Title:

Quantifying the potential for red blood cell β -adrenergic sodium-proton exchangers to protect oxygen transport in hypoxic and hypercapnic white seabass

Running title:

β -adrenergic protection of red blood cell oxygen transport

Authors:

Till S. Harter¹, Alexander M. Clifford and Martin Tresguerres²

Affiliations:

Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093, USA

¹corresponding author: tharter@ucsd.edu

²co-corresponding author: mtresguerres@ucsd.edu

Keywords:

β -NHE, Slc9a1b, carbon dioxide, hemoglobin, red tide, Bohr effect, Root effect, fish

ORCID IDs:

TSH 0000-0003-1712-1370

MT 0000-0002-7090-9266

Abstract:

White seabass (*Atractoscion nobilis*) are increasingly experiencing periods of low oxygen (O₂; hypoxia) and high carbon dioxide (CO₂, hypercapnia) due to climate change and eutrophication of the coastal waters of California. Hemoglobin (Hb) is the principal O₂ carrier in the blood and in many teleost fishes Hb-O₂ binding is compromised at low pH; however, the red blood cells (RBC) of some species regulate intracellular pH with adrenergically-stimulated sodium-proton-exchangers (β-NHE). We hypothesized that RBC β-NHEs in white seabass are an important mechanism that can protect the blood O₂-carrying capacity during hypoxia and hypercapnia. We determined the O₂-binding characteristics of white seabass blood, the response of RBCs to adrenergic stimulation, and quantified the protective effect of β-NHE activity on Hb-O₂ saturation. White seabass had typical teleost Hb characteristics, with a moderate O₂ affinity (PO₂ at half-saturation; P₅₀ 2.9 kPa) that was highly pH-sensitive (Bohr coefficient -0.92; Root effect 52%). The presence of RBC β-NHEs was confirmed by functional, molecular and bioinformatic data and super-resolution imaging revealed, for the first time, the subcellular location of β-NHE protein in vesicle-like structures and on the RBC membrane, and its translocation after adrenergic stimulation. The activation of RBC β-NHEs increased Hb-O₂ saturation by ~8% in normoxia at 1 kPa PCO₂, and by up to 20% in hypoxia. Our results confirm that RBC β-NHE activity in white seabass has great potential to protect arterial O₂ transport in environmentally relevant conditions of hypoxia and hypercapnia, but also reveal a potential vulnerability of fish to combinations of these stressors.

Introduction

White seabass (*Atractoscion nobilis*), a teleost fish species endemic to the coastal waters of California, are apex-predators with ecological significance, sought-after targets of recreational and commercial fisheries and are gaining importance in aquaculture. Their natural habitat along the kelp forests of the north-eastern Pacific is subject to strong seasonal fluctuations in water chemistry, due to the upwelling of deeper waters that are often depleted of oxygen (O_2 ; hypoxia), have a high carbon dioxide tension (CO_2 ; hypercapnia) and thus, a low pH (1). In addition, a steadily warming climate and growing anthropogenic nutrient loading are increasing the frequency of large algae blooms (“red tides”) in California’s coastal waters (2). When these algae blooms wane, the microbial decomposition of their biomass, consumes O_2 and produces CO_2 and other toxic and acidic by-products of biological decay, such as hydrogen sulfide, altogether creating large hypoxic and hypercapnic zones (3). Species that are highly mobile may be able to avoid these areas, but for many sedentary species, survival will depend on enduring these conditions. Climate change at large is also leading to more hypoxic and acidic oceans and, over generations, some species may adapt to cope with their altered habitats (4). However, the reoccurrence of upwelling and severe algae blooms may acutely expose animals to conditions that far exceed worst-case predictions for the end of the century, creating strong selective pressures for hypoxia and hypercapnia tolerance and perhaps overwhelming the rates at which some species can adapt to climate change.

The most recent severe red tide in Southern California occurred in April-May of 2020, when the water measurements at the pier of the Scripps Institution of Oceanography (SIO) in La Jolla (CA, USA) revealed average daily dissolved O_2 levels of $<2 \text{ mg l}^{-1}$ and pH as low as 7.06 (5). At a water temperature of 17°C and 35 ppt salinity, these values correspond to 5.3 kPa PO_2 (6) and 1.16 kPa PCO_2 (CO2SYS software; , 7). Equally alarming was the prolonged duration of hypoxia, where for nine consecutive days water PO_2 was below the threshold (4.6 mg l^{-1}) that is considered lethal for 90% of marine life (8). The SIO aquatics facility is supplied with water taken in at the pier, which resulted in hypoxic and hypercapnic exposures of all research animals, despite every effort to aerate the tanks. However unfortunate, this natural experiment revealed a remarkable tolerance of white seabass to these adverse water conditions, despite being deprived the behavioral avoidance of hypoxia that may be recruited in the wild. Therefore, the aim of the present study was to explore the O_2 -transport capacity of white seabass with a focus on the

cellular mechanisms at the level of the red blood cell (RBC) that may contribute to their hypoxia and hypercapnia tolerance.

For obligate aerobic animals, the challenge to surviving unavoidable environmental hypoxia is balancing the uptake and delivery of O₂ with its consumption in the mitochondria (9). Hemoglobin (Hb) is the principal O₂ carrier in the blood and therefore the cardiovascular O₂-carrying capacity is largely determined by the O₂-binding characteristics of Hb. As such, a higher Hb-O₂ affinity will favor the extraction of O₂ from hypoxic waters and thus, a lower Hb P₅₀ (the partial pressure of O₂ at which Hb is 50% saturated) is typically associated with hypoxia tolerance in fishes (10, 11); however, whether white seabass have high-affinity Hbs that would confer some hypoxia tolerance is currently unknown.

Hb-O₂ binding in teleost fishes is highly pH-sensitive, where a reduction in pH decreases Hb-O₂ affinity via the Bohr effect (12), and the Root effect prevents Hb from becoming fully O₂-saturated at low pH, even at super-atmospheric PO₂ (13, 14). The reduction in Hb-O₂ carrying capacity due to the Root effect is physiologically significant, as it enhances the unloading of O₂ at the eyes and the swimbladder of teleosts, where blood is acidified locally (15–17). In contrast, during a systemic blood acidosis that may occur during exercise or hypoxia, the pH-sensitive Hbs of teleosts may fail to become fully oxygenated at the gills, decreasing the O₂-carrying capacity of arterial blood and leading to hypoxemia at the tissues. Thus, a combined hypoxic and hypercapnic exposure may be especially dangerous for teleosts, as a reduced availability of O₂ in the environment is paired with the simultaneous reduction of Hb-O₂ affinity via the Bohr effect at low pH; however, whether white seabass have pH-sensitive Hbs is currently unknown.

Hb is housed within RBCs that, in teleosts, may prevent systemic hypoxemia by actively regulating their intracellular pH (pH_i) to protect Hb-O₂ binding during a reduction in extracellular pH (pH_e). In brief, a decrease in arterial PO₂ or pH leads to the release of catecholamines into the blood (18, 19), which bind to a β-adrenergic receptor on the RBC membrane and activate a sodium-proton-exchanger (β-NHE, Slc9a1b,) via the cyclic adenosine monophosphate (cAMP) pathway (20). The extrusion of H⁺ by the β-NHE raises pH_i above the equilibrium condition, which increases Hb-O₂ affinity and will promote the extraction of O₂ from hypoxic waters (21). The adrenergic stimulation of RBCs also causes an influx of Na⁺ and Cl⁻ that leads to osmotic swelling and that has been used as a marker to determine the presence of RBC β-NHEs in fish species (22, 23). A broader phylogenetic analysis indicates that most

teleosts, but not other fishes, have RBC β -NHEs (24); however, whether white seabass RBCs have β -NHE activity is currently unknown.

Based on these considerations, we hypothesized that β -NHE activity in white seabass is an important mechanism that can protect the blood O_2 -carrying capacity during environmentally relevant levels of hypoxia and hypercapnia ($PO_2 < 5.3$ kPa and $PCO_2 < 1.16$ kPa; see above). We tested this hypothesis in a series of *in vitro* experiments and predicted that: i) white seabass have a high Hb- O_2 affinity to maintain O_2 uptake under hypoxic conditions, which was addressed by generating oxygen equilibrium curves (OEC) over a range of PO_2 ; ii) white seabass display the large Bohr and Root effects that are typical of teleosts, which was addressed by generating OECs over a range of PCO_2 , and measuring pH_e and RBC pH_i ; iii) white seabass have a RBC β -NHE, which was addressed using molecular, bioinformatic and immunocytochemical techniques to establish its presence and localization, and by measuring RBC swelling after adrenergic stimulation and the inhibition of NHEs with amiloride; and finally iv) we quantified the protective effect of RBC β -NHE activity on blood O_2 -carrying capacity under environmentally relevant conditions, by measuring Hb- O_2 saturation at increasing levels a hypercapnia in normoxia and hypoxia.

Materials and Methods

Animals and husbandry

White seabass (*A. nobilis*, Ayres 1860) were obtained from the Hubbs Sea World Research Institute (HSWRI, Carlsbad, USA) and were held indoors at the SIO aquatics facility for several months before experiments. Photoperiod was set to a 12:12 h light-dark cycle and fish were housed in large fiberglass tanks (~3.5-10 m³) supplied with flow-through seawater from an inshore intake; the average water temperature at the time of experiments was 17°C. Aeration was provided to ensure normoxic conditions in all tanks (>90% air saturation of O₂) and these water parameters were monitored every day. All fish were fed twice a week with commercial dry pellets (Skretting; Classic Bass 9.5 mm; Stavanger, Norway) and feeding was suspended 48 h before blood sampling. The white seabass used for the determination of blood O₂-binding characteristics had an average weight of 1146±96 g (*N* = 8), while those used for the β-NHE experiments had an average weight of 357±27 g (*N* = 6). Animal husbandry and all experimental procedures were in strict compliance with the guidelines by the Institutional Animal Care and Use Committee (IACUC) and approved by the Animal Care Program at the University of California San Diego (Protocol no. S10320).

Blood sampling

White seabass were moved individually into darkened boxes supplied with air and flow-through seawater, 24 h prior to blood sampling. The next day the water supply was shut off and the fish were anesthetized by carefully pouring a diluted benzocaine solution (Fisher Scientific, Acros 150785000; Waltham, USA; concentrated stock made up in ethanol) into the box without disturbing the fish, for a final concentrations of 70 mg l⁻¹ benzocaine (<0.001% ethanol). After visible loss of equilibrium, fish were transferred to a surgery table, positioned ventral-side-up and their gills were perfused with water containing a maintenance dose of anesthetic (30 mg l⁻¹ benzocaine). Blood sampling was by caudal puncture and 3 ml of blood were collected into a heparinized syringe. This procedure ensured minimal disturbance of the fish (25), which can decrease blood pH due to air-exposure (respiratory acidosis) and due to anaerobic muscle contractions during struggling (metabolic acidosis). After sampling, the fish were recovered and returned to their holding tank, and each individual was only sampled once. In the lab, the blood was centrifuged at 500 g for 3 min to separate the plasma from the blood cells. The plasma was collected in a bullet tube and stored over-night at 4°C. To remove any catecholamines released

during sampling, the blood cells were rinsed three times in cold Cortland's saline (in mM: NaCl 147, KCl 5.1, CaCl 1.6, MgSO₄ 0.9, NaHCO₃ 11.9, NaH₂PO₄ 3, glucose 5.6; adjusted to the measured plasma characteristics in white seabass of 345 mOsm and pH 7.8) and the buffy coat was aspirated generously to remove white blood cells and platelets. Finally, the pellet was re-suspended in 10 volumes of fresh saline to allow the RBCs to return to a resting state and stored aerobically on a tilt-shaker, over-night, at 17°C (26).

