell proteomics update: is there more to discover? *Blood Transfus*. 2017;15(2):182-187.
88. Lange PF, Huesgen PF, Nguyen K, Overall CM. Annotating N termini for the human proteome project: N termini and N α -acetylation status differentiate stable cleaved protein species from degradation remnants in the human erythrocyte proteome. *J Proteome Res*. 2014;13(4):2028-2044.
89. Gallagher EJ, Harter TS, Wilson JM, Brauner CJ. The ontogeny of Na⁺ uptake in larval rainbow trout reared in waters of different Na⁺ content. *J Comp Physiol B*. 2021;191:29-42.
90. Wolf K. Physiological salines for fresh-water teleosts. *Prog Fish-Cult*. 1963;25:135-140.
91. Harter TS, Clifford AM, Tresguerres M. Adrenergically induced translocation of red blood cell β -adrenergic sodium-proton exchangers has ecological relevance for hypoxic and hypercapnic white seabass. *Am J Physiol Regul Integr Comp Physiol*. 2021;321(5):R655-R671.
92. Boron WF, De Weer P. Intracellular pH transients in squid giant axons caused by CO₂, NH₃, and metabolic inhibitors. *J Gen Physiol*. 1976;67(1):91-112.
93. Zeidler R, Kim HD. Preferential hemolysis of postnatal calf red cells induced by internal alkalization. *J Gen Physiol*. 1977;70(3):385-401.
94. Kiceniuk JW, Jones DR. The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. *J Exp Biol*. 1977;69:247-260.
95. Kauffman KM, Polz MF. Streamlining standard bacteriophage methods for higher throughput. *MethodsX*. 2018;5:159-172.
96. RStudioTeam. RStudio: Integrated Development Environment for R. 2021 <http://www.rstudio.com/>
97. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag; 2009.

SUPPORTING INFORMATION

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