Blood O₂-binding characteristics

The next day, RBCs were rinsed with saline three times and re-suspended in their native plasma at a hematocrit of 5%; this value was chosen based on preliminary trials and yields an optic density that allows for spectrophotometric measurements of Hb-O₂ binding characteristics (~0.6 mM Hb). A volume of 1.4 ml of blood was loaded into a glass tonometer at 17°C and equilibrated to arterial gas tensions (21 kPa PO₂, 0.3 kPa PCO₂ in N₂) from a custom-mixed gas cylinder (Praxair; Danbury, USA). After one hour, 2 µl of blood were removed from the tonometer and loaded into the diffusion chamber of a spectrophotometric blood analyzer (BOBS, Loligo Systems; Viborg, Denmark). The samples were equilibrated to increasing PO₂ tensions (0.5, 1, 2, 4, 8, 16 and 21 kPa PO₂) from a gas mixing system (GMS, Loligo), in two-minute equilibration steps and the absorbance was recorded once every second at 190-885 nm. At the beginning and end of each run, the sample was equilibrated to high (99.7 kPa PO₂, 0.3 kPa PCO₂ in N₂ for 8 min) and low (0 kPa PO₂, 0.3 kPa PCO₂ in N₂ for 8 min) PO₂ conditions; for the calculation of Hb-O₂ saturation from raw absorbance values, it was assumed that Hb was fully oxygenated or deoxygenated under the two conditions, which was confirmed by inspecting the absorption spectra (all raw data are deposited online). A PCO₂ of 0.3 kPa was maintained throughout these trials to prevent RBC pH_i from increasing above physiologically relevant levels and this value was chosen to match that measured in the arterial blood of rainbow trout (*Oncorhynchus mykiss*) *in vivo* (27). All custom gas mixtures were validated by measuring PO₂ with an FC-2 Oxzilla and PCO₂ with a CA-10 CO₂ analyzer (Sable Systems, North Las Vegas, USA) that were calibrated daily against high purity N₂, air, or 5% CO₂ in air.

An additional 250 µl of blood were removed from the tonometer to measure blood parameters as follows. Hematocrit (Hct) was measured in triplicate in microcapillary tubes (Drummond Microcaps, 15 µl; Parkway, USA), after centrifuging at 10,000 g for 3 min. Hb was measured in triplicate using the cyano-methemoglobin method (Sigma-Aldrich Drabkin's

D5941; St. Louis, USA) and an extinction coefficient of 10.99 mmol cm⁻¹ (28). Blood pH was measured with a thermostatted microcapillary electrode at 17°C (Fisher Accumet 13-620-850; Hampton, USA; with Denver Instruments UB-10 meter; Bohemia, USA), calibrated daily against precision pH buffers (Radiometer S11M007, S1M004 and S11M002; Copenhagen, Denmark). Thereafter, the blood was centrifuged to separate plasma and RBCs and total CO₂ content (TCO₂) of the plasma was measured in triplicate with a Corning 965 (Midland, USA). The RBCs in the pellet were lysed by three freeze-thaw cycles in liquid nitrogen and pH_i was measured in the lysate as described for pH_e (29). After completing these measurements, the PCO₂ in the tonometer was increased in steps from 0.3 to 2.5 kPa and, each time, OECs and blood parameters were measured as described above.

RBC swelling after β-adrenergic stimulation

After storage of blood samples over-night in saline, the RBCs were rinsed three times in fresh saline and re-suspended in their native plasma at a Hct of 25%. A volume of 1.8 ml was loaded into a tonometer and equilibrated to 3 kPa PO₂ and 1 kPa PCO₂ in N₂ at 17°C for one hour; similar hypoxic and acidotic conditions have been shown to promote β-NHE activity in other teleost species (30, 31). After one hour, an initial subsample of blood was taken and Hct, Hb, pH_e and pH_i were measured as described above. Thereafter, the blood was split into aliquots of 600 µl that were loaded into individual tonometers and treated with either: i) a carrier control (0.25% dimethyl sulfoxide, DMSO; VWR BDH 1115; Radnor, USA), ii) the β-adrenergic agonist isoproterenol (ISO; Sigma I6504; 10 µM final concentration, which stimulates maximal β-NHE activity in rainbow trout; , 19), or iii) ISO plus the NHE inhibitor amiloride (ISO+Am; Sigma A7410; 1 mM, according to 32). These treatments were staggered so that samples from each tonometer could be taken for the measurements of blood parameters at 10, 30 and 60 mins after drug additions.

To collect RBC samples for immunocytochemistry, the above tonometry trial was repeated with RBCs that were suspended in saline instead of plasma; this step was necessary as initial trials showed that plasma proteins interfered with the quality of cell fixations. Subsamples were removed from individual tonometers at the initial and 60 min time points. A volume of 60 µl was immediately re-suspended in 1.5 ml ice-cold fixative (3% paraformaldehyde, 0.175 % glutaraldehyde in 0.6 x phosphate buffered saline with 0.05 M sodium cacodylate buffer; made up from Electron Microscopy Sciences RT15949, Hatfield, USA) and incubated for 60 min on a

revolver rotator at 4°C. After fixation, cells were washed three times in 1 x phosphate Buffered Saline (PBS, Corning 46-013-CM, Corning, USA) and stored at 4°C for processing (after visual inspection of cell morphology the fixation resulted in satisfactory results for $N = 4$ out of 6 fish). An additional subsample of 100 µl was removed from the tonometers and centrifuged to remove the saline. The RBC pellet was re-suspended in 5 volumes of lysis buffer containing 1 mM DL-Dithiothreitol (DTT; Thermo Fisher R0861; Waltham, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma P7626) and 10 mM benzamidine hydrochloride hydrate (BHH; Sigma B6506) in PBS. The RBCs were lysed by three cycles of freeze-thawing in liquid nitrogen, the lysate was centrifuged at 500 g for 10 min at 4°C and the supernatant was frozen at -80°C for Western blot processing.

Hb-O₂ binding after β-adrenergic stimulation

An additional aliquot of RBCs was re-suspended in native plasma at a Hct of 5%. Volumes of 300 µl were loaded into one of four tonometers and equilibrated to arterial gas tensions at 17°C (as described previously) and treated with either: i) a carrier control (DMSO; 0.25%), ii) ISO (10 µM), or iii) ISO+Am (1 mM). These treatments were staggered to allow for standardized measurements at 60 min after drug additions, when 2 µl of blood were removed from the tonometer and loaded into the BOBS for real-time measurements of Hb-O₂ saturation during a respiratory acidosis. Therefore, the blood was exposed to stepwise increases in PCO₂ (0.3, 0.5, 1, 1.5, 2 and 3 kPa) allowing for two minutes of equilibration at each step; preliminary trials showed full equilibration to the new PCO₂ after ~1 min and absorbance remained constant thereafter. As described previously, this protocol also included initial and final calibration steps, at which the sample was fully O₂-saturated and then desaturated. A first trial was performed in normoxia (21 kPa PO₂) and then a second sample was loaded from the same tonometer for an additional run under hypoxic conditions (3 kPa PO₂). The PO₂ value in these hypoxic runs was chosen to yield a Hb-O₂ saturation close to P₅₀ and was informed from the previous measurements of Hb-O₂ binding characteristics. Finally, 250 µl of blood were removed from the tonometer for the measurement of blood parameters, as described previously.

Subcellular localization of RBC β-NHE

Fixed RBCs were permeabilized in 1.5 ml 0.1% triton-X100 (VWR Amresco 1421C243) in PBS for 15 min at room temperature on a revolver rotator. Thereafter, the RBCs were blocked for auto-fluorescence in 100 mM glycine in PBS for 15 min, after which the cells were rinsed

three times in PBS. For immunocytochemistry, 200 μ l of these fixed RBCs were re-suspended in a blocking buffer containing 3% bovine serum albumin (VWR 0332) and 1% normal goat serum (Lampire Biological Laboratories 7332500; Pipersville, USA) in PBS and incubated for six hours on a rotator. Primary antibodies were added directly into the blocking buffer and incubated on a rotator over-night at 4°C. A monoclonal mouse anti-*Tetrahymena* α -tubulin antibody (deposited by Frankel, J. and Nelsen, E.M at the Developmental Studies Hybridoma Bank, DSHB12G10; Iowa City, USA) was used at 0.24 μ g ml⁻¹ and a custom, polyclonal, affinity-purified, rabbit anti-rainbow trout β -NHE (epitope: MERRVSVMERMSH) was used at 0.02 μ g ml⁻¹. After primary incubations the RBCs were washed three times in PBS and incubated for three hours on a rotator at room temperature in blocking buffer containing secondary antibodies: 1:500 goat anti-mouse (AlexaFluor 568; Thermo Fisher Life Technologies A-11031), 1:500 goat anti-rabbit (AlexaFluor 488; A-11008) and 1:1000 4',6-diamidino-2-phenylindole (DAPI; Roche 10236276001; Basel, Switzerland). After secondary incubations, RBCs were washed three times and were re-suspended in PBS. To validate the β -NHE antibody, controls were performed by leaving out the primary antibody and by pre-absorbing the primary antibody with its pre-immune-peptide. All images were acquired with a confocal laser-scanning fluorescence microscope (Zeiss Observer Z1 with LSM 800, Oberkochen, Germany) and ZEN blue edition software v.2.6. For super-resolution imaging the cells were re-suspended in PBS with a mounting medium (Thermo Fisher Invitrogen ProLong P36980) and acquisition was with the Zeiss AiryScan detector system. To ensure that images were comparable, the acquisition settings were kept identical between the different treatments and between treatments and the controls. Optical sectioning and three-dimensional (3D) reconstructions of single RBCs from the different treatments were processed with the Imaris software v.9.0. (Oxford Instruments, Abingdon, UK) and rendered into movies.

Molecular β -NHE characterization

For Western blotting, RBC crude homogenates were thawed and centrifuged at 16,000 g for one hour at 4°C to obtain a supernatant containing the cytoplasmic fraction and a pellet containing a membrane-enriched fraction that was re-suspended in 100 μ l of lysis buffer. The protein concentration of all three fractions was measured with the Bradford's assay (BioRad 5000006; Hercules, USA). Samples were mixed 1:1 with Laemmli's sample buffer (BioRad 161-0737) containing 10% 2-Mercaptoethanol (Sigma M3148) and were heated to 75°C for 15 min.

Sample loading was at 5 μg protein from each fraction for the detection of β -NHE and 60 μg protein from crude homogenate for the detection of α -tubulin, into the lanes of a 5% stacking- and 10% separating polyacrylamide gel (Biorad, MiniProtean Tetra cell). The proteins were separated at 60 V for 30 min and 150 V until the Hb fraction (~ 16 kDa) ran out the bottom of the gel (~ 60 min); previous trials had shown that the high Hb content of these lysates may bind some antibodies non-specifically. The proteins were transferred onto a Immun-Blot polyvinylidene difluoride membrane (PVDF; BioRad) using a semi-dry transfer cell (Bio-Rad Trans-Blot SD) over-night, at 90 mA and 4°C . PVDF membranes were blocked over-night, on a shaker at 4°C in tris-buffered saline with 1% tween 20 (TBS-T; VWR Amresco ProPure M147) and 0.1 g ml^{-1} skim milk powder (Kroger; Cincinnati, USA). Primary antibodies were made up in blocking buffer and mixed on a shaker at 4°C , over-night, before applying to the membranes. The anti- α -tubulin antibody was used at 4.7 ng ml^{-1} , the anti- β -NHE antibody at 0.42 ng ml^{-1} , and controls at a peptide concentration exceeding that of primary antibody by 10:1. Primary incubations were for four hours on a shaker at room temperature and membranes were rinsed three times in TBS-T for 5 min. Secondary incubations were with either an anti-rabbit or mouse, horse-radish peroxidase conjugated secondary antibody (BioRad 1706515 and 1706516) for three hours on a shaker at room temperature. Finally, the membranes were rinsed three times in TBS-T for 5 min and the proteins were visualized by enhanced chemiluminescence (BioRad, Clarity 1705061) in a BioRad Universal III Hood with Image Lab software v.6.0.1. Protein sizes were determined relative to a precision dual-colour protein ladder (BioRad 1610374).

The white seabass β -NHE sequence was obtained by transcriptomics analysis of gill samples that were not perfused to remove the blood and these combined gill and RBC tissue samples were stored in RNA later for processing. Approximately 50 μg of sample were transferred into 1 ml of Trizol reagent (Thermo Fisher 15596026) and were homogenized on ice with a handheld motorized mortar and pestle (Kimble Kontes, Dusseldorf, Germany). These crude homogenates were centrifuged at 1000 g for 1 min and the supernatant was collected for further processing. RNA was extracted in RNA spin columns (RNAEasy Mini; Qiagen, Hilden, Germany) and treated with DNase I (ezDNase; Thermo Fisher, 11766051) to remove traces of genomic DNA. RNA quantity was determined by spectrophotometry (Nanodrop 2000; Thermo Fisher) and RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent; Santa Clara, USA). Poly-A enriched complementary DNA (cDNA) libraries were constructed using the

TruSeq RNA Sample Preparation Kit (Illumina; San Diego, USA). Briefly, mRNA was selected against total RNA using Oligo(dt) magnetic beads and the retained RNA was chemically sheared into short fragments in a fragmentation buffer, followed by first- and second-stand cDNA synthesis using random hexamer primers. Illumina adaptor primers (Forward P5-Adaptor, 5'AATGATACGGCGACCACCGAGA3'; Reverse P7-Adaptor 5' CGTATGCCGTCTTCTGCTTG 3') were then ligated to the synthesized fragments and subjected to end-repair processing. After agarose gel electrophoresis, 200-300 bp insert fragments were selected and used as templates for downstream PCR amplification and cDNA library preparation. The combined gill and RBC samples (1 µg RNA) were sent for RNAseq Poly-A sequencing with the Illumina NovoSeq™ 6000 platform (Novogene; Beijing, China) and raw reads are made available on NCBI (PRJNA722314).

RNAseq data was used to generate a *de novo* transcriptome assembly which was mined for white seabass isoforms of the Slc9a1 protein family using methods previously described (33). Briefly, raw reads were analyzed, trimmed of adaptor sequences, and processed with the OpenGene/fastp software (34), to remove reads: i) of low quality (PHRED quality score < 20), ii) containing >50% unqualified bases (base quality < 5), and iii) with >10 unknown bases. Any remaining unpaired reads were discarded from downstream analysis and quality control metrics were carried out before and after trimming (raw reads 80.07 x 10⁶; raw bases 12.01 Gb; clean reads 79.44 x 10⁶, clean bases 11.84 Gb, clean reads Q30 95.26%; GC content 46.67%). Thereafter, fastq files were merged into a single data set, normalized, and used for *de novo* construction of a combined gill and RBC transcriptome using the Trinity v2.6.6 software. Normalization and assembly were performed using the NCGAS (National Centre for Genome Analysis Support) *de novo* transcriptome assembly pipeline (github.com/NCAS/de-novo-transcriptome-assembly-pipeline/tree/master/Project_Carbonate_v4) on the Carbonate High Performance Computing cluster at Indiana University. For assembly, minimum kmer coverage was set to three and the minimum number of reads needed to glue two inchworm contigs together, was set to four (35). The resulting nucleotide FASTA file was translated into six protein reading frames using BBMap (36), which were mined for the NHE-like proteins using HMMER3 v.3.0 (hmmer.org) by querying the *de novo* assembly against a hidden markov model (HHM) homology matrix generated from 132 aligned protein sequences of the vertebrate NHE family (Slc9a1 – Slc9a9; for accession numbers see Table S1 of the supplement at:

doi.org/10.6084/m9.figshare.14934405.v1). Sequences were aligned using MUSCLE (37) in SeaView (38, 39), with NHE2 from *Caenorhabditis elegans* as an outgroup, and results were refined using GBlocks (40) according to the parameters specified previously (41). Phylogenetic analysis was conducted on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (42) using the RAxML software v.8.2.12 (43) with the LG evolutionary and GTRGAMMA models (44). Branch support was estimated by bootstraps with 450 replications and the constructed tree was edited in FigTree v.1.4.4. Finally, the open reading frame of the white seabass β -NHE sequence (predicted 747 amino acids), was analyzed for the presence of the Kozak nucleic acid motifs (5'-(gcc)gccRccAUGG-3'; 45) immediately upstream of putative start codons, using the ATGpr software (46).

To confirm the expression of the β -NHE in the RBCs of white seabass, an additional blood sample of 1 ml was collected and processed as described previously. RBCs were lysed by repeatedly passing them through a 23G needle and RNA extraction was on 50 μ g of RBCs by standard Trizol and chloroform extraction following the kit instructions. Isolated RNA was treated with DNase I and 1 μ g RNA was used to synthesize first-strand cDNA using SuperScript IV reverse transcriptase (Thermo Fisher 18090010). Full-length cDNA sequences were obtained in 35 cycles of PCR reactions with Phusion DNA polymerase (New England Biolabs, Ipswich, USA; MO531L) and specific primers designed against the sequence of the phylogenetically characterized white seabass β -NHE obtained from the combined gill and RBC transcriptome (Integrated DNA Technologies, Coralville, Iowa; F: 5'TCC CGT ACT ATC CTC ATC TTC A-3' R: 5'-CCT CTG CTC TCT GAA CTG TAA AT-3'). Amplicons were analyzed by gel electrophoresis (Bio-Rad ChemiDoc) that confirmed the presence of a single band (2372 bp; Fig. S1). A-overhangs were added to Phusion products with one unit Taq polymerase (New England Biolabs; MO267S) followed by 10 min incubation at 72°C. Products were cloned (TOPO TA Cloning Kit/pCR 2.1-TOPO Vector; Invitrogen; K4500) and the ligated product was transformed into TOP10 chemically competent *E. coli* cells (Invitrogen; K457501) according to manufacturer specifications. Following over-night incubation at 37°C, single colonies of transformants were grown in Luria-Bertani (LB) broth over-night on a shaking incubator (37°C, 220 rpm; Barnstead MaxQ 4000). Plasmid DNA was isolated (PureLink Quick Plasmid Miniprep kit; Invitrogen K210010) according to manufacturer specifications and inserts were sequenced to confirm their identity and uploaded to NCBI (MW962257).

Calculations and statistical analysis

All data were analyzed with R v.4.0.4 (47) in RStudio v.1.4.1106 (48) and figures were created with the ggplot2 package (49). Normality of the residuals was tested with the Shapiro-Wilk test (stat.desc function in R) and homogeneity of variances was confirmed with the Levene's test (leveneTest function in R). Deviations from these parametric assumptions were corrected by transforming the raw data. All R source code is made publicly available (doi.org/10.6084/m9.figshare.14934405.v1).

To determine the blood O₂-binding characteristics, Hb P₅₀ and n_H values were those determined with the BOBS software v.1.0.20 (Loligo) and oxygen equilibrium curves (OEC) were generated by fitting a two parameter Hill function to the mean P₅₀ and n_H for 8 individual fish. The main effects of PCO₂ on P₅₀ and n_H were analyzed with ACOVA ($P < 0.05$, $N = 8$). Plasma [HCO₃⁻] was calculated from TCO₂ by subtracting the molar [CO₂] calculated from the dissociation constant and solubility coefficients in plasma at 17°C and the corresponding sample pH (6). The Bohr effects relative to pH_i and pH_e, the relationship between RBC pH_i and pH_e and the non-bicarbonate buffer capacity of whole blood were determined by linear regression analysis, results of which are shown in detail in the supplement (Fig. S2A-D). The average values for these blood characteristics are shown in the main text and were calculated as the average slopes across all individuals.

In the RBC swelling trial, mean cell Hb was calculated as [Hb] divided by Hct as a decimal. Since Hb is a membrane impermeable solute, MCHC is used as a common indicator of RBC swelling. Main effects of drugs (DMSO, ISO and ISO+Am), time (10, 30 and 60 min) and their interaction (drug×time) on Hct, [Hb], MCHC, pH_e and pH_i were determined by two-way ANOVAs (lm and Anova functions in R; $N = 5-6$; $P < 0.05$) and multiple comparisons were conducted with t-tests (pairwise.t.test function in R) and controlling the false detection likelihood (FDR) with a Benjamini-Hochberg correction (p.adjust function in R).

The effect of RBC β-adrenergic stimulation on Hb-O₂ binding was assessed by analyzing the absorbance data from the BOBS in R (R source code available at github.com/tillharter/White-Seabass-beta-NHE). In brief, the absorbances recorded at 430 nm were divided by the isosbestic wavelength of 390 nm (where absorbance is independent of Hb-O₂ binding), and these ratios were used as the raw data for subsequent analyses. For each trace, the ten final absorbance ratios at each equilibration step were averaged (i.e. 10 s) and Hb-O₂ saturation was calculated relative

to the absorbance at the two initial calibration conditions (i.e. high: 99.7 kPa PO₂, 0.3 kPa PCO₂; and low: 0 kPa PO₂, 0.3 kPa PCO₂), assuming full saturation or desaturation of Hb, respectively. These calibration values were measured again at the end of each trial and a linear correction of drift was performed for each sample. The resulting values for Hb-O₂ saturation were plotted against PCO₂ and several non-linear models were fit to the data (Michaelis-Menten, Exponential and Hill). The model with the best fit (lowest AIC) was a three-parameter Hill function that was applied to each individual trace. The parameter estimates from this model yielded the maximal reduction in Hb-O₂ saturation (Max. ΔHb-O₂ sat.; %) in normoxia (21 kPa PO₂) and hypoxia (3 kPa PO₂), and the PCO₂ at which this response was half-maximal (EC₅₀PCO₂; kPa). The main effects of drugs (DMSO, ISO and ISO+Am), O₂ (normoxia and hypoxia) and their interaction (drug×O₂), on the parameter estimates from the Hill functions were determined by two-way ANOVAs (lm and Anova functions in R; *N* = 6; *P* < 0.05). When significant main effects were detected, multiple comparisons were conducted with t-tests (pairwise.t.test function in R) and controlling the false detection likelihood (FDR) with a Benjamini-Hochberg correction (p.adjust function in R). The Root effect was determined from the non-linear model, as the Max. ΔHb-O₂ sat. of the control treatment (DMSO). To quantify the relative changes in Hb-O₂ saturation (ΔHb-O₂ sat.) due to drug treatments, data were expressed relative to the paired measurements in the DMSO treatment for each individual fish and relative to the initial Hb-O₂ saturation at 0.3 kPa PCO₂ (i.e. 95.6 and 55.0% Hb-O₂ saturation in normoxia and hypoxia, respectively). All data are presented as means±s.e.m.

Results

Blood O₂-binding characteristics

The blood O₂-binding characteristics of white seabass are summarized in Figure 1. When PCO₂ was increased from arterial tension (0.3 kPa) to severe hypercapnia (2.5 kPa) Hb P₅₀ increased significantly from 2.9±0.1 to 11.8±0.3 kPa. At the same time, the cooperativity of Hb-O₂ binding, expressed by n_H, decreased significantly from 1.52±0.04 to 0.84±0.03, which was reflected in a change in the shape of the OECs from sigmoidal to hyperbolic. Over the tested range of PCO₂, white seabass displayed a Bohr coefficient of -0.92±0.13 when expressed relative to the change in pH_e and -1.13±0.11 when expressed relative to the change in RBC pH_i (Fig. S2A and B). In addition to the reduction in Hb-O₂ affinity at elevated PCO₂, white seabass blood also displayed a large Root effect, where the non-linear model predicted a maximal reduction of Hb-O₂ carrying capacity of 52.4±1.8%. The relationship between pH_i and pH_e had a slope of 0.67±0.07 (Fig. S2C), reflecting the higher buffer capacity of the intracellular space. The non-bicarbonate buffer capacity of white seabass whole blood was -2.43±0.56 mmol l⁻¹ pH_e⁻¹ at a Hct of 5% (Fig. S2D). By correcting this value for the higher Hct *in vivo*, according to Wood et al. (50), white seabass with a Hct of 25% are expected to have a whole blood non-bicarbonate buffer capacity of -9.68 mmol l⁻¹ pH_e⁻¹.

RBC swelling after β-adrenergic stimulation

The β-adrenergic stimulation of white seabass blood with ISO induced changes in the measured blood parameters (Fig. 2). Significant main effects of drug, time and their interaction (drug×time), indicate that Hct was affected by the experimental treatments (Fig. 2A). A large increase in Hct was observed in ISO-treated blood that was absent in ISO+Am and DMSO-treated RBCs. In addition, a main effect of drug treatments on MCHC indicated that the increase in Hct after ISO addition was due to swelling of the RBCs (Fig. 2B), whereas [Hb] was not affected by the treatments (Fig. S3). Significant main effects of drug and time were also detected for pH_e, where a large extracellular acidification was observed in ISO-treated blood, relative to the DMSO and ISO+Am treatments (Fig. 2C). No significant main effect of drug or time were observed on RBC pH_i, but multiple comparisons indicated a trend for a higher pH_i in ISO compared to DMSO treated blood (*P* = 0.081; Fig. 2D). Differential interference contrast (DIC) images confirmed a normal morphology of the RBCs at the beginning and the end of the trials, thus validating the fixation procedure. Swelling was observed in ISO-treated RBCs, relative to

initial measurements, or DMSO and ISO+Am-treated cells (Fig. 2E-H). The swelling of ISO-treated RBCs occurred largely along the z-axis of the cells (indicated by the arrows), whereas no visible distortion was observed in the x-y directions.

Molecular β -NHE characterization

The combined gill and RBC *de novo* transcriptome of white seabass contained nine Slc9a1 transcripts and phylogenetic analysis placed these sequences within well-supported clades of the NHE family tree (Fig. 3). Importantly, one of these white seabass transcripts grouped with the Slc9a1b sequences from other teleost fishes, supporting its classification as a white seabass β -NHE. Results from RT-PCR, cloning and sequencing confirmed the expression of β -NHE mRNA in isolated white seabass RBCs. A search for the Kozak nucleic acid motif in the open reading frame of the white seabass β -NHE sequence yielded the five most likely potential start codons, including one that would produce a 66 kDa protein (Table S2). This size closely matched the single band that was specifically recognized by the polyclonal β -NHE antibody in Western blots with crude homogenate, cytosolic and membrane-enriched fractions of a white seabass RBC lysate (Fig. S4A); whereas no immunoreactivity was observed in lanes where the antibody had been incubated with its pre-immune peptide. The anti- α -tubulin antibody detected a single band in the RBC crude homogenate, at the predicted size of 54 kDa (Fig. S4B). Finally, the white seabass β -NHE protein sequence shared seven consecutive amino acids with the peptide used to raise the polyclonal antibodies (Fig. S4C), which is sufficient for specific antibody binding (51). More importantly, the antigen peptide sequence was absent in the other eight white seabass NHE isoforms, ruling out non-specific antibody recognition of these NHEs.

Subcellular localization of RBC β -NHE

The subcellular location of β -NHE protein in white seabass RBCs was determined by immunofluorescence cytochemistry and super-resolution confocal microscopy (Fig. 4). In DMSO-treated RBCs, the β -NHE immunolabelling was most intense in intracellular vesicle-like structures, and weaker at the plasma membrane. There was substantial heterogeneity in the staining pattern for β -NHE in these control cells, with varying amounts of intracellular and membrane staining. In ISO-treated RBCs, the staining pattern for β -NHE was more homogeneous and most cells showed intense immunoreactivity for β -NHE in the membrane that co-localized with α -tubulin in the marginal band, and the intracellular, vesicle-like staining that was observed in the control cells was reduced (see Fig. S5 for an overview image with more

cells). In contrast, ISO-treated cells that were incubated without the primary antibody or where the antibody was pre-absorbed with its pre-immune-peptide showed no immunoreactivity for β -NHE (Fig. S6). Finally, optical sectioning and three-dimensional reconstruction of these RBCs confirmed that the membrane staining for β -NHE occurred in a single plane and co-localized with α -tubulin in the marginal band (see 3D movies S1 and S2).

Hb-O₂ binding after β -adrenergic stimulation

To characterize the protective effect of RBC β -NHE activation on Hb-O₂ binding, blood samples were first equilibrated to 21 kPa PO₂ and 0.3 kPa PCO₂ in tonometers and no significant effects of drug treatment (DMSO, ISO or ISO+Am) were observed on any of the measured blood parameters (Fig. S7); average values were: Hct $5.20 \pm 0.14\%$ ($P = 0.095$), [Hb] 0.178 ± 0.006 mM ($P = 0.889$), MCHC 3.46 ± 0.15 mM l⁻¹ RBC ($P = 0.490$), pH_e 7.848 ± 0.018 ($P = 0.576$), pH_i 7.464 ± 0.021 ($P = 0.241$). Thereafter, blood was loaded into the BOBS, where Hb-O₂ saturation was measured spectrophotometrically at increasing levels of a respiratory acidosis in normoxia (21 kPa PO₂). As expected from the pH-sensitivity of Hb-O₂ binding in white seabass, an increase in PCO₂ from 0.3 to 3 kPa caused a severe reduction in Hb-O₂ saturation in all treatments via the Root effect (Fig. 5). The raw data were analyzed by fitting a three-parameter Hill model to the individual observations within each treatment and significant differences were observed in the parameter estimates that describe these models. EC₅₀PCO₂ was affected by the experimental treatments, as shown in a significant main effect of drug (Fig. 6A). Multiple comparisons confirmed significant differences in EC₅₀PCO₂, which was 0.85 ± 0.06 kPa in DMSO, 0.91 ± 0.06 kPa in ISO+Am and 1.08 ± 0.06 kPa in ISO-stimulated blood. In contrast, the magnitude of the responses, Max. Δ Hb-O₂ sat., was not affected by the experimental treatments and no significant main effect of drug was detected; the average Max. Δ Hb-O₂ sat. across treatments was $-51.1 \pm 0.7\%$ (Fig. 6B).

When the same experiment was repeated under hypoxic conditions (3 kPa PO₂), an increase in PCO₂ likewise caused a severe reduction in Hb-O₂ saturation, indicating that in white seabass, a Root effect can also be expressed at saturations around P₅₀ (Fig. 5). A significant main effect of O₂ indicated that cells in the hypoxic condition required a lower EC₅₀PCO₂ to achieve Max. Δ Hb-O₂ sat., compared to the normoxic condition (Fig. 6A). There was also a significant main effect of drug on EC₅₀PCO₂ and multiple comparisons indicated a similar pattern in the individual drug effects as in the normoxic experiment, which was further confirmed by the

absence of a significant drug $\times$ O₂ interaction. Finally, a significant effect of O₂ on Max. Δ Hb-O₂ sat. (Fig. 6B) indicated a larger response magnitude in hypoxic blood, but that was unaffected by drug treatments, and the average Max. Δ Hb-O₂ sat. across treatments was $-74.1 \pm 0.1\%$.

To quantify the protective effect of RBC β -NHE activation on Hb-O₂ binding during a hypercapnic acidosis, Hb-O₂ saturation was expressed relative to the paired measurements in the DMSO treatment for each individual fish and relative to the initial Hb-O₂ saturation at 0.3 kPa PCO₂ (i.e. 95.6 and 55.0% Hb-O₂ saturation in normoxia and hypoxia, respectively). In normoxia, the benefit of β -NHE stimulation with ISO showed a bell-shaped relationship with a maximal Δ Hb-O₂ sat. of $7.8 \pm 0.02\%$ at 1 kPa PCO₂ (Fig. 7A). When NHEs were inhibited in ISO+Am blood, Δ Hb-O₂ sat. was only $1.9 \pm 0.4\%$ at 1 kPa PCO₂ and significantly lower compared to the other treatments; at higher PCO₂ the 95% confidence intervals overlapped with the DMSO values, indicating no difference from controls. In hypoxic blood, the ISO treatment had the largest effects on Δ Hb-O₂ sat. at 0.3 kPa PCO₂, with maximal values of $19.2 \pm 0.0\%$ that decreased towards higher PCO₂ (Fig. 7B). Whereas, in the ISO+Am treatment, Δ Hb-O₂ sat. was $6.4 \pm 0.0\%$ at 0.3 kPa PCO₂, and significant differences to the DMSO controls were only observed at PCO₂ below 1.5 kPa.

Discussion

In line with our initial hypothesis, RBC β -NHE activity in white seabass may greatly protect the blood O_2 -carrying capacity during environmentally relevant levels of hypoxia and hypercapnia. However, not all predictions were met as expected: white seabass did not have an unusually high Hb- O_2 affinity and thus, other aspects of their physiology are likely more important in determining their tolerance to hypoxia. Like other teleosts, white seabass had highly pH-sensitive Hbs, where a reduction in pH decreased both Hb- O_2 affinity via the Bohr effect and Hb- O_2 carrying capacity via the Root effect. Several lines of evidence corroborated the presence of a RBC β -NHE in white seabass and super-resolution imaging revealed, for the first time, the subcellular location of β -NHE protein in intracellular, vesicle-like structures and on the RBC membrane. Furthermore, adrenergic stimulation induced changes in the intracellular distribution of the β -NHE that may indicate a role of protein translocation in regulating β -NHE activity. A detailed quantification of the protective effects of RBC β -NHE activity, revealed the largest benefits at ~ 1 kPa PCO_2 in normoxia (21 kPa PO_2), where Hb- O_2 saturation increased by $\sim 8\%$. Whereas in hypoxia (3 kPa PO_2), β -NHE activity had its largest effect at arterial PCO_2 (0.3 kPa) and enhanced Hb- O_2 saturation by $\sim 20\%$; however, the benefits of β -NHE activation in hypoxia decrease rapidly at higher PCO_2 , revealing a potential vulnerability of white seabass to combinations of these stressors.

Many hypoxia tolerant vertebrates have evolved Hbs with a high affinity for O_2 (low Hb P_{50} values), which helps to extract the gas from the respiratory medium (9, 52, 53). White seabass in the present study had a Hb P_{50} of 2.9 ± 0.1 kPa (Fig. 1), which is higher than the values typically found in hypoxia tolerant fishes, such as carp (*Cyprinus carpio*) that have Hb P_{50} values as low as 0.5 kPa (54). In fact, the Hb P_{50} of white seabass resemble more closely the values in the well-studied rainbow trout, of 3.3 kPa (55), a cold-stream salmonid, of no noteworthy hypoxia tolerance. However, the O_2 -binding affinity of Hb must strike a balance between loading O_2 at the gas exchange surface and unloading O_2 at the tissues (56). Everything else being equal, a higher Hb P_{50} can sustain a higher PO_2 at the tissue capillaries, enhancing the diffusion gradient of O_2 to the mitochondria, which is of particular benefit to those species with a high scope for exercise (57). Thus, it seems that a high Hb- O_2 affinity is not part of the physiological mechanism that facilitates hypoxia tolerance in white seabass, but instead, a high tissue PO_2 may be important to sustain exercise performance in these active piscivores.

As in other teleosts, especially those in the highly-derived group of perciformes, Hb-O₂ binding in white seabass was highly pH-sensitive. An increase in PCO₂ from arterial levels (0.3 kPa) to severe hypercapnia (2.5 kPa), caused a significant right-shift of the OEC (Fig. 1) via the Bohr effect, increasing P₅₀ to 11.8±0.3 kPa. When considering the corresponding changes in pH_e (from 8.1 to 7.2 over the range of tested PCO₂; see Fig. S2A) the Bohr coefficient in white seabass was -0.92, and slightly higher, at -1.13, when considering the changes in RBC pH_i (from 7.7 to 7.0: Fig. S2B). Again, these Hb-O₂ binding characteristics resemble closely those of rainbow trout, where P₅₀ increases to 10 kPa at 2 kPa PCO₂, yielding a Bohr coefficient (relative to pH_e) of -0.87 (55). A normoxic increase in PCO₂ caused a significant reduction in Hb-O₂ saturation via the Root effect, and at PCO₂ above 3 kPa the O₂-carrying capacity of white seabass Hb was reduced by 52.4±1.8% (DMSO treatment; Fig. 6B). These results are in line with those of other teleosts, such as rainbow trout (~55%), tench (*Tinca tinca*; ~50%) and the European perch (*Perca fluviatilis*; ~70%), where the larger Root effect values may reflect the higher final PCO₂ used during those trials (58).

The Root effect is part of a specialized system of O₂ supply to the eye and the swimbladder of teleosts, where blood is acidified in a counter-current exchanger (the *rete mirabile*) to produce high PO₂ that bridge the large diffusion distances to the avascular retina of teleosts and inflate the swimbladder against large hydrostatic pressures (59). In the course of teleost evolution there have been numerous secondary losses of the *choroid* and swimbladder *retes*. While their presence has not directly been determined in white seabass, an ancestral state reconstruction predicts no secondary loss of either *rete* on the teleost branch leading up to the perciformes, which include the white seabass (24). In addition, all of the five independent losses of the *choroid rete* have coincided with a reduction of the Root effect below 40% (24, 60). Thus, the large Root effect of white seabass is consistent with the presence of a *choroid rete* and likely critical for maintaining a high ocular PO₂ that facilitates the visual acuity in these active predators (15).

Vertebrate Hbs are intracellular proteins and, as such, are affected by the microenvironment within the RBC cytoplasm. Teleost β-NHEs can actively modulate Hb-O₂ binding by controlling RBC pH_i and several lines of evidence in our study indicate the presence of functional β-NHEs in white seabass RBCs. A combined gill and RBC transcriptome detected nine sequences belonging to the vertebrate NHE (Slc9a1) family and phylogenetic analysis

classified the white seabass Slc9a1b transcript as belonging to the larger group of teleost RBC β -NHEs (Fig. 3). These findings were supported by the results from RT-PCR, confirming the expression of the β -NHE in white seabass RBCs. Western blots with a polyclonal anti-trout β -NHE antibody recognized a single band at 66 kDa in white seabass RBC homogenates (Fig. S4A), which is smaller than the 84 kDa predicted based on the longest possible mRNA transcript (Table S2). However, a search for Kozak motifs revealed the five most likely potential start codons in the open reading frame of the white seabass β -NHE mRNA sequence, one of which predicted a protein size of 66 kDa that matches the protein size detected in Western blots. This predicted β -NHE isoform lacks 158 amino acids on the N-terminus, which, according to structural NHE-protein models (61), are not essential for the transporter's activity, but may determine a differential sensitivity to inhibitors (61, 62). NHE isoforms from other teleosts have also been shown to separate in Western blotting with a similar size discrepancy (63), and show differential sensitivity to amiloride and its derivatives compared to mammalian NHEs (64).

Adrenergic stimulation of white seabass RBCs with ISO caused a ~25% volume increase during the 60 min trials, whereas no changes in RBC volume were detected in DMSO treated cells. The swelling response was corroborated by a significant reduction in MCHC and by visually confirming the increase in cell volume under a microscope, and these results closely match previous reports of RBC swelling after adrenergic stimulation in other teleosts (23, 65, 66). In addition, the ISO-induced swelling was abolished by the inhibition of NHEs in ISO+Am-treated RBCs, providing additional pharmacological support for the presence of a RBC β -NHE in white seabass. In ISO-treated RBCs, but not those treated with DMSO or ISO+Am, we observed a decrease in pH_e , which is the direct result of H^+ excretion by NHE activity. Corresponding changes in RBC pH_i are typically smaller, due to the higher buffer capacity of the intracellular space ($\text{pH}_i = 0.67 \pm 0.07 \times \text{pH}_e$; Fig. 1), and the additional freeze-thaw steps and plasma removal increase the variability of these measurements. Consequently, we were not able to resolve significant treatment effects on RBC pH_i , but a non-significant trend may point towards a small increase in RBC pH_i . Another interesting observation in these RBC swelling trials were the changes in cell morphology due to adrenergic stimulation. The increase in cell volume was largely due to an expansion along the z-axis of the cells, whereas the dimensions in the x-y axis apparently remained unaffected. The nucleated RBCs of non-mammalian vertebrates, including fish, have a marginal band, a structural component of their cytoskeleton formed by strands of α -

tubulin that maintains their elliptical shape in the face of shear and osmotic disturbances (67). The stiffness of this marginal band (68) may be a major impediment to swelling along the x-y axis forcing the cells to widen in the z-direction.

Fixed RBCs from these swelling trials were studied in more detail by super-resolution microscopy and by immunolabelling β -NHE and α -tubulin (Fig. 4). All RBCs showed β -NHE immunoreactivity, corroborating the presence of β -NHE protein in these cells. In control RBCs, β -NHE protein was detected intracellularly and appeared to be confined to vesicles, while weaker staining was detected on the plasma membrane (Fig. 4B and S5). A similar staining pattern has been described for a NHE1-like protein in the RBCs of winter flounder (*Pseudopleuronectes americanus*; Pedersen et al., 2003). However, the immunolabelling of this NHE was with polyclonal antibodies raised against a region of the human NHE1 sequence (aa 631-746) that is highly conserved with both the teleost Slc9a1a and Slc9a1b. Therefore, these previous results likely include staining of several NHE isoforms including the flounder β -NHE. The antibody used in the present study showed a high specificity for the white seabass β -NHE (Fig. S4) and a confounding detection of other RBC NHE isoforms is unlikely.

An important finding of our work was that the intracellular localization of β -NHE protein changed after adrenergic stimulation of the RBCs. In ISO-treated cells the staining pattern for β -NHE was more homogeneous compared to controls, with strong signal at the plasma membrane, and weaker intracellular signal (Fig. 4F and S5). Optical sectioning and 3D reconstructions of these cells clearly showed that the intense membrane staining for β -NHE was confined to a single plane, colocalizing with α -tubulin in the marginal band, and that this staining was mostly absent in DMSO-treated cells (Movies S1 and S2). Furthermore, the use of super-resolution microscopy allowed us to discern the subcellular orientation the β -NHE signal, which was extracellular relative to α -tubulin (Fig. 4H), thus, indicating a direct contact with the blood plasma that is essential for regulating pH_i via NHE activity. Combined, these observations may point towards an adrenergically-induced translocation of β -NHE protein from the cytoplasm into the membrane of white seabass RBCs. Intracellular translocation of NHEs in response to various stimuli has been reported in other systems, such as the gills of acid infused hagfish (70), insulin-treated rat cardiomyocytes (71), isolated mammalian cells after acidification (72) or the initiation of Na^+ -glucose co-transport in intestinal epithelial cells (73). Studies on rainbow trout RBCs found that the abundance of radio-labelled β -NHE protein in the membrane increased after

hypoxic incubation (1.2 kPa for 30 mins, 74), which generally supports a mechanism of protein translocation. However, we incubated all RBCs in hypoxia and hypercapnia, and observed β -NHE translocation after adrenergic stimulation. Clearly, there are still many open questions regarding the well-studied β -NHE response of teleosts, and additional work is required to characterize the cellular mechanisms underlying the translocation of β -NHE protein and its regulation by catecholamines, PO_2 , PCO_2 or pH. If substantiated, these findings may open new avenues in the research of RBC pH_i regulation in teleosts and perhaps other vertebrates.

The activation of RBC β -NHEs has been shown to raise pH_i above the equilibrium condition and plays an important role in protecting Hb- O_2 binding in teleosts (21). Previous work has characterized the resulting left-shift in the OEC (75–77) and the changes in arterial O_2 -carrying capacity due to adrenergic stimulation of the RBCs (78–80). In the present study we quantified the protective effect of β -NHE activation on Hb- O_2 saturation in white seabass under environmentally relevant levels of hypercapnia and hypoxia. As expected, Hb- O_2 saturation decreased significantly, due to the Root effect, when PCO_2 was increased from 0.3–3 kPa (Fig. 5). Adrenergic stimulation of the RBCs with ISO significantly delayed the reduction in Hb- O_2 saturation to higher $EC_{50}PCO_2$ that were 1.08 ± 0.06 kPa in ISO compared to 0.85 ± 0.06 kPa in DMSO-treated blood (Fig. 6A). In ISO+Am-treated RBC, the $EC_{50}PCO_2$ decreased significantly to 0.91 ± 0.06 kPa, compared to ISO-treated cells, corroborating the involvement of the RBC β -NHE in the response. However, the $EC_{50}PCO_2$ of ISO+Am-treated RBCs was still significantly higher compared to DMSO controls, perhaps indicating that 1 mM amiloride did not lead to a full inhibition of the β -NHE under the tested conditions, or that other, amiloride insensitive transporters, play a role in elevating RBC pH_i after adrenergic stimulation.

While β -NHE activity shifted the reduction in Hb- O_2 saturation to a higher PCO_2 , the magnitude of the Root effect was not affected by adrenergic stimulation (Fig. 6B). No significant differences were observed in Max. Δ Hb- O_2 sat. in any of the tested treatments and therefore, a severe acidosis generated by high PCO_2 can overwhelm the physiological capacity of the β -NHE to protect RBC pH_i . The H^+ extrusion by the β -NHE is secondarily active and driven by the trans-membrane Na^+ gradient created by the RBC $Na^+-K^+-ATPase$ (NKA). While both NKA activity (81) and the RBC rate of O_2 consumption ($\dot{M}O_2$) increase after adrenergic stimulation (82), it is possible that the capacity of the NKA to maintain the larger Na^+ gradients required to compensate for a greater reduction in pH_i is limited, as could be the availability of ATP to fuel

the exchange. In addition, H^+ that are extruded by the β -NHE will react with HCO_3^- in the plasma to form CO_2 that can, once again, diffuse into the cells. This re-acidification of the cells via CO_2 is part of the Jacobs-Stewart cycle and typically rate-limited by the formation of CO_2 in the plasma of teleosts (83). However, as pH_e decreases, the pool of plasma H_2CO_3 becomes larger (84), accelerating the Jacobs-Stewart cycle and the re-acidification of the cells, which may explain, in part, why β -NHE activity is ineffective at very high PCO_2 .

The benefit of β -NHE activity on Hb- O_2 saturation was non-linear over the range of PCO_2 tested, and in normoxia the bell-shaped response had a maximum at ~ 1 kPa PCO_2 (Fig. 7A). The observed relationship is likely dependent on the sigmoidal shape of the OEC, where a left-shift due to β -NHE activity has only marginal effects when Hb- O_2 saturation is high and the curve is flat (85). In addition, β -NHE activity in many teleosts is stimulated by high intracellular $[H^+]$ and inhibited by high extracellular $[H^+]$ as pH_e decreases, yielding a bell-shaped relationship between β -NHE activity and pH (86). The ecological implications are noteworthy, as the protective effect of β -NHE activity on Hb- O_2 binding is greatest over the range of PCO_2 that wild white seabass are currently experiencing during severe red-tide or upwelling events. The increase in Hb- O_2 saturation at these PCO_2 is $\sim 8\%$, and the effect can be harnessed continuously with every pass of the RBCs through the gills. Everything else being equal, an increase in arterial O_2 content can sustain a proportionally higher $\dot{M}O_2$, increasing the scope for activity or reducing the requirements for anaerobic pathways of ATP production that can lead to a toxic accumulation of metabolic by-products, such as lactate and H^+ . Thus, for fish that experience a potentially life-threatening surge in PCO_2 , an 8% increase in arterial O_2 content could make the difference between escaping into less-noxious waters or perishing in the attempt.

In the hypoxic trials, DMSO treated blood at arterial PCO_2 (0.3 kPa) had a Hb- O_2 saturation of $55.0 \pm 3.3\%$, which was close to the target value around Hb P_{50} (Fig. 5). As in normoxia, an increase in PCO_2 caused a significant reduction in Hb- O_2 saturation, indicating the presence of a Root effect in hypoxia, which further decreased Hb- O_2 saturation, even below the level of the maximal normoxic Root effect. Consequently, H^+ binding to Hb must occur over nearly the entire range of the OEC, which stands in contrast to previous findings in rainbow trout where the Bohr effect and H^+ binding to Hb occurred largely in the upper half of the OEC (87, 88). The possibility of inter-specific differences in the interaction between Hb- O_2 and H^+ binding cannot be resolved from the present data. However, it seems more likely that the kinetics of H^+

binding that induce the Bohr effect are different from those of the Root effect, which is supported by previous work indicating different molecular mechanisms for the two effects (89, 90). The interacting kinetics of O₂ and H⁺ binding to the Root effect Hbs of teleosts remain a worthwhile avenue for future research and studying a broader range of environmental and metabolic scenarios, in more species, may strengthen the important ecological implications of the present work.

As in normoxic blood, adrenergic activation of the β -NHE in hypoxia, increased Hb-O₂ saturation during a hypercapnic acidosis. This protective effect of the β -NHE was reflected in a significantly higher EC₅₀PCO₂ in ISO- compared to DMSO or ISO+Am-treated RBCs (Fig. 6A). A significant main effect of O₂ on EC₅₀PCO₂, would indicate that in hypoxic blood a lower PCO₂ is required to desaturate Hb, compared to normoxic blood. However, this parameter estimate is influenced by the combined effects of PO₂ and PCO₂ on Hb-O₂ saturation (by taking into account the full scale from 0-100%), which is not easily untangled statistically. Importantly, there was no drug×O₂ interaction, indicating that the effect of the drugs was similar under normoxia and hypoxia, highlighting the benefit of β -NHE activation under both conditions.

In hypoxia, the benefit of β -NHE activation on Hb-O₂ saturation was also non-linear over the tested range of PCO₂ (Fig. 7B). β -NHE activation in hypoxic blood caused the largest increase in Hb-O₂ saturation at arterial PCO₂ (0.3 kPa) and the benefits decreased markedly towards higher levels of hypercapnia; likely due to the flattening of the OEC at low Hb-O₂ saturations and perhaps some inhibition of the transporter by the increasing extracellular [H⁺]. The effect of β -NHE activity on Hb-O₂ binding was larger in hypoxia compared to normoxia, and at 0.3 kPa PCO₂ Hb-O₂ saturation increased by 11±0.4%. This effect is even greater when considering that the available O₂-carrying capacity is lower in hypoxia and when expressed relative to the available Hb-O₂ saturation (55% in DMSO treated blood), the relative benefit of β -NHE activity was 19.2±0.0%. Many teleost β -NHEs are O₂-sensitive (91) and the larger effects of β -NHE activity on Hb-O₂ saturation in hypoxia may be related to a partial inhibition of the transporter in normoxia; whereas, the effect appears to be less severe in white seabass compared to other species (30, 66, 92). The nearly 20% increase in Hb-O₂ saturation due to β -NHE activity is of great ecological significance and could be a principal pathway to safeguard arterial O₂ transport and facilitate hypoxic survival of white seabass in the wild. However, the present data also indicate a diminishing benefit of the β -NHE response when PCO₂ increases; thus, revealing

744 a potential vulnerability of white seabass to the combined stressors of hypoxia and hypercapnia;
745 surviving these conditions likely requires additional behavioral and metabolic adjustments, that
746 are yet to be determined.

Conclusion

The present results provide a thorough characterization of the Hb-O₂ binding system of white seabass, a non-model marine teleost with great ecological and economic importance in Southern California. Several lines of evidence confirmed the presence of a RBC β -NHE and super-resolution microscopy may point towards a regulation of the transporter's activity via intracellular translocation, a potentially novel pathway that deserves a more thorough investigation. In white seabass, the activity of the RBC β -NHE may provide significant protection of Hb-O₂ binding during hypercapnic conditions with maximal benefits around the ecologically relevant level of ~1 kPa PCO₂. Large benefits of β -NHE activation were also observed in hypoxia, however, with a greater sensitivity to increases in PCO₂. Combined, these data indicate that RBC function plays a critical role in modulating the O₂-binding characteristics of the pH-sensitive Hbs in white seabass and is likely part of the suite of physiological responses that determines their hypoxia and hypercapnia tolerance. Finally, these results also highlight a potential vulnerability of white seabass to combinations of these stressors and further research is needed to study the implications for wild fish conservation along the steadily warming and eutrophicated California coast and in high density aquaculture.

Acknowledgements

Thanks are due to Mark Drawbridge and the Hubbs SeaWorld Research Institute (HSWRI) for generously providing the white seabass and Phil Zerofski, Jessica Hallisey, Garfield Kwan and Daniel Jio for their help with animal care.

767 **Grants**

768 TSH was supported, and the study was funded by a National Science Foundation (NSF)
769 grant to MT (award no. 1754994), and AMC was supported by a SIO Postdoctoral Scholar
770 Fellowship.

771 **Disclosures**

772 The authors declare no competing interests.

773 **Endnotes**

774 The supplemental materials are available through figshare
775 (doi.org/10.6084/m9.figshare.14934405.v1) as well as all raw data and R source code
776 (doi.org/10.6084/m9.figshare.14944293.v1), and sequence data is available through NCBI (see
777 detailed accession numbers in manuscript and supplement Table S1).

References

1. **Frieder CA, Nam SH, Martz TR, Levin LA.** High temporal and spatial variability of dissolved oxygen and pH in a nearshore California kelp forest. *Biogeosciences* 9: 3917–3930, 2012. doi: 10.5194/bg-9-3917-2012.
2. **Van Dolah, F M.** Marine algal toxins: origins, health effects, and their increased occurrence. *Environ Health Perspect* 108: 133–141, 2000. doi: 10.1289/ehp.00108s1133.
3. **Diaz RJ, Rosenberg R.** Spreading Dead Zones and Consequences for Marine Ecosystems. *Science* 321: 926–929, 2008. doi: 10.1126/science.1156401.
4. **Harley CDG, Hughes AR, Hultgren KM, Miner BG, Sorte CJB, Thornber CS, Rodriguez LF, Tomanek L, Williams SL.** The impacts of climate change in coastal marine systems. *Ecol Lett* 9: 228–241, 2006. doi: <https://doi.org/10.1111/j.1461-0248.2005.00871.x>.
5. **Clemens S, Smith JE.** *SOAR Monitoring Report*. 2020.
6. **Boutilier RG, Heming TA, Iwama GK.** Physicochemical parameters for use in fish respiratory physiology. In: *Fish Physiology*, edited by Hoar W. S., Randall DJ. New York: Academic Press, 1984, p. 403–426.
7. **Lewis E, Wallace DWR.** *Program Developed for CO₂ System Calculations*. Oak Ridge Natl. Lab., Oak Ridge, Tenn.: ORNL/CDIAC-105, Carbon Dioxide Inf. Anal. Cent., 1998.
8. **Vaquer-Sunyer R, Duarte CM.** Thresholds of hypoxia for marine biodiversity. *PNAS* 105: 15452–15457, 2008. doi: 10.1073/pnas.0803833105.
9. **Hughes GM.** Respiratory responses to hypoxia in fish. *Am Zool* 13: 475–489, 1973.
10. **Jensen FB, Weber RE.** Respiratory properties of tench blood and hemoglobin. Adaptation to hypoxic-hypercapnic water. *Mol Physiol* 2: 235–250, 1982.
11. **Mandic M, Todgham AE, Richards JG.** Mechanisms and evolution of hypoxia tolerance in fish. *Proc R Soc Lond, Ser B: Biol Sci* 276: 735–744, 2009.
12. **Bohr C, Hasselbalch K, Krogh A.** About a new biological relation of high importance that the blood carbonic acid tension exercises on its oxygen binding. *Skand Arch Physiol* 16: 402–412, 1904.
13. **Root RW.** The respiratory function of the blood of marine fishes. *Biol Bull* 61: 427–456, 1931. doi: 10.2307/1536959.
14. **Scholander PF, Van Dam L.** Secretion of gases against high pressures in the swimbladder of deep sea fishes. I. Oxygen dissociation in blood. *Biol Bull* 107: 247–259, 1954. doi: 10.2307/1538611.

- 811 15. **Damsgaard C, Lauridsen H, Harter TS, Kwan GT, Thomsen JS, Funder AM,**
812 **Supuran CT, Tresguerres M, Matthews PG, Brauner CJ.** A novel acidification
813 mechanism for greatly enhanced oxygen supply to the fish retina. *eLife* 9: e58995, 2020.
814 doi: 10.7554/eLife.58995.
- 815 16. **Pelster B.** Buoyancy at depth. In: *Fish Physiology*, edited by Randall D, Farrell A. New
816 York: Academic Press, 1997, p. 195–238.
- 817 17. **Wittenberg JB, Wittenberg BA.** Active secretion of oxygen into the eye of fish. *Nature*
818 194: 106–107, 1962. doi: 10.1038/194106a0.
- 819 18. **Randall DJ, Perry SF.** Catecholamines. In: *Fish Physiology*, edited by Hoar WS, Randall
820 DJ, Farrell AP. New York: Academic Press, 1992, p. 255–300.
- 821 19. **Tetens V, Lykkeboe G.** Potency of adrenaline and noradrenaline for b-adrenergic proton
822 extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J Exp Biol* 134: 267–280, 1988.
- 823 20. **Mahe Y, Garciaromeu F, Motais R.** Inhibition by amiloride of both adenylate-cyclase
824 activity and the Na⁺/H⁺ antiporter in fish erythrocytes. *Eur J Pharmacol* 116: 199–206,
825 1985. doi: 10.1016/0014-2999(85)90154-2.
- 826 21. **Nikinmaa M.** Membrane transport and control of hemoglobin-oxygen affinity in nucleated
827 erythrocytes. *Physiol Rev* 72: 301–21, 1992.
- 828 22. **Rummer JL, Roshan-Moniri M, Balfry SK, Brauner CJ.** Use it or lose it? Sablefish,
829 *Anoplopoma fimbria*, a species representing a fifth teleostean group where the βNHE
830 associated with the red blood cell adrenergic stress response has been secondarily lost. *J*
831 *Exp Biol* 213: 1503–1512, 2010. doi: 10.1242/jeb.038844.
- 832 23. **Shu JJ, Harter TS, Morrison PR, Brauner CJ.** Enhanced hemoglobin-oxygen unloading
833 in migratory salmonids. *J Comp Physiol B* 7: 1–11, 2017. doi: 10.1007/s00360-017-1139-9.
- 834 24. **Berenbrink M, Koldkjaer P, Kepp O, Cossins AR.** Evolution of oxygen secretion in
835 fishes and the emergence of a complex physiological system. *Science* 307: 1752–1757,
836 2005. doi: 10.1126/science.1107793.
- 837 25. **Montgomery DW, Simpson SD, Engelhard GH, Birchenough SNR, Wilson RW.** Rising
838 CO₂ enhances hypoxia tolerance in a marine fish. *Sci Rep* 9: 15152, 2019. doi:
839 10.1038/s41598-019-51572-4.
- 840 26. **Caldwell S, Rummer JL, Brauner CJ.** Blood sampling techniques and storage duration:
841 Effects on the presence and magnitude of the red blood cell beta-adrenergic response in
842 rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol, A: Mol Integr Physiol* 144:
843 188–195, 2006. doi: 10.1016/j.cbpa.2006.02.029.
- 844 27. **Brauner CJ, Thorarensen H, Gallagher P, Farrell AP, Randall DJ.** CO₂ transport and
845 excretion in rainbow trout (*Oncorhynchus mykiss*) during graded sustained exercise. *Resp*
846 *Physiol* 119: 69–82, 2000.

- 847 28. **van Kampen EJ, Zijlstra WG.** Spectrophotometry of Hemoglobin and Hemoglobin
848 Derivatives. In: *Advances in Clinical Chemistry*, edited by Latner AL, Schwartz MK.
849 Elsevier, p. 199–257.
- 850 29. **Zeidler R, Kim HD.** Preferential hemolysis of postnatal calf red cells induced by internal
851 alkalization. *J Gen Physiol* 70: 385–401, 1977. doi: 10.1085/jgp.70.3.385.
- 852 30. **Motais R, Garcia-Romeu F, Borgese F.** The control of Na^+/H^+ exchange by molecular
853 oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer. *J gen Physiol*
854 90: 197–207, 1987. doi: 10.1085/jgp.90.2.197.
- 855 31. **Salama A, Nikinmaa M.** Species differences in the adrenergic responses of fish red cells:
856 studies on whitefish, pikeperch, trout and carp. *Fish Physiol Biochem* 6: 167–173, 1989.
- 857 32. **Borgese F, Sardet C, Cappadoro M, Pouyssegur J, Motais R.** Cloning and expression of
858 a cAMP-activated Na^+/H^+ exchanger: evidence that the cytoplasmic domain mediates
859 hormonal regulation. *PNAS* 89: 6765–9, 1992.
- 860 33. **Clifford AM, Weinrauch AM, Edwards SL, Wilkie MP, Goss GG.** Flexible ammonia
861 handling strategies using both cutaneous and branchial epithelia in the highly ammonia-
862 tolerant Pacific hagfish. *Am J Physiol Regul, Integr Comp Physiol* 313: R78–R90, 2017.
863 doi: 10.1152/ajpregu.00351.2016.
- 864 34. **Chen S, Zhou Y, Chen Y, Gu J.** fastp: an ultra-fast all-in-one FASTQ preprocessor.
865 *Bioinformatics* 34: i884–i890, 2018. doi: 10.1093/bioinformatics/bty560.
- 866 35. **Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X,**
867 **Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind**
868 **N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A.** Full-
869 length transcriptome assembly from RNA-Seq data without a reference genome. *Nat*
870 *Biotechnol* 29: 644–652, 2011. doi: 10.1038/nbt.1883.
- 871 36. **Bushnell B.** BBMap: A Fast, Accurate, Splice-Aware Aligner [Online]. Lawrence Berkeley
872 National Lab. (LBNL), Berkeley, CA (United States). <https://www.osti.gov/biblio/1241166>
873 [30 Mar. 2021].
- 874 37. **Edgar RC.** MUSCLE: multiple sequence alignment with high accuracy and high
875 throughput. *Nucleic Acids Res* 32: 1792–1797, 2004. doi: 10.1093/nar/gkh340.
- 876 38. **Galtier N, Gouy M, Gautier C.** SEAVIEW and PHYLO_WIN: two graphic tools for
877 sequence alignment and molecular phylogeny. *Bioinformatics* 12: 543–548, 1996. doi:
878 10.1093/bioinformatics/12.6.543.
- 879 39. **Gouy M, Guindon S, Gascuel O.** SeaView Version 4: A Multiplatform Graphical User
880 Interface for Sequence Alignment and Phylogenetic Tree Building. *Mol Biol Evol* 27: 221–
881 224, 2010. doi: 10.1093/molbev/msp259.

- 882 40. **Castresana J.** Selection of Conserved Blocks from Multiple Alignments for Their Use in
883 Phylogenetic Analysis. *Mol Biol Evol* 17: 540–552, 2000. doi:
884 10.1093/oxfordjournals.molbev.a026334.
- 885 41. **Talavera G, Castresana J.** Improvement of Phylogenies after Removing Divergent and
886 Ambiguously Aligned Blocks from Protein Sequence Alignments. *Systematic Biology* 56:
887 564–577, 2007. doi: 10.1080/10635150701472164.
- 888 42. **Miller MA, Pfeiffer W, Schwartz T.** Creating the CIPRES Science Gateway for Inference
889 of Large Phylogenetic Trees. Gateway Computing Environments Workshop (GCE). New
890 Orleans, LA: 2010, p. 1–8.
- 891 43. **Stamatakis A.** RAxML version 8: a tool for phylogenetic analysis and post-analysis of
892 large phylogenies. *Bioinformatics* 30: 1312–1313, 2014. doi:
893 10.1093/bioinformatics/btu033.
- 894 44. **Le SQ, Gascuel O.** An Improved General Amino Acid Replacement Matrix. *Mol Biol Evol*
895 25: 1307–1320, 2008. doi: 10.1093/molbev/msn067.
- 896 45. **Kozak M.** An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs.
897 *Nucleic Acids Res* 15: 8125–8148, 1987. doi: 10.1093/nar/15.20.8125.
- 898 46. **Nishikawa T, Ota T, Isogai T.** Prediction whether a human cDNA sequence contains
899 initiation codon by combining statistical information and similarity with protein sequences.
900 *Bioinformatics* 16: 960–967, 2000. doi: 10.1093/bioinformatics/16.11.960.
- 901 47. **RCoreTeam.** R: A language and environment for statistical computing [Online]. R
902 Foundation for Statistical Computing. <https://www.R-project.org/>.
- 903 48. **RStudioTeam.** RStudio: Integrated Development Environment for R [Online]. RStudio,
904 Inc. <http://www.rstudio.com/>.
- 905 49. **Wickham H.** *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag,
906 2009.
- 907 50. **Wood CM, McDonald DG, McMahon BR.** The influence of experimental anemia on
908 blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*)
909 and the rainbow trout (*Salmo gairdneri*). *J Exp Biol* 96: 221–237, 1982.
- 910 51. **Dunn C, O’Dowd A, Randall RE.** Fine mapping of the binding sites of monoclonal
911 antibodies raised against the Pk tag. *J Immunol Methods* 224: 141–150, 1999. doi:
912 10.1016/s0022-1759(99)00017-4.
- 913 52. **Mairbäurl H.** Red blood cell function in hypoxia at altitude and exercise. *Int J Sports Med*
914 15: 51–63, 1994.
- 915 53. **Tenney SM.** Hypoxia and the Brain: Functional significance of differences in mammalian
916 hemoglobin affinity for oxygen. In: *Proceeding of the 9th International Hypoxia*

- 917 *Symposium*, edited by Sutton, J. R, Houston C. S., Coates G. Lake Louise, Canada: Queen
918 city printers, Burlington, Vt., 1995, p. 57–68.
- 919 54. **Brauner CJ, Wang T, Val AL, Jensen FB.** Non-linear release of Bohr protons with
920 haemoglobin-oxygenation in the blood of two teleost fishes; carp (*Cyprinus carpio*) and
921 tambaqui (*Colossoma macropomum*). *Fish Physiol Biochem* 24: 97–104, 2001.
- 922 55. **Rummer JL, Brauner CJ.** Root effect haemoglobins in fish may greatly enhance general
923 oxygen delivery relative to other vertebrates. *PloS one* 10: e0139477, 2015. doi:
924 10.1371/journal.pone.0139477.
- 925 56. **Brauner CJ, Wang T.** The optimal oxygen equilibrium curve: a comparison between
926 environmental hypoxia and anemia. *Am Zool* 37: 101–108, 1997.
- 927 57. **Mairbäurl H, Weber RE.** Oxygen transport by hemoglobin. *Compr Physiol* 2: 1463–1489,
928 2012.
- 929 58. **Berenbrink M, Koldkjaer P, Wright EH, Kepp O, da Silva AJ.** Magnitude of the Root
930 effect in red blood cells and haemoglobin solutions of fishes: a tribute to August Krogh.
931 *Acta Physiol* 202: 583–592, 2011. doi: 10.1111/j.1748-1716.2010.02243.x.
- 932 59. **Pelster B, Randall DJ.** Physiology of the Root effect. In: *Fish Physiology*, edited by Perry
933 SF, Tufts BL. New York: Academic Press, 1998, p. 113–140.
- 934 60. **Berenbrink M.** Historical reconstructions of evolving physiological complexity: O₂
935 secretion in the eye and swimbladder of fishes. *J Exp Biol* 210: 1641–1652, 2007. doi:
936 10.1242/jeb.003319.
- 937 61. **Landau M, Herz K, Padan E, Ben-Tal N.** Model structure of the Na⁺/H⁺ exchanger 1
938 (NHE1): functional and clinical implications. *J Biol Chem* 282: 37854–37863, 2007. doi:
939 10.1074/jbc.M705460200.
- 940 62. **Lee BLLL, Sykes BDS, Fliegel LF.** Structural analysis of the Na⁺/H⁺ exchanger isoform
941 1 (NHE1) using the divide and conquer approach. *Biochem Cell Biol* , 2011. doi:
942 10.1139/O10-140.
- 943 63. **Chen XL, Zhang B, Chng YR, Ong JLY, Chew SF, Wong WP, Lam SH, Ip YK.**
944 Na⁺/H⁺ Exchanger 3 Is Expressed in Two Distinct Types of Ionocyte, and Probably
945 Augments Ammonia Excretion in One of Them, in the Gills of the Climbing Perch Exposed
946 to Seawater. *Front Physiol* 8, 2017. doi: 10.3389/fphys.2017.00880.
- 947 64. **Blair S, Li X, Dutta D, Chamot D, Fliegel L, Goss G.** Rainbow Trout (*Oncorhynchus*
948 *mykiss*) Na⁺/H⁺ Exchangers tNhe3a and tNhe3b Display Unique Inhibitory Profiles
949 Dissimilar from Mammalian NHE Isoforms. *Int J Mol Sci* 22: 2205, 2021. doi:
950 10.3390/ijms22042205.

- 951 65. **Nikinmaa M, Huestis WH.** Adrenergic swelling of nucleated erythrocytes - cellular
952 mechanisms in a bird, domestic goose, and 2 teleosts, striped bass and rainbow trout. *J Exp*
953 *Biol* 113: 215–224, 1984.
- 954 66. **Weaver YR, Kiessling K, Cossins AR.** Responses of the Na⁺/H⁺ exchanger of European
955 flounder red blood cells to hypertonic, beta-adrenergic and acidotic stimuli. *J Exp Biol* 202:
956 21–32, 1999.
- 957 67. **Joseph-Silverstein J, Cohen WD.** The cytoskeletal system of nucleated erythrocytes. III.
958 Marginal band function in mature cells. *J Cell Biol* 98: 2118–2125, 1984.
- 959 68. **Dmitrieff S, Alsina A, Mathur A, Nédélec FJ.** Balance of microtubule stiffness and
960 cortical tension determines the size of blood cells with marginal band across species. .
- 961 69. **Pedersen SF, King SA, Rigor RR, Zhuang Z, Warren JM, Cala PM.** Molecular cloning
962 of NHE1 from winter flounder RBCs: activation by osmotic shrinkage, cAMP, and
963 calyculin A. *Am J Physiol Cell Physiol* 284: C1561–C1576, 2003.
- 964 70. **Parks SK, Tresguerres M, Goss GG.** Blood and gill responses to HCl infusions in the
965 Pacific hagfish (*Eptatretus stoutii*). *Can J Zool* 85: 855–862, 2007.
- 966 71. **Lawrence SP, Holman GD, Koumanov F.** Translocation of the Na⁺/H⁺ exchanger 1
967 (NHE1) in cardiomyocyte responses to insulin and energy-status signalling. *Biochem J* 432:
968 515–523, 2010. doi: 10.1042/BJ20100717.
- 969 72. **Gens JS, Du H, Tackett L, Kong S-S, Chu S, Montrose MH.** Different ionic conditions
970 prompt NHE2 and NHE3 translocation to the plasma membrane. *Biochim Biophys Acta,*
971 *Biomembr* 1768: 1023–1035, 2007. doi: 10.1016/j.bbamem.2007.01.003.
- 972 73. **Zhao H, Shiue H, Palkon S, Wang Y, Cullinan P, Burkhardt JK, Musch MW, Chang**
973 **EB, Turner JR.** Ezrin regulates NHE3 translocation and activation after Na⁺-glucose
974 cotransport. *PNAS* 101: 9485–9490, 2004. doi: 10.1073/pnas.0308400101.
- 975 74. **Reid SD, Perry SF.** Quantification of presumptive Na⁺/H⁺ antiporters of the erythrocytes
976 of trout and eel. *Fish Physiol Biochem* 12: 455–463, 1994.
- 977 75. **Jensen BJ, Nikinmaa M, Weber RE.** Effects of exercise stress on acid-base balance and
978 respiratory function in blood of the teleost *Tinca tinca*. *Resp Physiol* 51: 291–301, 1983.
- 979 76. **Nikinmaa M.** Adrenergic regulation of hemoglobin oxygen-affinity in rainbow trout red
980 cells. *J Comp Physiol* 152: 67–72, 1983.
- 981 77. **Nikinmaa M, Soivio A.** Oxygen dissociation curves and oxygen capacities of blood of a
982 freshwater fish, *Salmo gairdneri*. *Ann Zool Fenn* 16: 217–221, 1979.
- 983 78. **Cossins AR, Richardson PA.** Adrenaline-induced Na⁺/H⁺ exchange in trout erythrocytes
984 and its effects upon oxygen carrying capacity. *J Exp Biol* 118: 229–246, 1985.

- 985 79. **Ferguson RA, Tufts BL, Boutilier RG.** Energy metabolism in trout red cells:
986 consequences of adrenergic stimulation *in vivo* and *in vitro*. *J Exp Biol* 143: 133–147, 1989.
- 987 80. **Nikinmaa M, Cech JJ, McEnroe M.** Blood oxygen transport in stressed striped bass
988 (*Morone saxatilis*): role of β -adrenergic responses. *J Comp Physiol* 154: 365–369, 1984.
- 989 81. **Bourne PK, Cossins AR.** On the instability of K^+ influx in erythrocytes of the rainbow
990 trout, *Salmo gairdneri*, and the role of catecholamine hormones in maintaining *in vivo*
991 influx activity. *J Exp Biol* 101: 93–104, 1982.
- 992 82. **Boutilier RG, Ferguson RA.** Nucleated red cell function: metabolism and pH regulation.
993 *Can J Zool* 67: 2986–2993, 1989.
- 994 83. **Jacobs MH, Stewart DR.** The role of carbonic anhydrase in certain ionic exchanges
995 involving the erythrocyte. *J gen Physiol* 25: 539–552, 1942.
- 996 84. **Motais R, Fievet B, Garcia-Romeu F, Thomas S.** Na^+-H^+ exchange and pH regulation in
997 red blood cells: role of uncatalyzed $H_2CO_3^-$ dehydration. *Am J Physiol* 256: C728–C735,
998 1989.
- 999 85. **Kobayashi M, Ishigaki K, Kobayashi M, Imai K.** Shape of the haemoglobin-oxygen
1000 equilibrium curve and oxygen transport efficiency. *Resp Physiol* 95: 321–328, 1994.
- 1001 86. **Borgese F, Garcia-Romeu F, Motais R.** Ion movements and volume changes induced by
1002 catecholamines in erythrocytes of rainbow trout: effect of pH. *J Physiol* 382: 145–157,
1003 1987.
- 1004 87. **Brauner CJ, Gilmour KM, Perry SF.** Effect of haemoglobin oxygenation on Bohr proton
1005 release and CO_2 excretion in the rainbow trout. *Resp Physiol* 106: 65–70, 1996.
- 1006 88. **Brauner CJ, Thorarensen H, Gallagher P, Farrell AP, Randall DJ.** The interaction
1007 between O_2 and CO_2 exchange in rainbow trout during graded sustained exercise. *Resp*
1008 *Physiol* 119: 83–96, 2000.
- 1009 89. **Brittain T.** The Root effect. *Comp Biochem Physiol* 86B: 473–481, 1987.
- 1010 90. **Perutz MF, Brunori M.** Stereochemistry of cooperative effects in fish and amphibian
1011 haemoglobins. *Nature* 299: 421–426, 1982.
- 1012 91. **Gibson J, Cossins A, Ellory J.** Oxygen-sensitive membrane transporters in vertebrate red
1013 cells. *J Exp Biol* 203: 1395–1407, 2000.
- 1014 92. **Salama A, Nikinmaa M.** The adrenergic responses of carp (*Cyprinus carpio*) red cells:
1015 effects of PO_2 and pH. *J Exp Biol* 136: 405–416, 1988.

Figure legends

Figure 1 Oxygen binding characteristics of white seabass whole blood. Oxygen equilibrium curves showing hemoglobin-oxygen saturation (Hb-O₂ sat.; %) as a function of the partial pressure of oxygen (PO₂; kPa) at five partial pressures of carbon dioxide (PCO₂). The PO₂ that yields 50% Hb-O₂ saturation (P₅₀) and the cooperativity coefficient of Hb-O₂ binding (Hill coefficient, n_H) are shown for each curve. The main effects of PCO₂ on P₅₀ and n_H were analyzed with ACOVA ($P < 0.05$, $N = 8$). The Bohr coefficients (B) relative to extracellular (pH_e) and intracellular (pH_i), the relationship between pH_e and pH_i and the non-bicarbonate buffer capacity of the blood (at 5% Hct) were determined by linear regressions (see Fig. S2). The Root effect (R) was determined at 21 kPa PO₂ from the model parameters shown for the DMSO treatment in Fig. 6B. All data are means±s.e.m.

Figure 2 Changes in blood parameters after adrenergic stimulation of white seabass whole blood. A) Hematocrit (%), B) mean cell hemoglobin content (MCHC; mM hemoglobin l⁻¹ red blood cells), C) extracellular pH (pH_e) and D) intracellular pH (pH_i). Blood was equilibrated in tonometers at 3 kPa PO₂ and 1 kPa PCO₂ and treated with either: i) a carrier control (DMSO; 0.25%), ii) the β-adrenergic agonist isoproterenol (ISO; 10 μM), or iii) ISO plus amiloride (ISO+Am; 1 mM), an inhibitor of sodium-proton exchangers (NHE). The dotted line indicates initial values for each parameter and changes were recorded over 60 min. The main effects of drug, time, and their interaction term (drug×time) were analyzed with a two-way ANOVA ($P < 0.05$, $N = 6$ and $N = 5$ for ISO+Am). There were no significant changes in hemoglobin concentration ([Hb]; mM) throughout the trials. Multiple comparisons were with paired t-tests with a Benjamini-Hochberg correction and superscript letters that differ indicate significant differences between treatments at 60 min. Individual datapoints and means±s.e.m. Inserts E-H) differential interference contrast (DIC, 60x) images of red blood cells fixed at the beginning (ini) and the end of the trial (T60). Cell swelling was visually confirmed in ISO-treated cells, but not in other treatments, and mostly along the z-axis of the cells (arrows), while the x-y-axis seemed largely unaffected.

Figure 3: Phylogenetic analysis of nine NHE-like protein sequences in the *de novo* assembly of a combined white seabass gill and red blood cell transcriptome. Novel white seabass sequences are highlighted in blue and the β-NHE in red (Slc9a1b). Shadings delineate sub-families of the Slc9a1 gene family. The tree was rooted against the NHE2 from *Caenorhabditis elegans* in orange. Accession numbers for all species are those reported in Table S1.

Figure 4 Immunocytochemical localization of the β-adrenergic sodium proton exchanger (β-NHE) in white seabass red blood cells. Blood was equilibrated in tonometers at 3 kPa PO₂ and 1 kPa PCO₂ for 60 mins (see Fig. 2 for details) in the presence of either: A-D) a carrier control (DMSO; 0.25%), or E-H) the β-adrenergic agonist isoproterenol (ISO; 10 μM). Fixed cells were immuno-stained with a monoclonal α-tubulin antibody to visualize the marginal band (red), with DAPI to visualize the cell nuclei (A and E), and with a polyclonal anti-β-NHE

antibody (green, B and F). D and H) Magnified view of the insets in the merged images, where arrows indicate weak or absent β -NHE immunoreactivity on the membrane of Ctrl cells and intense staining in ISO-treated cells. These responses were representative and repeatable ($N = 4$) and images showing a larger number of cells are available in the supplement (Fig. S5).

Figure 5 Hemoglobin-oxygen saturation (Hb-O₂ sat.; %) during hypercapnic acidification of white seabass whole blood. Hematocrit was set to 5%, blood was equilibrated in tonometers at 21 kPa PO₂ and 0.3 kPa PCO₂ and treated with either: i) a carrier control (DMSO; 0.25%), ii) the β -adrenergic agonist isoproterenol (ISO; 10 μ M), or iii) ISO plus amiloride (ISO+Am; 1 mM), an inhibitor of sodium-proton exchangers (NHE). For each sample, runs were performed in normoxia (21 kPa PO₂; solid symbols) or hypoxia (3 kPa PO₂; open symbols). Individual datapoints and means $\pm$ s.e.m. ($N = 6$).

Figure 6 Parameter estimates describing the changes in hemoglobin-oxygen saturation during hypercapnic acidification of white seabass whole blood. A) The PCO₂ that elicits a half-maximal reduction in Hb-O₂ saturation (EC₅₀PCO₂; kPa). and B) the maximal reduction in Hb-O₂ saturation due to acidification (Max. Δ Hb-O₂ sat.; %). Treatments were: i) a carrier control (DMSO; 0.25%), ii) the β -adrenergic agonist isoproterenol (ISO; 10 μ M), or iii) ISO plus amiloride (ISO+Am; 1 mM) an inhibitor of sodium-proton exchangers (NHE). For each sample, runs were performed in normoxia (21 kPa PO₂; solid symbols) or hypoxia (3 kPa PO₂; open symbols). The main effects of drug treatments (drug), oxygen (O₂) and their interaction term (drug $\times$ O₂) were analyzed with a two-way ANOVA ($P < 0.05$, $N = 6$). Multiple comparisons were with paired t-tests and a Benjamini-Hochberg correction and superscript letters that differ indicate significant differences between treatments for each O₂ tension. Individual datapoints and means $\pm$ s.e.m. ($N = 6$).

Figure 7 Relative changes in hemoglobin-oxygen saturation (Δ Hb-O₂ sat.; %) during hypercapnic acidification of white seabass whole blood. A) in normoxia (21 kPa PO₂; solid symbols) or B) hypoxia (3 kPa PO₂; open symbols). Treatments were: i) a carrier control (DMSO; 0.25%), ii) the β -adrenergic agonist isoproterenol (ISO; 10 μ M), or iii) ISO plus amiloride (ISO+Am; 1 mM), an inhibitor of sodium-proton exchangers (NHE). Individual datapoints, means $\pm$ s.e.m. and 95% confidence intervals ($N = 6$).

Ctrl

A

2 μ m

B

2 μ m

C

2 μ m

D

0.5 μ m

ISO

E

2 μ mNucleus
 α -tubulin

F

2 μ m β -NHE

G

2 μ m

Merged

H

0.5 μ m